

# 1 **Title:** Evolution of Carbon Isotope Fractionation in Cyanobacteria

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28

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31

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## 33 **Abstract**

34 The history of Earth's carbon cycle reflects trends in atmospheric composition  
35 convolved with the evolution of photosynthesis. Fortunately, key parts of the carbon  
36 cycle have been recorded in the carbon isotope ratios of sedimentary rocks. The  
37 dominant model used to interpret this record as a proxy for ancient atmospheric CO<sub>2</sub> is  
38 based on carbon isotope fractionations of modern photoautotrophs, and longstanding  
39 questions remain about how their evolution might have impacted the record. We  
40 interrogated the intersection of environment and evolution by measuring both biomass  
41 ( $\epsilon_p$ ) and enzymatic ( $\epsilon_{\text{Rubisco}}$ ) carbon isotope fractionations of a cyanobacterial strain  
42 (*Synechococcus elongatus* PCC 7942) solely expressing a putative ancestral Form 1B  
43 rubisco dating to  $\gg 1$  Ga. This strain, nicknamed ANC, grows in ambient pCO<sub>2</sub> and

44 displays larger  $\epsilon_p$  values than WT, despite having a much smaller  $\epsilon_{\text{Rubisco}}$  ( $17.23 \pm$   
45  $0.61\%$  vs.  $25.18 \pm 0.31\%$ , respectively). Measuring both enzymatic and biomass  
46 fractionation revealed a surprising result—ANC  $\epsilon_p$  exceeded ANC  $\epsilon_{\text{Rubisco}}$  in all  
47 conditions tested, violating prevailing models of cyanobacterial carbon isotope  
48 fractionation. However, these models were corrected by accounting for cyanobacterial  
49 physiology, notably the  $\text{CO}_2$  concentrating mechanism (CCM). Our modified model  
50 indicated that powered inorganic carbon uptake systems contribute to  $\epsilon_p$ , and this effect  
51 is exacerbated in ANC. These data suggested that understanding the evolution of both  
52 the CCM and rubisco is critical for interpreting the carbon isotope record, and that large  
53 fluctuations in the record may reflect the evolving efficiency of carbon fixing  
54 metabolisms as well as changes in atmospheric  $\text{CO}_2$ .

55

## 56 **Significance Statement**

57 Fossils record the past, but so too do modern organisms via comparative biology.  
58 Rubisco is the most abundant protein on the planet, and is a keystone enzyme in  
59 photosynthesis. To understand how this process has co-evolved with changes in the  
60 abundance of atmospheric carbon dioxide, we reconstructed an ancestral rubisco (>one  
61 billion years old), and generated a mutant Cyanobacteria strain that must rely on this  
62 ancient protein for growth. By measuring the carbon isotope fractionation *in vitro* and *in*  
63 *vivo* we found that prevailing models of carbon flow in Cyanobacteria could be corrected  
64 by accounting for known aspects of cyanobacterial physiology. This highlighted the  
65 value of considering both evolution and physiology for comparative biological  
66 approaches to understanding Earth history.

