Version of Record: https://www.sciencedirect.com/science/article/pii/S1096717616300623
2f176dc253628dba690184527eaec8c9

CRISPR interference as a titratable, *trans*-acting regulatory tool for metabolic engineering in the cyanobacterium *Synechococcus sp*. strain PCC 7002

Authors and Affiliations:

Gina C. Gordon[a,b], Travis C. Korosh[a,c], Jeffrey C. Cameron[d,e], Andrew L. Markley[a], Matthew B. Begemann[a,b], Brian F. Pfleger[a,b,#]

a. Department of Chemical and Biological Engineering, University of Wisconsin-Madison, 1415 Engineering Drive, Madison, WI 53706

b. Microbiology Doctoral Training Program, University of Wisconsin-Madison, 1550 Linden Drive, Madison, WI 53706

c. Department of Environmental Chemistry and Technology, University of Wisconsin-Madison, 660 N Park St, Madison, WI 53706

d. Department of Chemistry and Biochemistry, University of Colorado-Boulder, 596 UCB, Boulder, CO 80309

e. Renewable and Sustainable Energy Institute, University of Colorado-Boulder, 27 UCB, Boulder, CO 80309

Corresponding Author – 3629 Engineering Hall, 1415 Engineering Dr., Madison, WI 53706, United States. Phone: +1 608 890 1940. Fax: +1 608 262-5434. E-mail address: pfleger@engr.wisc.edu.

© 2019. This manuscript version is made available under the Elsevier user license
https://www.elsevier.com/open-access/userlicense/1.0/

Abstract

Trans-acting regulators provide novel opportunities to study essential genes and regulate metabolic pathways. We have adapted the clustered regularly interspersed palindromic repeats (CRISPR) system from *Streptococcus pyogenes* to repress genes *in trans* in the cyanobacterium *Synechococcus sp.* strain PCC 7002 (hereafter PCC 7002). With this approach, termed CRISPR interference (CRISPRi), transcription of a specific target sequence is repressed by a catalytically inactive Cas9 protein recruited to the target DNA by base-pair interactions with a single guide RNA that is complementary to the target sequence. We adapted this system for PCC 7002 and achieved conditional and titratable repression of a heterologous reporter gene, yellow fluorescent protein. Next, we demonstrated the utility of finely tuning native gene expression by downregulating the abundance of phycobillisomes. In addition, we created a conditional auxotroph by repressing synthesis of the carboxysome, an essential component of the carbon concentrating mechanism cyanobacteria use to fix atmospheric CO_2. Lastly, we demonstrated a novel strategy for increasing central carbon flux by conditionally downregulating a key node in nitrogen assimilation. The resulting cells produced 2-fold more lactate than a baseline engineered cell line, representing the highest photosynthetically generated productivity to date. This work is the first example of titratable repression in cyanobacteria using CRISPRi, enabling dynamic regulation of essential processes and manipulation of flux through central carbon metabolism. This tool facilitates the study of essential genes of unknown function and enables groundbreaking metabolic engineering capability, by providing a straightforward approach to redirect metabolism and carbon flux in the production of high-value chemicals.

Keywords

CRISPRi, tunable, cyanobacteria, lactate, chemical production, carboxysome, phycobilisome, synthetic biology

1 **1.1 Introduction**

2 The ability to manipulate and predictably control gene expression is an essential tool for
3 engineering metabolism and biology. Modern gene expression toolboxes include promoter
4 libraries for initiating transcription at desired rates (Alper et al., 2005; Markley et al., 2015),
5 transcriptional regulator/operator pairs for creating dynamic switches and circuits (Stanton et al.,
6 2013; Zhang et al., 2012), transcription terminators for insulating neighboring expression
7 cassettes (Chen et al., 2013) models of translation initiation for engineering ribosome binding
8 sites (Espah Borujeni et al., 2014; Salis et al., 2010), codon optimization algorithms for
9 optimizing gene sequences (Puigbò et al., 2007), and RNA structures that tune mRNA turnover,
10 termination, and translation initiation (Pfleger et al., 2006). These tools have enhanced the study
11 of natural systems and enabled the creation of engineered microbes for addressing societal
12 challenges such as sustainable chemical production (Chubukov et al., 2014; Hara et al., 2014;
13 Liao et al., 2016; Lynch and Gill, 2012; Smanski et al., 2016). Unfortunately, differences in how
14 microbes recognize promoters, regulate gene expression, and translate protein do not allow
15 genetic circuits and tools to be universally moved between organisms with the desired outcome.
16 Instead synthetic biology tools must be validated in new hosts and then frequently adapted and
17 refined for optimal functionality (Keasling, 2012).

18 The majority of established tools regulate genes in *cis* and require replacement of native
19 expression cassettes with heterologous sequences to alter the level of gene expression. Replacing
20 sequences has the unfortunate side-effect of removing native regulation that has often evolved to
21 optimize the protein abundance needed for a given set of natural physiological states. In many
22 instances, the native context is ideal for one state but needs to be altered for a new unnatural state
23 (e.g. chemical production instead of growth). *Trans* acting tools that supersede native regulation

24 in specific environments are therefore desirable additions to the synthetic biology toolbox
25 (Copeland et al., 2014). One such tool, termed CRISPR interference (CRISPRi), takes advantage
26 of the adaptive RNA-based defense system that in many bacteria targets and cleaves foreign
27 nucleic acids such as viruses and plasmids (Qi et al., 2013). Diverse CRISPR-Cas systems exist
28 in bacteria with altered locus architecture, components, maturation processes, and functions
29 (Makarova et al., 2015). The type II system from *Streptococcus pyogenes* has been the most
30 widely adapted to manipulate gene expression. A complex of a single guide RNA (sgRNA)
31 containing a sequence complementary to the target and the protein Cas9 DNA nuclease protein
32 can initiate double-stranded breaks. Point mutations in the two active sites can be used to create a
33 nuclease deficient or dead Cas9 (dCas9). The complex of the sgRNA and dCas9 is still able to
34 bind target DNA and can be used to either repress or activate gene expression (Bikard et al.,
35 2013). There is strong evidence that repression is caused by the dCas9-sgRNA complex
36 sterically blocking RNA polymerase elongation (Gilbert et al., 2013). The use of CRISPRi in
37 *Escherichia coli* has led to efficient repression of targets as high as 300-fold when the sgRNA
38 was targeted to the non-template strand near the 5' end of the gene with no detectable off-target
39 affects (Qi et al., 2013). This repression was reversible and can be multiplexed to target several
40 genes simultaneously.
41 CRISPRi has become an increasingly attractive alternative to other *trans*-acting
42 regulators repressor proteins such as trans-activator-like effectors (TALEs) or zinc fingers, due
43 to the simplicity of design and ease of synthesis. By altering just 20 nucleotides of the sgRNA,
44 the system can be designed to repress any gene of interest. However, it is necessary to choose a
45 target site with a protospacer adjacent motif (PAM) which is 5'-NGG-3' for *S. pyogenes* Cas9,
46 but engineered Cas9 nucleases are reducing this constraint (Kleinstiver et al., 2015). Despite this

47 limitation this technology has been used to repress reporter genes as well as enhance titers of
48 products in various organisms: flavonoids in *E. coli* (Wu et al., 2015) and L-lysine and L-
49 glutamate in *Corneybacterium glutamicum* (Cleto et al., 2016).

50 Here, we adapted this technology to an industrially relevant cyanobacterial strain,
51 *Synechococcus sp. strain* PCC 7002 (PCC 7002) (Devroe et al., 2010; Reppas and Ridley, 2011).
52 Cyanobacteria are an attractive chassis for chemical production that enables direct conversion of
53 carbon dioxide and sunlight into useful products (Oliver and Atsumi, 2014). PCC 7002 is a
54 promising strain of cyanobacteria because it grows rapidly, is halotolerant, is naturally
55 transformable, can tolerate high light conditions, and has a growing synthetic biology toolbox
56 (Markley et al., 2015; Zess et al., 2016). To add to the PCC 7002 toolbox, we developed a
57 tunable gene repression system using CRISPRi. The system is novel because targets can be
58 repressed to varying degrees by controlling the expression of CRISPRi components with an
59 inducer, anhydrotetracycline (aTc). Most CRISPRi systems including one developed for another
60 cyanobacterium *Synechocystis sp.* PCC 6803, can be turned on and off but the ability to titrate
61 the levels of expression was not reported (Yao et al., 2015). Attempts have been made to tune
62 repression by introducing mismatches in the sgRNA, but this involves making numerous strains
63 to reach a variety of repression levels (Qi et al., 2013).

64 The ability to finely tune native gene expression will be critical for manipulating essential
65 genes and to achieve growth regimes that decouple biomass and chemical production for
66 maximum yield (Xu et al., 2014). In many cases, intermediate levels of enzyme expression result
67 in maximum product titer (Freed et al., 2015; Pitera et al., 2007). With the described CRISPRi
68 technology we can lower expression of essential cyanobacterial genes, decrease but not abolish
69 flux towards competing products, and manipulate cellular processes to varying degrees. Here,

70 we demonstrate the utility of finely tuning native gene expression by downregulating the
71 abundance of phycobilisomes. In addition, we create a conditional auxotroph by repressing
72 synthesis of the carboxysome, an essential component of the carbon concentrating mechanism
73 cyanobacteria use to fix atmospheric CO_2. Lastly, we demonstrate a novel strategy for increasing
74 central carbon flux by conditionally downregulating a key node in nitrogen assimilation
75 pathways. The resulting cells produced 2-fold more lactate than a baseline engineered cell line,
76 representing the highest photosynthetically generated productivity to date.

77 **1.2 Materials and Methods**

78 **1.2.1 *Chemicals, reagents, and media***

79 Strains were grown and maintained on media A+ media (Stevens et al., 1973) with 1.5%
80 (w/v) Bacto-Agar (Fisher). Strains with antibiotic resistance markers were selected on media
81 with antibiotics (kanamycin, 100 μg/mL; gentamicin, 30 μg/mL) and strains with cassettes
82 introduced in the *acsA* locus were plated on 100 μM acrylic acid. Strains were grown in glass
83 culture tubes (2 x 15 cm) with 20 mL media A+ and bubbled with either air (0.04% CO_2) or high
84 CO_2 (10% CO_2). Temperature was maintained at 38°C and light intensity was approximately 150
85 umol photons $m^{-2}s^{-1}$. Optical density was measured in a Genesys 20 spectrophotometer (Thermo
86 Scientific) in 1-cm cuvette.
87

88 **1.2.2 *Strain Construction***

89 All strains, plasmids, and plasmid sequence used in this study are in the supplementary
90 material. Plasmids were cloned in *Escherichia coli* DH5α. The sequences of chromosomal
91 cassettes are available as supplementary material. Genes and sgRNA were transformed onto the
92 chromosome of wild-type PCC 7002 using homologous recombination. dCas9 was introduced at

93 the *acsA* locus (Begemann et al., 2013) and the sgRNA and a kanamycin resistance marker were
94 introduced at the NS1 site (Davies et al., 2014). In some strains EYFP or the LDH with the
95 $P_{cLac143}$ inducible promoter system were placed in the *glpK* pseudogene along with a gentamicin
96 resistance marker (Begemann et al., 2013). Constructs were made using Gibson assembly
97 (Gibson et al., 2009) with regions of homology added in the 5' end of the primers. Site specific
98 mutations in LDH enzymes was based on the V39R mutation described for the L-LDH of *B.*
99 *subtilis* strain 168 (Richter et al., 2011). In the case of the *B. subtilis* LDH, the point mutation
100 was made by site-directed mutagenesis protocol (Ho et al., 1989) with overlap extension PCR
101 and specific primers, followed by a DpnI treatment and transformation to chemically competent
102 *E. coli* DH5α. Some sgRNA were instead made by adding the 20 nucleotide target sequence in
103 the 5' region of an oligonucleotide primer and amplifying all of the way around a previously
104 constructed plasmid to create one linear piece. After purification, PCR products were
105 phosphorylated with T4 polynucleotide kinase and ligated with T4 DNA ligase (NEB) to form
106 circular plasmids. sgRNAs targeting the non-template strand were picked with DNA 2.0's gRNA
107 designer by entering a gene of interest. Potential target sequences with a NGG PAM sequence
108 within the first 500 nucleotides from the start codon were used in a BLAST search against the
109 wild-type PCC 7002 genome to ensure no substantial off target effects. Binding sites of sgRNA
110 can be found in the supplementary material.

111 **1.2.3** ***Measurement of Fluorescence***

112 Cultures (20 mL media A+) were bubbled with air for 24 hours before being diluted in
113 triplicate to OD_{730nm} = 0.5 in 10 mL of media A+. Cultures were bubbled for 24 hours to an
114 OD_{730nm} ~ 1 where 1.5 OD_{730nm}-mLs were spun down at 1,400xg for 10 minutes. Cell lysis with

115 Bugbuster and fluorescence measurements on a Tecan M1000 plate reader were performed as
116 described previously (Markley et al., 2015).

117 **1.2.4** ***Reversibility Test***

118 Cultures (20 mL media A+) were inoculated from fresh plates and bubbled with air for 48
119 hours. Cultures were diluted to ~ 0.1 OD_{730nm}.mL in 20 mL A+ in quadruplicate and 1 μg/mL
120 aTc was added where indicated. A fluorescence measurement of 200 μl of cells was taken in a
121 Tecan M1000 plate reader with a gain of 200. Cultures were spun down at 5000xg for 10 min in
122 a Beckman Coulter Allegra X-15R Centrifuge, washed once with A+ media, and resuspended in
123 the same volume of A+ media to maintain a constant cell density pre- and post-wash.

124 **1.2.5** ***Measurement of Absorbance***

125 Cultures (20 mL media A+) were bubbled with air for 24 hours before being diluted in
126 triplicate to OD_{730nm} = 0.5 in 10 mL of media A+ with aTc added where indicated. Cultures were
127 bubbled for 24 hours with air before an absorbance scan (300-750 nm) was conducted in 96 well
128 plates with a working volume of 200 ul using a Tecan M1000 plate reader.

129 **1.2.6** ***Spot Plating***

130 Cultures (20 mL media A+) were inoculated from plates and grown with 10% CO_2 for 24
131 hours. Cultures were diluted to OD_{730nm} = 0.5 in 20 mL A+, aTc was added where indicated, and
132 bubbled for another 24 hours with 10% CO_2. Samples were suspended at OD_{730nm} = 0.01 as
133 measured by a Genesys 20 spectrophotometer, 10-fold serially diluted in media A+, and spotted
134 onto solid media (7μl/spot).

135 **1.2.7** ***Fluorescence Microscopy***

136 Cultures (20 mL media A+) were inoculated from plates and bubbled with 10% CO_2 for
137 24 hours with aTc where indicated. Cultures were diluted to OD_{730nm} = 0.05 in 20 mL media A+

138 and aTc was added where indicated, and bubbled for another 24 hours with 10% CO_2. Cultures
139 were diluted to OD_{730nm} = 0.05 in 20 mL A+ with aTc and grown with 10% CO_2 for 5 hours
140 before visualizing. Cells (2 µl) were spotted onto 1% (w/v in media A+) agarose pads in a 16-
141 well chamber slide (Nunc™ Lab-Tek™, Scotts Valley, CA), air-dried, and covered with a 0.17
142 mm coverslip. Images were acquired on a Zeiss Axioimager Z2 for GFP (excitation: BP 470/40
143 nm; beam splitter: FT 495 nm; emission: BP 525/50 nm), and brightfield using a 100X oil-
144 immersion objective (NA = 1.3). Images were analyzed with ImageJ (Abràmofff et al., 2005).