67

## 68 **Main Text**

### 69 Introduction

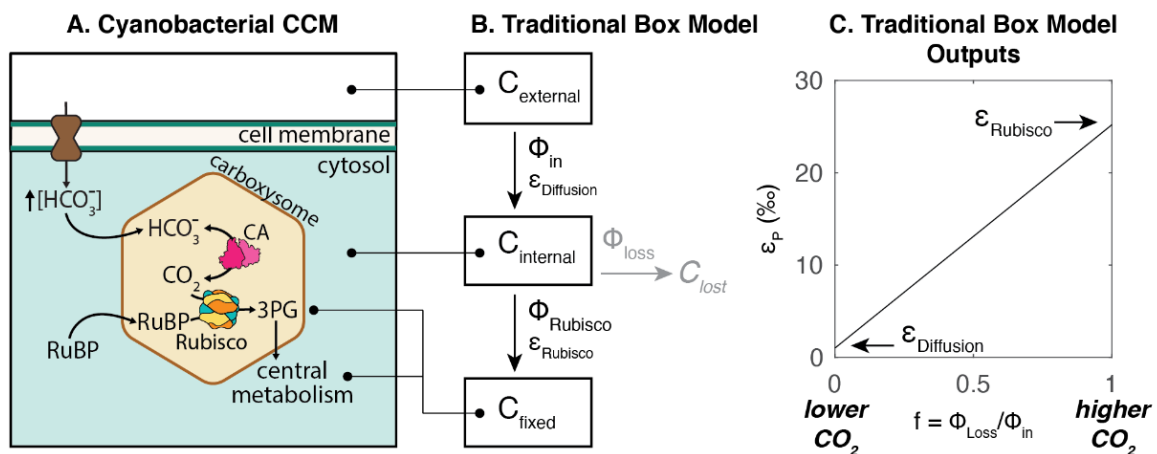
70 Throughout Earth's history, autotrophic cells have had to take external, oxidized  
71 inorganic carbon ( $\text{C}_i$ ) and 'fix' it into reduced organic carbon ( $\text{C}_o$ ) to create biomass and grow.  
72 The challenge is particularly acute for aquatic autotrophs because the diffusion constant of  $\text{CO}_2$   
73 in air is  $\sim 10,000$ -fold greater than it is in water. In addition, in waters at pH 7-8,  $\text{HCO}_3^-$  is 10-100  
74 times more abundant than  $\text{CO}_2$  but  $\text{HCO}_3^-$  is much less membrane permeable (1). Therefore,  
75 the chemistry of  $\text{C}_i$  imposes inherent constraints that carbon-fixing organisms must work within.

76 Today, and for much of Earth's history, the most widespread strategy for carbon fixation  
77 is the Calvin-Benson-Bassham (CBB) Cycle, where the key carbon fixation step is catalyzed by  
78 ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (rubisco) (2, 3). But rubisco's central  
79 role in the CBB cycle and oxygenic photosynthesis poses a conundrum because it is usually  
80 considered to be a non-specific and slow enzyme. The first issue concerns rubisco's dual  
81 carboxylase and oxygenase activities: the RuBP intermediate (enediolate) is susceptible to both  
82  $\text{O}_2$  and  $\text{CO}_2$  attack (4). Consequently, instead of fixing a  $\text{CO}_2$  molecule during photosynthesis,  
83 rubisco can instead assimilate  $\text{O}_2$  to yield 2-phosphoglycolate (2-PG), which is not part of the

84 CBB cycle and therefore must be salvaged through photorespiratory pathways that consume  
 85 ATP, reducing power, and carbon (5). The second issue concerns rubisco's maximum  
 86 carboxylation rate ( $k_{cat}$ ), which is  $\approx 7$ -10 times slower than other central metabolic enzymes (6),  
 87 and displays very limited variation across large phylogenetic distances (7).

88 Both issues—its dual carboxylase / oxygenase activity and limited maximum  
 89 carboxylation rate—are typically rationalized by considering the evolutionary history of this  
 90 enzyme in the context of long-term changes in environmental  $\text{CO}_2$  and  $\text{O}_2$  concentrations.  
 91 Rubisco is thought to have evolved at a time when there was trace  $\text{O}_2$  and much higher  $\text{CO}_2$   
 92 concentrations in the atmosphere, in contrast to the modern atmosphere where  $\text{O}_2$  is roughly  
 93 20% while  $\text{CO}_2$  is only about 0.04% by partial pressure. Rubisco is also thought to have been  
 94 the primary carboxylating enzyme of global photosynthesis since the Great Oxygenation Event,  
 95 and potentially far prior (8).