145 **1.2.8** ***Lactate Quantification***

146 Cultures (10 mL A+ media A+) were inoculated from plates and bubbled with air for 24
147 hours before being diluted in duplicate to OD_{730nm} = 0.05 in 15 mL of media A+, induced with 1
148 mM IPTG, and bubbled with either air (0.04% CO_2) or high CO_2 (1% CO_2). Temperature was
149 maintained at 38°C and light intensity was approximately 250 umol photons $m^{-2}s^{-1}$. Samples
150 were withdrawn periodically for biomass and lactate measurements, centrifuged, and the
151 supernatant was stored at -20°C until quantification by HPLC (Shimadzu Co., Columbia, MD,
152 USA) equipped with a quaternary pump, autosampler, vacuum degasser, photodiode array and
153 refractive index detector. HPLC separations were performed using an Ultra Aqueous C18
154 column (Restek). The HPLC operating conditions were as follows: mobile phase: 50 mM
155 KH_2PO_4 (pH 2.5 with 1% Acetonitrile), flow rate 0.100 mL/min, column temperature 30°C,
156 photodiode array detector at 210 nm, run time: 10 minutes, injection volume 10 µL. Organic
157 acids were quantified by comparison with peaks generated by known amounts of lactate sodium
158 salt (Sigma) in Media A^+. Dry cell weight concentrations were estimated from OD_{730nm} values
159 using a standard curve created from lyophilized cell pellets (1 OD_{730nm} = 1.409 g/L; R^2=0.9852).
160 Biomass productivity was calculated over the time course using a linear fit. Lactate

161 concentrations for each individual culture are plotted with respect to time and a linear fit was
162 applied.
163

164 **1.3 Results and Discussion**

165 **1.3.1** ***Optimization of YFP Repression with CRISPRi***

166 To demonstrate a functional CRISPRi system in PCC 7002 we integrated three
167 expression cassettes into the chromosome: 1) a fluorescent reporter, 2) an inducible dCas9, and
168 3) a constitutively expressed sgRNA (**Figure 1a**). We constructed a reporter cassette consisting
169 of a heterologous yellow fluorescence protein (EYFP) expressed from a strong constitutive
170 promoter and a gentamicin resistance marker. The resulting cassette was integrated into a neutral
171 site in the genome by replacing the *glpK* pseudogene and selecting transformants on gentamicin.
172 We integrated dCas9 under the control of the anhydrotetracycline (aTc) induction system, EZ3
173 (Zess et al., 2016) into the *acsA* locus using acrylic acid as a counter selection marker
174 (Begemann et al., 2013). The aTc-controlled promoter was critical to construct the system; no
175 transformants were obtained when strains were transformed with a plasmid containing dCas9
176 expressed from the stronger cLac94 IPTG-inducible promoter (Markley et al., 2015). A single
177 guide RNA (sgRNA) was designed to target the non-template strand of YFP at a position ~175
178 nucleotides downstream of the start codon. An expression cassette containing the sgRNA
179 expressed from the strong constitutive promoter, P_{J23119}, was integrated onto the chromosome at
180 neutral site 1 (NS1) with a kanamycin resistance marker (Davies et al., 2014). We measured bulk
181 YFP fluorescence in extracts from these strains and found that the fluorescence levels were
182 unaffected in strains containing only dCas9 or the sgRNA (**Figure 1b**) even when expression of
183 dCas9 was induced with aTc (**Supplemental Figure 1**). In strains containing all three cassettes,

184 YFP fluorescence was reduced to 0.05% of the unregulated control. In the presence of 1 μg/mL
185 aTc (to induce dCas9 expression), YFP fluorescence was further reduced to 0.02% of the control.
186 These data confirm that CRISPRi functions in PCC 7002, however the limited dynamic range of
187 the system limits the utility in many applications.

188 In effort to increase the dynamic range of the CRISPRi system, we hypothesized that
189 decreasing expression of the sgRNA and dCas9 would reduce repression in the absence of aTc.
190 Therefore, we replaced the promoter controlling expression of dCas9 with weaker alternatives,
191 but saw no reduction in repression in the absence of aTc (**Supplemental Figure 2**). Similarly,
192 we changed the promoter strength of the sgRNA from a strong promoter, P_{J23119}, to a medium
193 constitutive promoter, P_{J23108}, a weak constitutive promoter, P_{J23117}, and the same aTc-inducible
194 system (Zess et al., 2016) controlling dCas9 expression (**Figure 2**). We found that decreasing the
195 sgRNA promoter strength reduced the repression of YFP in the uninduced culture (from 7% of
196 the control for P_{J23119}, to 11% for P_{J23108}, and 70% for P_{J23117}), but also in the induced samples
197 (from 3% of the control for P_{J23119}, to 6% for P_{J23108}, and 45% for P_{J23117}). In contrast, expression
198 of the sgRNA under control of the aTc-inducible system (Zess et al., 2016) already controlling
199 dCas9, generated the largest dynamic range. The YFP fluorescence of the uninduced sample was
200 reduced to 46% of the control and the induced sample maintained strong repression of YFP (4%
201 of the control).

202 Due to the required *tet* operator, the resultant sgRNA from the aTc-inducible system has
203 an additional 7 nucleotides at the 5' end. When those same 7 nucleotides were added to the 5' of
204 the sgRNA expressed from P_{J23119} there was no change in YFP repression (**Supplemental**
205 **Figure 3**). This finding suggests that CRISPRi in PCC 7002 may be tolerant to mis-matched

206 extra nucleotides at the 5' end of the sgRNA in contrast to examples in other bacteria where 5'
207 extensions decrease the efficiency of repression (Larson et al., 2013).

208 We hypothesize that minimizing both dCas9 and sgRNA concentrations relieve the
209 unintended repression when dCas9 is uninduced. In other words, very small concentrations of
210 dCas9 may be capable of effectively silencing YFP expression when excess sgRNA is present.
211 Attempts to reduce dCas9 expression using an alternate promoter and by deleting the promoter
212 region were unsuccessful, suggesting that residual or promiscuous expression of dCas9 is
213 sufficient for full repression (**Supplemental Figure 3**).

214 We next sought to improve this tunable repression by decreasing repression of the target
215 gene in the absence of aTc. We hypothesized that reducing the expression level of dCas9 by
216 altering its ribosome binding site (RBS) would meet this goal. Using the RBS Library Calculator
217 (Farasat et al., 2014) we designed five additional RBS with predicted decreasing translation
218 initiation rates (labeled A-F). We achieved a range of repression in the uninduced samples
219 although they did not correspond to the predicted translation initiation rate of dCas9 (**Figure
220 3A**). Altering the dCas9 RBS allowed us to obtain minimal repression without aTc (~30%) while
221 maintaining repression when maximally induced (~90%). In an effort to further optimize the
222 system, we created 5' truncations of the sgRNA as this has been shown to lower repression in
223 other systems ((Qi et al., 2013; Xu et al., 2015). Here, we observed a drastic decrease in
224 repression between 15 nucleotides and 12 nucleotides, but overall dynamic range was not
225 improved relative to the original 20 nucleotide sgRNA system (**Supplemental Figure 4**). We
226 proceeded to characterize the strain with the "F" RBS controlling dCas9 expression (there was
227 no statistical difference in YFP fluorescence between uninduced samples of "C" and "F" RBS).
228 With the uninduced samples normalized to 100%, we were able to titrate YFP fluorescence to

229 13% when fully induced (**Figure 3B**). There is still some repression in the uninduced sample
230 (~30% of no CRISPRi control). Last, we designed sgRNA to target different locations within
231 YFP and found similar results for sgRNA targeting both the template and non-template strands
232 near the beginning of the transcript, with slightly reduced repression when fully induced when
233 targeting the 3' end of the transcript (**Supplemental Figure 5**).
234

235 **1.3.2** ***Reversibility of repression***

236 One advantage of trans-acting regulators is the potential to dynamically regulate
237 expression of target genes between on and off states in the same culture. To examine the
238 potential of the CRISPRi system in this regard, we performed a series of timecourse experiments
239 wherein aTc levels were either added or removed as specific timepoints and YFP fluorescence
240 was monitored. In the absence of aTc, YFP fluorescence increased over time (**Figure 4a**) as
241 expected. When cultures were induced, (**Figure 4b**), YFP fluorescence decreased over time as
242 cells grew (filled in markers). Cultures were centrifuged, washed, and resuspended in fresh
243 media with or without aTc. In the absence of aTc, YFP fluorescence returned, albeit at a slower
244 rate than the completely uninduced cultures (**Figure 4a**). In contrast, induced cultures maintained
245 a lower level of YFP fluorescence for the remainder of the experiment. The increasing
246 fluorescence in the samples without aTc shows that CRISPRi can be turned on and off by the
247 addition or removal of aTc. This tool enables the study of PCC 7002 genes allowing the knock-
248 down of genes to intermediate levels as well as the ability to track physiological changes
249 throughout time when repressing or depressing genes of interest.
250

251 **1.3.3** ***Repression of native genes***

252 We tested whether CRISPRi could be used to rapidly modulate two key processes in
253 photosynthetic organisms: light harvesting and CO_2 fixation. First, we designed a system to
254 target and repress expression of core phycobilisome genes. Phycobilisomes are multi-subunit
255 pigment-protein complexes that function as light harvesting antennae in cyanobacteria by
256 transferring light energy to the photosynthetic reaction centers, photosystem I and photosystem II
257 (Grossman et al., 1993; Liu et al., 2013). The phycobilisome is comprised of an allophycocyanin
258 core attached to peripheral rods assembled from disks of phycocyanin separated by linker
259 polypeptides. In PCC 7002, the *cpcBACDEF* operon encodes the major components of the
260 peripheral rods including the α- (CpcA) and β-subunits (CpcB) of phycocyanin, the rod linkers
261 (CpcC and CpcD), and two proteins involved in attachment of the phycocyanobilin pigment to
262 the α-phycocyanin subunit (CpcE and CpcF) (Bryant et al., 1990). Since reduction of antennae
263 size has been a promising strategy for increasing light penetration into high-density
264 cyanobacterial cultures (Kirst et al., 2014), we designed an sgRNA to target *cpcB* and inhibit
265 transcription of the entire *cpcBACDEF* operon. Others have shown that when the rod proteins are
266 deleted there is a decrease in absorbance corresponding to phycocyanin at 635 nm (Lea-Smith et
267 al., 2014). We hypothesized that repression of this operon would lead to an observable
268 phenotype – a decrease in absorbance in the phycocyanin absorbance peak at 635 nm relative to
269 the 680 nm chlorophyll peak (Alvey et al., 2011).
270 Following induction of the dCas9 in the strain containing the sgRNA targeting cpcB, we
271 observed a significant decrease in the absorption peak at 635 nm, indicating a severe depletion
272 phycobilisomes compare to the control strain (**Figure 5**). Dynamic modulation of the light-
273 harvesting complex, as shown by our CRISPRi strategy, could be useful for improving growth at
274 high cell density (Kirst et al., 2014) without affecting initial biomass scale-up that could be

275 reduced in a mutant lacking phycobilisomes. Moreover, CRISPRi may be advantageous
276 compared to making traditional deletion strains due to challenges in isolating homoplasmic
277 strains, especially when the deletions have impacts on cellular fitness; for example, we were
278 unable to generate a fully segregated *cpcB* strain, but could phenocopy this trait using CRISPRi
279 (Lea-Smith et al., 2014).

280 Next, we tested whether essential genes could be repressed using the CRISPRi system. In
281 all free-living cyanobacteria and some chemoautotrophic bacteria, CO_2-fixation occurs within
282 the carboxysome, a bacterial microcompartment comprised entirely of protein that functions as a
283 primitive organelle (Yeates et al., 2008). The carboxysome is comprised of a thin, multi-subunit,
284 semi-permeable protein shell that encapsulates the key carboxylase, Ribulose 1,5-bisphosphate
285 carboxylase/oxygenase (RuBisCO), and carbonic anhydrase in a unique sub-cellular environment
286 (Cameron et al., 2013). The carboxysome functions at a key component of the cyanobacterial
287 CO_2 concentrating mechanism, which increases the local concentration of CO_2 at the site of CO_2-
288 fixation within the carboxysome.

289 While carboxysomes are essential for growth in atmospheric CO_2 levels (0.04%), models
290 predict that increased productivities could be achieved using un-encapsulated RuBisCO during
291 growth in industrial conditions that provide high-CO_2 (Clark et al., 2014), including flue gas
292 from a coal-fired power plant (~15%). In PCC 7002, the *ccm* operon (*ccmK*$_1$*K*$_2$*LMN*) encodes the
293 hexameric shell proteins (CcmK1 and CcmK2), the pentameric vertex protein (CcmL), and the
294 interior scaffold proteins (CcmM and CcmN) that make up the major structural subunits of the
295 carboxysome (Ludwig et al., 2000). We designed a sgRNA to target *ccmK1*, with the prediction
296 that inhibition of the *ccm* operon would inhibit formation of the carboxysome, resulting in a

297 strain with a high-CO_2 requiring (HCR) phenotype and RuBisCO localized to the cytoplasm
298 instead of being encapsulated within the carboxysome shell (Cameron et al., 2013).

299 We tested this hypothesis by comparing growth of a mutant lacking the $ccmK_1K_2LMN$
300 (Δ*ccm*) operon with a CRISPRi strain targeting *ccmK* and a control containing the sgRNA but no
301 dCas9 on solid medium in air or elevated CO_2. Cultures were grown overnight in elevated
302 (~10%) CO_2, diluted and re-grown in the absence or presence of aTc, and then serially diluted
303 and spot-plated onto solid media placed in either elevated CO_2 or air. As expected, the control
304 strain with only the sgRNA could grow in both high CO_2 and air whereas Δ*ccm* could only grow
305 with high CO_2 (**Figure 6a**). While the undinduced CRISPRi strain could grow in both air and
306 high CO_2, the presence of aTc in the pre-culture resulted in a HCR phenotype, indicating
307 functional repression of carboxysome assembly.

308 To investigate whether assembly of the carboxysome was being inhibited, we expressed a
309 C-terminal sfGFP fusion of the RuBisCO large subunit, *rbcL*, as a visual marker in the test
310 strains. The uninduced CRISPRi strain resembled wild type (WT) with discrete fluorescent foci,
311 indicating the presence of carboxysomes (**Figure 6b**), whereas the Δ*ccm* strain exhibited diffuse
312 fluorescence. The induced strain resembled an intermediate between the WT and Δ*ccm* strains,
313 with polar fluorescent foci and diffuse GFP signal. We suspect that the foci represent an
314 intermediate in carboxysome assembly pathway initiated by molecular scaffolding of RuBisCO
315 by CcmM; this intermediate is the result of insufficient shell proteins and exhibits an HCR
316 phenotype (Cameron et al., 2013). Based on the HCR and visual phenotypes of the induced
317 strains, we hypothesize that repression of the entire *ccm* operon is incomplete, but sufficient to
318 inhibit assembly of functional carboxysomes.