96 Likely in response to these changing environmental concentrations, many aquatic  
 97 photoautotrophs have evolved  $\text{CO}_2$  concentrating mechanisms (CCMs) that concentrate  $\text{CO}_2$   
 98 around rubisco in order to enhance carboxylation and suppress oxygenation. Currently, all  
 99 known Cyanobacteria have CCMs, as do many bacterial chemolithoautotrophs, many aquatic  
 100 algae, and some plants (9). The bacterial CCM has two main components: i) an  $\text{C}_i$  pump  
 101 producing high cytosolic  $\text{HCO}_3^-$ , and ii) co-encapsulation of carbonic anhydrase (CA) and  
 102 rubisco inside proteinaceous organelles known as carboxysomes (Figure 1A) (10, 11). The  
 103 timing of carboxysome CCM evolution is uncertain, but it likely arose sometime during the  
 104 Proterozoic Eon (9). Therefore, for roughly half of Earth's history, bacterial rubiscos have  
 105 functioned in concert with a system that pumps  $\text{C}_i$  into and around the cell.  
 106



107  
 108 **Figure 1: Cyanobacterial  $\text{CO}_2$  Concentrating Mechanism (CCM) compared to traditional box model**  
 109 **architecture and outputs.** A) Cyanobacterial CCMs rely on i) active  $\text{HCO}_3^-$  uptake into the cell; ii) co-  
 110 encapsulation of carbonic anhydrase (CA) and rubisco within the carboxysome. These components  
 111 together produce a high carboxysomal  $\text{CO}_2$  concentration that enhances  $\text{CO}_2$  fixation by rubisco and  
 112 suppresses oxygenation. B) Architecture of the traditional box model based on (12–15) mapped onto a  
 113 cyanobacterial CCM; see Supplemental for full discussion of this model. Boxes denote carbon pools of  
 114 interest, and fluxes between boxes are denoted by  $\Phi$ . Each flux has its own isotopic fractionation denoted  
 115 by  $\epsilon$ ; no fractionation is assumed for  $\Phi_{\text{loss}}$ . Model assumes an infinitely large external carbon pool, that  
 116 carbon not fixed by rubisco ( $\text{C}_{\text{lost}}$ ) returns to this pool, and that fluxes are at steady state. Note that this

117 architecture does not include a box for the carboxysome, or fluxes via powered inorganic carbon uptake.  
118 C) Model solution is  $\epsilon_P = (1-f)\epsilon_{\text{Diffusion}} + f\epsilon_{\text{Rubisco}}$ , where  $\epsilon_P$  is defined as the difference in  $\delta^{13}\text{C}$  of  $C_{\text{external}}$   
119 and  $C_{\text{fixed}}$ , and  $f$  is defined as the ratio of  $\Phi_{\text{loss}}/\Phi_{\text{in}}$ . For this illustration,  $\epsilon_{\text{Rubisco}} = 25\text{‰}$  and  $\epsilon_{\text{Diffusion}} = 1\text{‰}$  is  
120 assumed. When  $f = 0$ ,  $\epsilon_P = \epsilon_{\text{Diffusion}}$ , and when  $f = 1$ ,  $\epsilon_P = \epsilon_{\text{Rubisco}}$ .