319

320 **1.3.4** ***Improving lactate production with CRISPRi***

321 Trans-acting regulators also have the potential to implement elegant regulatory strategies in
322 cell factories. Here, we used CRISPRi to increase the production of lactate. Lactate is an
323 excellent model compound for studying metabolic engineering strategies in photoautotrophic
324 bacteria because its synthesis pathway is short (one step removed from central metabolism) and
325 consumes cofactors within the range generated by photosynthesis during linear electron transport
326 (Oliver and Atsumi, 2014). PCC 7002 has an endogenous lactate dehydrogenase, but autotrophic
327 cultures produce negligible levels of lactate under optimal laboratory conditions. Therefore we
328 expressed an engineered version of the *Bacillus subtilis* lactate dehydrogenase (LDH) with a
329 previously described single amino acid substitution (Richter et al., 2011), that switches the
330 cofactor requirement of the enzyme from NADH to NADPH during conversion of pyruvate to
331 lactate. An expression cassette with LDH under the control of the $P_{cLac143}$ IPTG-inducible system
332 (Markley et al., 2015) was integrated into the chromosome at the *glpK* pseudogene locus along
333 with a gentamicin resistance marker to generate the base strain, CC133.

334 In conjunction with elevated expression of optimized LDH, we predicted that limited flux
335 from carbon fixation to pyruvate would limit lactate production. Enhanced pyruvate excretion
336 and attenuation of phycobilisome and chlorophyll *a* degradation has been demonstrated under
337 nitrogen starvation in several strains of cyanobacteria when glycogen synthesis is disrupted
338 (Davies et al., 2014, Gründel et al., 2012; Hickman et al., 2013). We hypothesized that moderate
339 reduction of glutamine synthetase I (*glnA*) expression would slow the rate of nitrogen
340 assimilation through the GS-GOGAT pathway (Muro-Pastor and Florencio, 2003). Slowing the
341 rate of nitrogen assimilation would thereby lead to increased intracellular accumulation of α-
342 ketoglutarate, activating the global nitrogen transcriptional activator, NtcA, a transcription factor

343 from the cyclic AMP receptor protein class (Herrero et al., 2001). This in turn, modulates a suite
344 of metabolic processes related to glycogen degradation and glycolysis, thereby enhancing the
345 flux of fixed carbon to pyruvate (Osanai et al., 2006). However, severe nitrogen limitation will
346 further increase intracellular α-ketoglutarate levels, facilitating complex formation between
347 active NtcA and regulatory factor, PipX (Espinosa et al., 2006), negatively affecting de-novo
348 protein synthesis and overall photosynthesis rates (Espinosa et al., 2014; Krasikov et al., 2012).
349 Therefore, we chose to titrate the degree of repression of *glnA* using the CRISPRi approached
350 described above (**Figure 7**). As additional controls, a sgRNA targeting YFP or *glnA* were
351 integrated in the NS1 locus under the control of the aTc-inducible system, to create strains
352 CC142 and CC130, respectively. dCas9 with the F RBS was integrated in CC130 to create strain
353 CC131.

354 Interestingly, we found enhanced lactate production, as well as delayed phycobilisome and
355 chlorophyll *a* degradation (**Supplemental Figure 6)**, in both strains harboring the *glnA* sgRNA
356 when compared to the control strains in 1% CO_2 (**Figure 8b**) or air (**Figure 8d**). Integration of
357 *glnA* sgRNA improved production rates from 0.079 ± 0.001 mM/hr in CC130 to 0.092 ± 0.001
358 mM/hr in CC131, both significantly improved to control strains CC133 and CC142, with rates of
359 0.045 ± 0.002 mM/hr and 0.029 ± 0.002 mM/hr, respectively, indicating that this effect was *glnA*
360 sgRNA specific. This *glnA* sgRNA mediated effect may be due to a basal level of repression
361 through cross-talk with the native CRISPR-Cas systems, unlike the other native genes targeted
362 previously, given the difference in transcript abundance between *glnA, ccmK,* and *cpcB* (Ludwig,
363 2012; Ludwig, 2011). There was no apparent difference in growth rate between the any of the
364 producing strains in 1% CO_2 (**Figure 8a**) or air (**Figure 8c**). This suggests that *glnA* repression or
365 LDH expression did not severely impact amino acid synthesis or biomass generation. Given the

366 significant increase in carbon products, the data also suggests that carbon storage polymers were
367 rerouted to product, or carbon fixation rates were enhanced in CC131, analogous to cells
368 engineered to secrete sucrose (Ducat et al., 2012).

369 **1.4 Conclusions**

370 CRISPRi provides a straightforward method to repress native genes of interest. We
371 showed the functionality of this system genes in the fast-growing cyanobacterium,
372 *Synechococcus sp.* strain PCC 7002 by repressing heterologous YFP and three native genes. We
373 found that leaky expression of the system components was sufficient to achieve strong repression
374 of its target and therefore the system required significant optimization to achieve the desired
375 dynamic range of repression. We found that expression of dCas9 and sgRNA from aTc-inducible
376 promoters allowed for a functional range of repression compared to expressing the sgRNA from
377 constitutive promoters of various strengths. Leakyness of the system was further improved by
378 screening RBS variants to modulate the levels of dCas9. The resulting platform enables the rapid
379 and tritratable repression of gene expression in an industrial relevant cyanobacterium. As a proof
380 of concept, we demonstrated repression of a YFP reporter construct, the native ligh-harvesting
381 phycobilisome (*cpcBACDEF*), and the carboxysome ($ccmK_1K_2LMN$). Repression was titratable
382 and reversible allowing genes to be turned off and on via the addition or removal of aTc.

383 CRISPRi is a synthetic biology tool that facilitates both the study of natural physiology as
384 well as engineering of strains for applied purposes. Current methods for increasing titers of
385 products include deleting genes in competing pathways, but maximum yields might be achieved
386 by lowering expression to some intermediate level. With CRISPRi technology we can lower
387 expression of essential genes, decrease but not abolish flux towards competing products, and
388 manipulate cellular processes to varying degrees. Here, we improved lactate production from

389 0.045 ± 0.002 mM lactate/hr to 0.092 ± 0.001 mM lactate/hr by repressing glutamine synthetase,
390 *glnA*. This was achieved without reducing autotrophic growth rates or mutating chromosomal
391 genes. We anticipate that this approach could be similarly effective in increasing photosynthetic
392 flux to other chemical products, especially those derived from pyruvate.

393 **1.5 Supporting Information**

394 Supporting information is attached and is composed of 6 figures highlighting
395 supplementary aspects of CRISPRi in PCC 7002.

396 **1.6 Author Information**

397 Department of Chemical and Biological Engineering, University of Wisconsin-Madison
398 1415 Engineering Dr., Madison, WI 53706, United States.
399

400 **1.7 Acknowledgements**

401 This work was supported by the US Department of Energy through grant DE-SC0010329; the
402 National Science Foundation grant EFRI-1240268; and the William F. Vilas Trust. GCG and
403 TCK are recipients of NIH Biotechnology Training Fellowships (NIGMS - 5 T32 GM08349).

1.8 References

1. Abràmofff, M.D., Magalhães, P.J., Ram, S.J., 2005. Image processing with ImageJ Part II. Biophotonics Int. 11, 36–43. doi:10.1117/1.3589100
2. Alper, H., Fischer, C., Nevoigt, E., Stephanopoulos, G., 2005. Tuning genetic control through promoter engineering. Proc. Natl. Acad. Sci. 102, 12678–12683. doi:10.1073/pnas.0504604102
3. Alvey, R.M., Biswas, A., Schluchter, W.M., Bryant, D.A., 2011. Effects of Modified Phycobilin Biosynthesis in the Cyanobacterium Synechococcus sp. Strain PCC 7002. J. Bacteriol. 193, 1663–1671. doi:10.1128/JB.01392-10
4. Begemann, M.B., Zess, E.K., Walters, E.M., Schmitt, E.F., Markley, A.L., Pfleger, B.F., 2013. An organic acid based counter selection system for cyanobacteria. PLoS One 8, e76594. doi:10.1371/journal.pone.0076594
5. Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F., Marraffini, L.A., 2013. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic Acids Res. 41, 7429–37. doi:10.1093/nar/gkt520
6. Bryant, D.A., Zhou, J., Gasparich, G.E., Lorimier, D., Guglielmi, G., Stirewalt, V.L., 1990. G. Drews et al. (eds.), 129–141.
7. Cameron, J.C., Wilson, S.C., Bernstein, S.L., Kerfeld, C.A., 2013. Biogenesis of a bacterial organelle: the carboxysome assembly pathway. Cell 155, 1131–40. doi:10.1016/j.cell.2013.10.044
8. Chen, Y.-J., Liu, P., Nielsen, A.A.K., Brophy, J.A.N., Clancy, K., Peterson, T., Voigt, C.A., 2013. Characterization of 582 natural and synthetic terminators and quantification of their design constraints. Nat. Methods 10, 659–64. doi:10.1038/nmeth.2515
9. Chubukov, V., Gerosa, L., Kochanowski, K., Sauer, U., 2014. Coordination of microbial metabolism. Nat. Rev. Microbiol. 12, 327–340. doi:10.1038/nrmicro3238
10. Clark, R.L., Cameron, J.C., Root, T.W., Pfleger, B.F., 2014. Insights into the industrial growth of cyanobacteria from a model of the carbon-concentrating mechanism. AIChE J. 60, 1269–1277. doi:10.1002/aic.14310
11. Cleto, S., Jensen, J.V., Wendisch, V.F., Lu, T.K., 2016. Corynebacterium glutamicum Metabolic Engineering with CRISPR Interference (CRISPRi). ACS Synth. Biol. 5, 375–85. doi:10.1021/acssynbio.5b00216
12. Copeland, M.F., Politz, M.C., Pfleger, B.F., 2014. Application of TALEs, CRISPR/Cas and sRNAs as trans-acting regulators in prokaryotes. Curr. Opin. Biotechnol. 29, 46–54. doi:10.1016/j.copbio.2014.02.010
13. Davies, F.K., Work, V.H., Beliaev, A.S., Posewitz, M.C., 2014. Engineering Limonene and Bisabolene Production in Wild Type and a Glycogen-Deficient Mutant of Synechococcus sp. PCC 7002. Front. Bioeng. Biotechnol. 2, 1–11. doi:10.3389/fbioe.2014.00021
14. Devroe, E.J., Kosuri, S., Berry, D.A., Afeyan, N.B., Skraly, F.A., Robertson, D.E., Green, B., Ridley, C.P., 2010. Hyperphotosynthetic organisms.
15. Ducat, D.C., Avelar-Rivas, J.A., Way, J.C., Silver, P.A., 2012. Rerouting carbon flux to enhance photosynthetic productivity. Appl. Environ. Microbiol. 78, 2660–8. doi:10.1128/AEM.07901-11
16. Espah Borujeni, A., Channarasappa, A.S., Salis, H.M., 2014. Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and

sliding at upstream standby sites. Nucleic Acids Res. 42, 2646–59. doi:10.1093/nar/gkt1139
17. Espinosa, J., Forchhammer, K., Burillo, S., Contreras, A., 2006. Interaction network in cyanobacterial nitrogen regulation: PipX, a protein that interacts in a 2-oxoglutarate dependent manner with PII and NtcA. Mol. Microbiol. 61, 457–69. doi:10.1111/j.1365-2958.2006.05231.x
18. Espinosa, J., Rodríguez-Mateos, F., Salinas, P., Lanza, V.F., Dixon, R., de la Cruz, F., Contreras, A., 2014. PipX, the coactivator of NtcA, is a global regulator in cyanobacteria. Proc. Natl. Acad. Sci. U. S. A. 111, E2423–30. doi:10.1073/pnas.1404097111
19. Farasat, I., Kushwaha, M., Collens, J., Easterbrook, M., Guido, M., Salis, H.M., 2014. Efficient search, mapping, and optimization of multi-protein genetic systems in diverse bacteria. Mol. Syst. Biol. 10, 731. doi:10.15252/msb.20134955
20. Freed, E.F., Winkler, J.D., Weiss, S.J., Garst, A.D., Mutalik, V.K., Arkin, A.P., Knight, R., Gill, R.T., 2015. Genome-Wide Tuning of Protein Expression Levels to Rapidly Engineer Microbial Traits.
21. Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343–5. doi:10.1038/nmeth.1318
22. Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., Stern-Ginossar, N., Brandman, O., Whitehead, E.H., Doudna, J.A., Lim, W.A., Weissman, J.S., Qi, L.S., 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154, 442–51. doi:10.1016/j.cell.2013.06.044
23. Grossman, A.R., Schaefer, M.R., Chiang, G.G., Collier, J.L., 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. Microbiol. Rev. 57, 725–49.
24. Gründel, M., Scheunemann, R., Lockau, W., Zilliges, Y., 2012. Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium Synechocystis sp. PCC 6803. Microbiology 158, 3032–43. doi:10.1099/mic.0.062950-0
25. Hara, K.Y., Araki, M., Okai, N., Wakai, S., Hasunuma, T., Kondo, A., 2014. Development of bio-based fine chemical production through synthetic bioengineering. Microb. Cell Fact. 13, 173. doi:10.1186/s12934-014-0173-5
26. Herrero, A., Muro-Pastor, A.M., Flores, E., 2001. Nitrogen control in cyanobacteria. J. Bacteriol. 183, 411–25.
27. Hickman, J.W., Kotovic, K.M., Miller, C., Warrener, P., Kaiser, B., Jurista, T., Budde, M., Cross, F., Roberts, J.M., Carleton, M., 2013. Glycogen synthesis is a required component of the nitrogen stress response in Synechococcus elongatus PCC 7942. Algal Res. 2, 98–106. doi:10.1016/j.algal.2013.01.008
28. Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., Pease, L.R., 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77, 51–59. doi:10.1016/0378-1119(89)90358-2
29. Keasling, J.D., 2012. Synthetic biology and the development of tools for metabolic engineering. Metab. Eng. 14, 189–195. doi:10.1016/j.ymben.2012.01.004
30. Kirst, H., Formighieri, C., Melis, A., 2014. Maximizing photosynthetic efficiency and culture productivity in cyanobacteria upon minimizing the phycobilisome light-harvesting antenna size. Biochim. Biophys. Acta - Bioenerg.