121  
122 Over geological timescales, trends in the environmental  $\text{CO}_2$  concentrations and the  
123 evolutionary history of carbon fixation and its role in the carbon cycle have been captured, in  
124 some noisy manner, in the carbon isotope record. This record is composed of measurements of  
125 the relative ratios of  $^{13}\text{C}$  to  $^{12}\text{C}$  isotopes in C-bearing phases in sedimentary rocks over time  
126 (reported using the delta notation ( $\delta^{13}\text{C}$ ) and expressed in per mil (‰); see Methods for detail).  
127 The carbon isotope record established a time series of touchpoints between biological and  
128 geological processes that—if interpreted correctly—might be read to understand the evolution of  
129 carbon-fixing processes through time. One key to reading the isotope record is the observation  
130 that rubisco displays a kinetic isotope effect (KIE) where it preferentially fixes  $^{12}\text{CO}_2$  over  $^{13}\text{CO}_2$   
131 due to the  $k_{\text{cat}}$  being slightly faster for  $^{12}\text{CO}_2$  than  $^{13}\text{CO}_2$  (16). This results in the reaction product,  
132 3-phosphoglycerate (3-PGA), being relatively depleted in  $^{13}\text{C}$  by several percent (tens of per  
133 mil ‰) compared to the overall isotopic composition of the initial  $\text{CO}_2$  substrate. The difference  
134 in  $\delta^{13}\text{C}$  of the  $\text{CO}_2$  substrate and the 3-PGA product is typically reported as  $\epsilon_{\text{Rubisco}}$ , and has  
135 been measured to vary between 18-30‰ for most extant rubiscos (17, 18), with the exception of  
136 rubisco from the coccolithophore *Emiliania huxleyi* at 11‰ (19). Because all resulting biomass is  
137 synthesized from 3-PGA in autotrophs utilizing the CBB cycle, biomass is depleted in  $^{13}\text{C}$   
138 compared to external  $C_i$  pools. The magnitude of this difference is called  $\epsilon_P$ .  $C_i$  pools are  
139 preserved in the rock record in the form of carbonate salts (in limestones and dolomites), while  
140 biomass and  $C_o$  pools are preserved in organic phases (typically kerogen) in a myriad of  
141 lithologies and are measured as rock total organic carbon (TOC). There is an additional  
142 fractionation factor associated with the preservation of biomass and  $C_i$  as rocks, so the  
143 magnitude of fractionation between  $C_i$  and  $C_o$  pools is termed  $\epsilon_{\text{TOC}}$  and varies slightly from  $\epsilon_P$   
144 (20). Overall, if one can accurately convert from  $\epsilon_{\text{TOC}}$  to  $\epsilon_P$ , and then from  $\epsilon_P$  to  $\epsilon_{\text{Rubisco}}$ , one may  
145 be able to learn about photosynthetic physiology over time from the rock record.

146 Much work has been done to address two main observations seen in the carbon isotope  
147 record: i) there is variation in  $\epsilon_{\text{TOC}}$  despite its general consistency around 25‰ (18), and ii)  
148 organic matter is more  $^{13}\text{C}$ -depleted further back in time compared to today, particularly in  
149 Precambrian-age rocks (20–23). If one accepts that these trends are not due to chemical  
150 alteration of sediment post-deposition (diagenesis) or other preservation effects (which are  
151 expected to have the opposite sign (24)), then one looks for mechanisms that might explain  
152 such a change. To date, those efforts have focused on environmental changes (e.g.  
153 temperature, pH,  $p\text{CO}_2$ ) that might have led to larger carbon isotope fractionations. Typically,  
154 model autotrophs are grown in different conditions, and then a proxy is calibrated that relates  
155 carbon isotope fractionation to the chosen environmental condition. Doing so, the community  
156 has found that in both lab cultures of photosynthetic algae (25, 26), and from field data of  
157 marine algae (13, 27, 28), that increased levels of dissolved  $\text{CO}_2$  in solution corresponded to an  
158 increased value of  $\epsilon_P$ . This general observation has been modified and honed by later studies to  
159 account for environmental parameters like the effects of temperature and pH on dissolved  $\text{CO}_2$   
160 concentrations, and physiological characteristics like growth rate, cell geometry/type, and

161 species/strain-type (25, 26, 29). However, even when taking these factors into account, it is still  
162 expected that increased concentrations of CO<sub>2</sub> will cause increased values of  $\epsilon_P$  (see (18) for  
163 review of factors affecting  $\epsilon_P$ ).