doi:10.1016/j.bbabio.2014.07.009

31. Kleinstiver, B.P., Prew, M.S., Tsai, S.Q., Topkar, V. V., Nguyen, N.T., Zheng, Z., Gonzales, A.P.W., Li, Z., Peterson, R.T., Yeh, J.-R.J., Aryee, M.J., Joung, J.K., 2015. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 523, 481–485. doi:10.1038/nature14592
32. Krasikov, V., Aguirre von Wobeser, E., Dekker, H.L., Huisman, J., Matthijs, H.C.P., 2012. Time-series resolution of gradual nitrogen starvation and its impact on photosynthesis in the cyanobacterium Synechocystis PCC 6803. Physiol. Plant. 145, 426–39. doi:10.1111/j.1399-3054.2012.01585.x
33. Larson, M.H., Gilbert, L.A., Wang, X., Lim, W.A., Weissman, J.S., Qi, L.S., 2013. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat. Protoc. 8, 2180–96. doi:10.1038/nprot.2013.132
34. Lea-Smith, D.J., Bombelli, P., Dennis, J.S., Scott, S.A., Smith, A.G., Howe, C.J., 2014. Phycobilisome-Deficient Strains of Synechocystis sp. PCC 6803 Have Reduced Size and Require Carbon-Limiting Conditions to Exhibit Enhanced Productivity. Plant Physiol. 165, 705–714. doi:10.1104/pp.114.237206
35. Liao, J.C., Mi, L., Pontrelli, S., Luo, S., 2016. Fuelling the future: microbial engineering for the production of sustainable biofuels. Nat. Rev. Microbiol. doi:10.1038/nrmicro.2016.32
36. Liu, H., Zhang, H., Niedzwiedzki, D.M., Prado, M., He, G., Gross, M.L., Blankenship, R.E., 2013. Phycobilisomes supply excitations to both photosystems in a megacomplex in cyanobacteria. Science 342, 1104–7. doi:10.1126/science.1242321
37. Ludwig, M., Bryant, D.A., 2012. Acclimation of the Global Transcriptome of the Cyanobacterium Synechococcus sp. Strain PCC 7002 to Nutrient Limitations and Different Nitrogen Sources. Front. Microbiol. 3, 145. doi:10.3389/fmicb.2012.00145
38. Ludwig, M., Bryant, D.A., 2011. Transcription Profiling of the Model Cyanobacterium Synechococcus sp. Strain PCC 7002 by Next-Gen (SOLiD^{TM}) Sequencing of cDNA. Front. Microbiol. 2, 41. doi:10.3389/fmicb.2011.00041
39. Ludwig, M., Sultemeyer, D., Price, G.D., 2000. Isolation of ccmKLMN genes from the marine cyanobacterium, Synechococcus sp. PCC7002 (Cyanobacteria), and evidence that CcmM is essential for carboxysome assembly. J. Phycol. 36, 1109–1118. doi:10.1046/j.1529-8817.2000.00028.x
40. Lynch, S.A., Gill, R.T., 2012. Synthetic biology: New strategies for directing design. Metab. Eng. 14, 205–211. doi:10.1016/j.ymben.2011.12.007
41. Makarova, K.S., Wolf, Y.I., Alkhnbashi, O.S., Costa, F., Shah, S.A., Saunders, S.J., Barrangou, R., Brouns, S.J.J., Charpentier, E., Haft, D.H., Horvath, P., Moineau, S., Mojica, F.J.M., Terns, R.M., Terns, M.P., White, M.F., Yakunin, A.F., Garrett, R.A., van der Oost, J., Backofen, R., Koonin, E. V., 2015. An updated evolutionary classification of CRISPR-Cas systems. Nat. Rev. Microbiol. 13, 722–736. doi:10.1038/nrmicro3569
42. Markley, A.L., Begemann, M.B., Clarke, R.E., Gordon, G.C., Pfleger, B.F., 2015. A synthetic biology toolbox for controlling gene expression in the cyanobacterium Synechococcus sp. PCC 7002. ACS Synth. Biol. 4, 595–603. doi:10.1021/sb500260k
43. Muro-Pastor, M.I., Florencio, F.J., 2003. Regulation of ammonium assimilation in cyanobacteria. Plant Physiol. Biochem. 41, 595–603. doi:10.1016/S0981-9428(03)00066-4
44. Oliver, J.W.K., Atsumi, S., 2014. Metabolic design for cyanobacterial chemical

synthesis. Photosynth. Res. 120, 249–61. doi:10.1007/s11120-014-9997-4
45. Osanai, T., Imamura, S., Asayama, M., Shirai, M., Suzuki, I., Murata, N., Tanaka, K., 2006. Nitrogen induction of sugar catabolic gene expression in Synechocystis sp. PCC 6803. DNA Res. 13, 185–95. doi:10.1093/dnares/dsl010
46. Pfleger, B.F., Pitera, D.J., Smolke, C.D., Keasling, J.D., 2006. Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. Nat. Biotechnol. 24, 1027–32. doi:10.1038/nbt1226
47. Pitera, D.J., Paddon, C.J., Newman, J.D., Keasling, J.D., 2007. Balancing a heterologous mevalonate pathway for improved isoprenoid production in Escherichia coli. Metab. Eng. 9, 193–207. doi:10.1016/j.ymben.2006.11.002
48. Puigbò, P., Guzmán, E., Romeu, A., Garcia-Vallvé, S., 2007. OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. Nucleic Acids Res. 35, W126–31. doi:10.1093/nar/gkm219
49. Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., Lim, W.A., 2013. Repurposing CRISPR as an RNA-γuided platform for sequence-specific control of gene expression. Cell 152, 1173–1183. doi:10.1016/j.cell.2013.02.022
50. Reppas, N.B., Ridley, C.P., 2011. Methods and compositions for the recombinant biosynthesis of n-alkanes.
51. Richter, N., Zienert, A., Hummel, W., 2011. A single-point mutation enables lactate dehydrogenase from Bacillus subtilis to utilize NAD+ and NADP+ as cofactor. Eng. Life Sci. 11, 26–36. doi:10.1002/elsc.201000151
52. Salis, H.M., Mirsky, E. a, Voigt, C. a, 2010. Automated Design of Synthetic Ribosome Binding Sites to Precisely Control Protein Expression. Nat Biotechnol 27, 946–950. doi:10.1038/nbt.1568.Automated
53. Smanski, M.J., Zhou, H., Claesen, J., Shen, B., Fischbach, M.A., Voigt, C.A., 2016. Synthetic biology to access and expand nature's chemical diversity. Nat. Rev. Microbiol. 14, 135–49. doi:10.1038/nrmicro.2015.24
54. Stanton, B.C., Nielsen, A.A.K., Tamsir, A., Clancy, K., Peterson, T., Voigt, C.A., 2013. Genomic mining of prokaryotic repressors for orthogonal logic gates. Nat. Chem. Biol. 10, 99–105. doi:10.1038/nchembio.1411
55. Stevens, S.E., Patterson, C.O.P., Myers, J., 1973. THE PRODUCTION OF HYDROGEN PEROXIDE BY BLUE-GREEN ALGAE: A SURVEY. J. Phycol. 9, 427–430. doi:10.1111/j.1529-8817.1973.tb04116.x
56. Wu, J., Du, G., Chen, J., Zhou, J., 2015. Enhancing flavonoid production by systematically tuning the central metabolic pathways based on a CRISPR interference system in Escherichia coli. Sci. Rep. 5, 13477. doi:10.1038/srep13477
57. Xu, H., Xiao, T., Chen, C.-H., Li, W., Meyer, C.A., Wu, Q., Wu, D., Cong, L., Zhang, F., Liu, J.S., Brown, M., Liu, X.S., 2015. Sequence determinants of improved CRISPR sgRNA design. Genome Res. 25, 1147–1157. doi:10.1101/gr.191452.115
58. Xu, P., Li, L., Zhang, F., Stephanopoulos, G., Koffas, M., 2014. Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. Proc. Natl. Acad. Sci. U. S. A. 111, 11299–304. doi:10.1073/pnas.1406401111
59. Yao, L., Cengic, I., Anfelt, J., Hudson, E.P., 2015. Multiple gene repression in cyanobacteria using CRISPRi. ACS Synth. Biol. doi:10.1021/acssynbio.5b00264
60. Yeates, T.O., Kerfeld, C.A., Heinhorst, S., Cannon, G.C., Jessup, M., 2008. Todd O. Yeates, Cheryl A. Kerfeld, Sabine Heinhorst, Gordon C. Cannon & Jessup M. Shively.