164 This supposition undergirds the traditional box model of carbon isotope fractionation in  
165 algae (12, 13), which was based on a model of carbon isotope fractionation in C<sub>3</sub> plants (14,  
166 15), which have no CCM (Figure 1B). This is often referred to as a “diffusion-based” model  
167 because CO<sub>2</sub> is assumed to passively diffuse in and out of aquatic cells (like gas exchange  
168 through leaf stomata) due to a concentration gradient of external vs. internal CO<sub>2</sub>. A “leakiness”  
169 term,  $f$ , can be defined as the ratio of fluxes ( $\Phi$ ) of carbon exiting or entering the cell based on  
170 this concentration gradient ( $f = \Phi_{out}/\Phi_{in} = [C_{int}]/[C_{ext}]$ ). In this simplified model,  $\epsilon_P$  is determined by  
171 the isotopic effect of two distinct steps: i) the diffusion of CO<sub>2</sub> into the cell ( $\epsilon_{Diffusion}$ ; <1‰ in water  
172 at steady state (30)); and ii) the carbon fixation step catalyzed by rubisco ( $\epsilon_{Rubisco}$ ; typically 18-  
173 30‰). The model is usually solved by assuming steady state, which results in a linear  
174 relationship between  $\epsilon_P$  and  $f$  so that experimentally measured values of  $\epsilon_P$  can be used to solve  
175 for  $f$ . This model also sets the minimum and maximum  $\epsilon_P$  values ( $\epsilon_{Diffusion}$  and  $\epsilon_{Rubisco}$  respectively,  
176 Fig. 1C) with corresponding physiological interpretations: when  $\epsilon_P \approx \epsilon_{Diffusion}$ , nearly all carbon  
177 entering the cell is used and with this mass balance constraint rubisco’s <sup>12</sup>C preference is not  
178 expressed; conversely, when  $\epsilon_P \approx \epsilon_{Rubisco}$ , very little of the carbon entering the cell is fixed ( $f \approx 1$ ),  
179 rubisco can “choose” between <sup>12</sup>C and <sup>13</sup>C substrates, and the KIE of rubisco can be fully  
180 expressed. Therefore, given the assumption that C<sub>i</sub> is taken up passively, it is possible to derive  
181 an increasing relationship between C<sub>ext</sub> and  $\epsilon_P$  from this model (see Supplemental and (13)).

182 One reason that Earth scientists are interested in models of biological carbon isotope  
183 fractionation is that it may help constrain historical pCO<sub>2</sub> concentrations. The ice core record,  
184 which provides direct observations of the Earth’s atmosphere, extends back only ≈1 million  
185 years (31), so, for the remaining four and half billion years of Earth history, model-driven proxies  
186 are used (32, 33). However, under the simplified diffusional model,  $\epsilon_P$  cannot exceed  $\epsilon_{Rubisco}$   
187 (Figure 1C), yet the largest  $\epsilon_P$  values observed in the Archaean Eon exceed 30‰ (21, 34) and  
188 also exceed all existing measurements of  $\epsilon_{Rubisco}$ . In addition, recent studies in dinoflagellates  
189 that showed that  $\epsilon_P$  can regularly exceed  $\epsilon_{Rubisco}$  under certain growth conditions argue that  $\epsilon_P$   
190 reflects additional isotopic fractionations that may occur with carbon uptake processes. This has  
191 motivated an updated model of Eukaryotic algae accounting for the estimated isotopic  
192 fractionations of different C<sub>i</sub> uptake mechanisms (17).

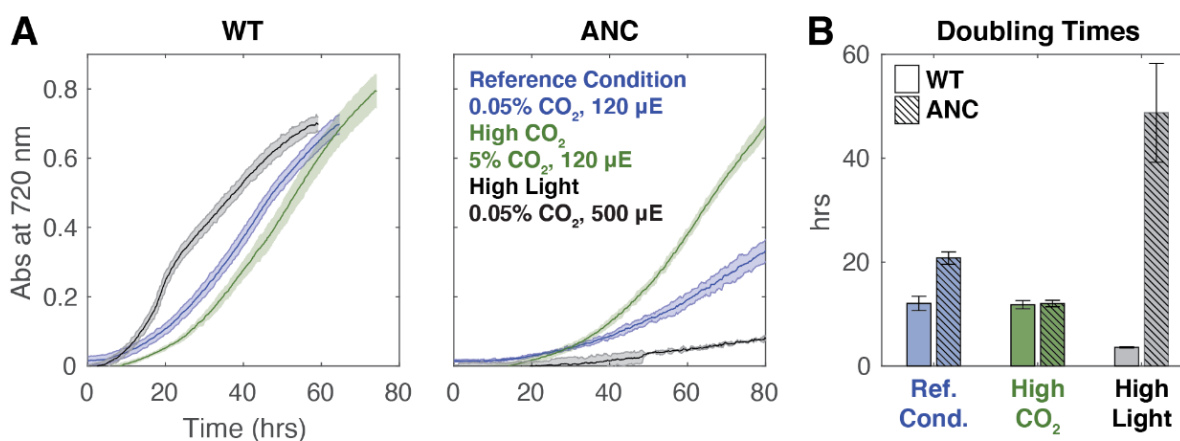
193 In addition to taking modern physiology into account, it is important to understand how  
194 the evolution of rubisco and the CCM may have also affected the carbon isotope composition of  
195 biomass. Recent studies have addressed this issue directly by testing model organisms that  
196 may better resemble an ancestral counterpart, including a cyanobacterial strain that lacks a  
197 CCM (22), a cyanobacterial strain that overexpresses rubisco (35), and a cyanobacterial strain  
198 expressing an inferred ancestral rubisco dating from ≈1-3 Ga in age (36, 37).

199 Here, we measured the  $\epsilon_P$  of a control strain of *S. elongatus* PCC 7942 expressing the  
200 wild-type rubisco (NS2-KanR, referred to as ‘WT’), as well as a mutant expressing an inferred  
201 ancestral Form 1B rubisco dating to >1 Ga (referred to as ‘ANC’) (38) in varied CO<sub>2</sub> and light

202 conditions. In addition, we measured the KIEs of the present-day and ancestral rubiscos  
203 ( $\epsilon_{\text{Rubisco}}$ ) *in vitro*. We observed that: i)  $\epsilon_p$  is greater for ANC than for its WT counterpart for all  
204 conditions, even though ANC  $\epsilon_{\text{Rubisco}}$  ( $17.23 \pm 0.61\text{‰}$ ) is considerably less than WT  $\epsilon_{\text{Rubisco}}$   
205 ( $25.18 \pm 0.31\text{‰}$ ); ii) ANC  $\epsilon_p$  exceeds  $\epsilon_{\text{Rubisco}}$  in all tested conditions even though the traditional  
206 model sets the maximum possible  $\epsilon_p = \epsilon_{\text{Rubisco}}$ ; iii) ANC  $\epsilon_p$  increases with higher light while WT  $\epsilon_p$   
207 increases with higher  $\text{CO}_2$ ; iv) ANC displays a growth defect at ambient  $\text{pCO}_2$  that is rescued at  
208 high  $\text{pCO}_2$ ; and v) ANC growth is severely inhibited in high light conditions. ANC  $\epsilon_p$  exceeding  
209  $\epsilon_{\text{Rubisco}}$  implies that the traditional box model is incomplete and additional processes and  
210 fractionation factors are needed. In addition, other aspects of cyanobacterial physiology beyond  
211 the CBB cycle must be taken into account to explain how  $\epsilon_p$  can vary independently of  $\text{CO}_2$ . We  
212 posit additional factors related to  $\text{C}_i$  uptake that might explain fractionation measurements that  
213 deviate from box model predictions in both extant and ancient organisms.

## 214 Results & Discussion

### 215 Ancestral Rubisco strain grows at ambient $\text{CO}_2$ concentrations



216 **Figure 2: Growth curves for WT and ANC strains across experimental conditions.** A) Averaged  
217 growth curves shown for WT and ANC strains to 80 hours, colored by growth condition as indicated in  
218 figure. Data was smoothed with a rolling median (Methods); see full ANC growth curves in Supplemental.  
219 B) Average doubling times with standard deviation for growth curves on the left. Doubling times were  
220 calculated using a Markov Chain Monte Carlo (MCMC) approach; see Supplemental for details. ANC  
221 displayed a growth defect relative to the WT at the reference condition, which was rescued at high  $\text{CO}_2$ .  
222 Across all conditions, ANC grew the slowest at high light, while WT grew the fastest.  
223