Nat. microviol Rev. 681–691.
61. Zess, E.K., Begemann, M.B., Pfleger, B.F., 2016. Construction of new synthetic biology tools for the control of gene expression in the cyanobacterium Synechococcus sp. strain PCC 7002. Biotechnol. Bioeng. 113, 424–32. doi:10.1002/bit.25713
62. Zhang, F., Ouellet, M., Batth, T.S., Adams, P.D., Petzold, C.J., Mukhopadhyay, A., Keasling, J.D., 2012. Enhancing fatty acid production by the expression of the regulatory transcription factor FadR. Metab. Eng. 14, 653–60. doi:10.1016/j.ymben.2012.08.009

Figure Captions:

Figure 1: Repression of YFP reporter with CRISPRi. (a) Cartoon illustrating how the dCas9-sgRNA complex interferes with RNA polymerase, thereby repressing transcription. (b) Schematic of the three cassettes used to implement CRISPRi in PCC 7002. (c) YFP fluorescence of PCC 7002 extracts containing dCas9 expressed from the aTc-inducible promoter and sgRNA targeting YFP from constitutive P_{J23119}. Repression requires the presence of both dCas9 and sgRNA but is not dependent on the presence of the inducer, aTc. Induction of dCas9 caused a significant decrease in YFP fluorescence ($p = 0.0008$, unpaired two-tailed t-test with equal standard deviation). Numbers above each bar report the percent fluorescence relative to the YFP only strain. Error bars represent standard deviation of fluorescence measured from four biological replicates.

Figure 2: The dynamic range of the system is improved when both dCas9 and sgRNA are controlled by EZ3 *tet* promoters. Bars show YFP fluorescence of extracts containing dCas9 expressed from the aTc-inducible promoter and sgRNA targeting YFP from constitutive promoters of varying strength and an aTc-inducible promoter. Promoter strengths were previously characterized in PCC 7002 (Markley et al., 2015). The percent fluorescence relative to the YFP only strain is displayed above the bar. Averages of strains grown in quadruplicate are shown (error bars show standard deviation, NM# indicates not measured).

Figure 3: Decreasing RBS strength of dCas9 maximizes the dynamic range and enables titration of repression. (a) Bars show YFP fluorescence of cultures with different ribosome binding sites (RBS) controlling translation of dCas9. RBS' were designed to be in decreasing strength, A to F. A one-way ANOVA showed statistical significance between uninduced samples ($p < 0.0001$), but not when C and F RBS were removed. There was no statistical difference

between uninduced samples of C and F RBS ($p = 0.115$ unpaired two-tailed t-test with equal standard deviation). Data are the average of biological quadruplicates and error bars show standard deviation. (b) Titration of strain with F RBS with increasing concentrations of aTc. Averages of strains grown in triplicate are shown with the uninduced sample normalized to 100% (error bars show standard deviation).

Figure 4: CRISPRi repression can be reversed. (a) YFP fluorescence increases over time in cultures grown without aTc. (b) YFP fluorescence increases over time before the addition of aTc (marked with arrow), where YFP fluorescence begins to decrease. Upon removal of aTc by centrifugation and washing, YFP fluorescence begins to increase again in cells without aTc (open boxes), but is maintained at a lower level when aTc was re-added (dark diamonds). Experiments were performed in quadruplicate with the average fluorescence values marked and the standard deviation shown as the errors bars. Data for panels A & B were collected at the same time using the same detection settings.

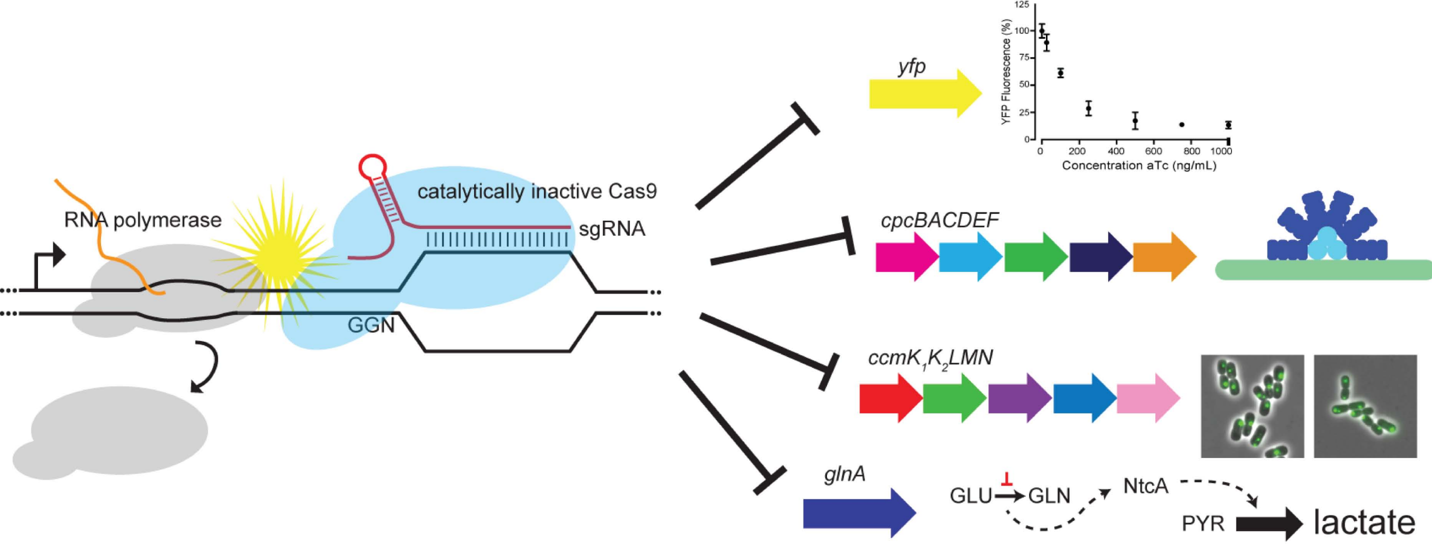
Figure 5: Tunable repression of the phycobilisome operon *cpcBACDEF*. A portion of the absorption spectra of PCC 7002 (CC86) cultures induced with different levels of aTc shows a decrease in the peak at 635 nm corresponding to the phycocyanin maximum absorbance peak. Average spectra are shown and were normalized to $OD_{730nm} = 0.4$ (standard deviations displayed with shading). The inset shows the absorbance at 635 nm with different concentrations of aTc.

Figure 6: Repression of the *ccm* operon. (a) Spot plates of control and *ccmK* repression strains grown in enriched CO_2 and ambient CO_2. Liquid cultures were grown with or without aTc with ~10% CO_2 and normalized before spot-plating on solid media (without aTc). Δ*ccm* is able to grow with enriched CO_2 but is unable to grow with ambient CO_2 The uninduced *ccmK*

repression strain could survive in air but those induced could not. (b) RbcL-sfGFP was used to visualize carboxysomes in control and *ccmK* repression strains.

Figure 7: Schematic of proposed mechanism for increased lactate production. Moderate accumulation of α-ketoglutarate by *glnA* repression leads to NtcA activation and subsequent activation of catabolic genes.

Figure 8: Repression of glutamine synthetase improves lactate production. Growth (a) and (b) lactate production of strains grown in 1% CO_2. Growth (c) and (d) lactate production of strains grown in air. Averages of strains grown in duplicate are shown (error bars show standard deviation).



RNA polymerase
catalytically inactive Cas9
sgRNA
GGN
yfp
YFP Fluorescence (%)
Concentration aTc (ng/mL)
cpcBACDEF
ccmK1K2LMN
glnA
GLU
GLN
NtcA
PYR
lactate