224  
225 Working in *S. elongatus* PCC 7942, we produced a mutant strain lacking the native Form  
226 1B rubisco, expressing instead an ancestral Form 1B rubisco produced by computational  
227 ancestral sequence reconstruction (Methods). This putative ancestral rubisco was previously  
228 purified and characterized *in vitro* (38). We compared the growth of this mutant strain, which we  
229 termed 'ANC', to the parent strain (wild-type or 'WT') across several light fluxes and  $\text{CO}_2$   
230 concentrations: i) Reference condition (ambient  $\text{pCO}_2$  (0.05% (v/v)), standard light flux (120  
231  $\mu\text{E}$ ); ii) High  $\text{CO}_2$  (5%  $\text{pCO}_2$  (v/v), 120  $\mu\text{E}$ ); iii) High light (0.05%  $\text{pCO}_2$  (v/v), 500  $\mu\text{E}$ ).  
232 Remarkably, the ANC strain managed to grow at ambient  $\text{pCO}_2$  and standard light conditions  
233 (Figure 2A), even though the ancestral rubisco has a carboxylation rate ( $V_c$ ) roughly half that of

234 WT (Table 1). This result is in contrast to another historical analogue strain (lacking CCM  
235 genes) that only grew in elevated  $p\text{CO}_2$  (22), but is similar to another ancestral analogue (with a  
236 predicted Precambrian Form 1B rubisco) that did grow in under ambient conditions (36).

237 The difference in  $V_C$  between the ancestral and modern rubiscos was mirrored in the  
238 doubling times of WT and ANC strains (Figure 2B, Table S3), where the doubling time of ANC  
239 was roughly twice that of WT in the reference condition ( $20.8 \pm 1.2$  vs.  $12.0 \pm 1.4$  hours  
240 respectively (avg.  $\pm$  s.d.). In addition, the carboxylation rate normalized to the Michaelis-Menten  
241 constant for  $\text{CO}_2$  in air ( $V_C/K_C^{\text{Air}}$ ) for the ancestral Form 1B rubisco, which measures the  
242 enzyme's ability to function at low  $\text{CO}_2$  concentrations, is roughly half that of the modern Form  
243 1B rubisco (Table 1; see (38) for full characterization of enzyme kinetics). This suggested that  
244 ANC's growth was limited by its ability to fix  $\text{CO}_2$  in a manner proportional to rubisco's  
245 carboxylation rate. This growth defect was ameliorated at high  $p\text{CO}_2$ , where doubling times for  
246 both strains were the same within uncertainty (WT  $11.8 \pm 0.8$  hours; ANC  $12.0 \pm 0.6$  hours),  
247 though we observed a longer lag phase for ANC. WT doubling times were the same within  
248 uncertainty for the reference and high  $\text{CO}_2$  conditions ( $12.0 \pm 1.4$  vs.  $11.8 \pm 0.8$  hours  
249 respectively) in contrast to ANC, whose doubling times did change in response to elevated  $\text{CO}_2$ .  
250 This suggested that  $\text{CO}_2$  availability is a growth-limiting factor for ANC but not WT, and further  
251 suggested that the differences in their growth kinetics are related to the efficacy of their CCMs.  
252 Consistent with our results, a similar ancestral Form 1B analogue displayed total carboxylase  
253 activity roughly half that of the modern Form 1B (39).

254 We observed the greatest differences in doubling times between ANC and WT when the  
255 strains were grown at high light (Figure 2, Table S3). In these conditions, WT cultures were a  
256 dark, blue-green color typical of healthy cyanobacterial cells while ANC cultures were yellow-  
257 green (Fig. S9), suggesting that ANC cultures were cannibalizing their photosynthetic antennae  
258 via a known starvation pathway to reduce the cell's capacity for light harvesting and  
259 photochemical electron transport (40). It is likely that ANC could not match its rate of  $\text{CO}_2$   
260 fixation to the rate of light harvesting, and hence invoked this regulatory pathway. WT, in  
261 contrast, grew rapidly in the high light condition, ostensibly because its rate of  $\text{CO}_2$  fixation could  
262 match the higher light-harvesting rate.

### 263 **Ancestral rubisco enzyme fractionates less than WT rubisco enzyme**

264 We measured the *in vitro* fractionations of the WT and ANC rubisco using the substrate  
265 depletion method ((41–44); see Methods and Supplemental for more details). Previous work on  
266 rubisco isotope kinetics predicted that  $\epsilon_{\text{Rubisco}}$  should correlate positively with specificity ( $S_{\text{C/O}}$ ), a  
267 unitless measure of the relative preference for  $\text{CO}_2$  over  $\text{O}_2$  (45). We therefore expected ANC  
268 and WT  $\epsilon_{\text{Rubisco}}$  values to be the same within uncertainty because of their similar  $S_{\text{C/O}}$  values, but  
269 we found that ANC  $\epsilon_{\text{Rubisco}}$  ( $17.23 \pm 0.61\text{‰}$ ) fractionated carbon isotopes during carboxylation  
270 about 8‰ less than that of WT  $\epsilon_{\text{Rubisco}}$  ( $25.18 \pm 0.31\text{‰}$ ) (Table 1).

271

Rubisco	$\epsilon_{\text{Rubisco}}$ (‰)	$V_C$ ( $\text{s}^{-1}$ )	$K_C^{\text{Air}}$ ( $\mu\text{M}$ )	$V_C/K_C^{\text{Air}}$ ( $\text{s}^{-1}\text{mM}^{-1}$ )	$S_{\text{C/O}}$
Ancestral Form 1B	$17.23 \pm 0.61$	$4.72 \pm 0.14$	168.7	28	$49.6 \pm 1.8$
Modern Form 1B	$25.18 \pm 0.31^*$	$9.78 \pm 0.48^*$	$184.1^*$	$53.1^*$	$50.3 \pm 2.0^*$

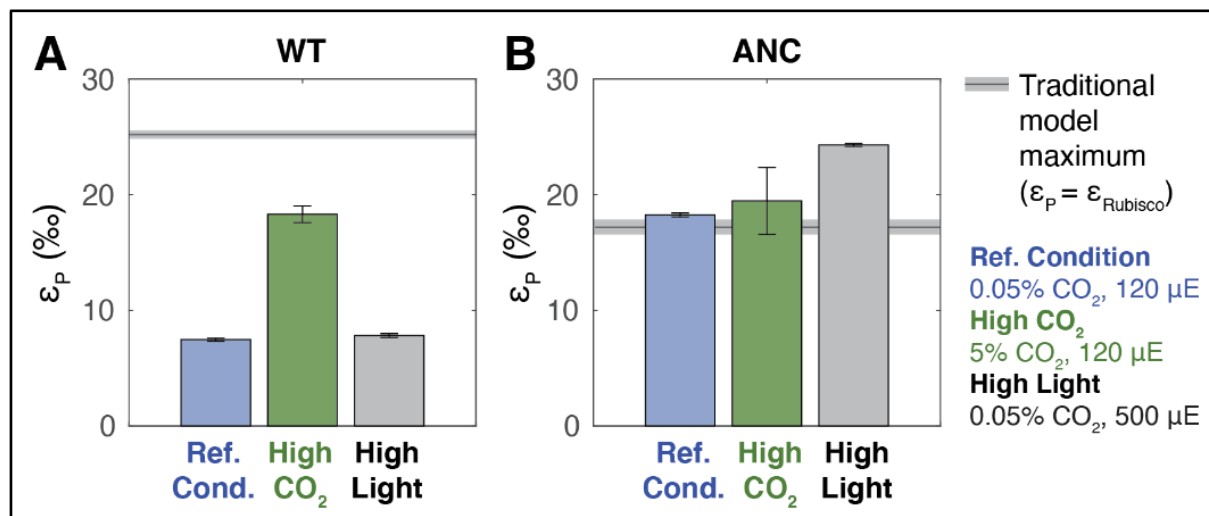
272  
273 **Table 1: Rubisco characteristics.** Relevant enzyme characteristics for the ancestral vs. modern Form  
274 1B rubisco. Starred values (\*) for the modern Form 1B were measured in rubiscos purified from  
275 *Synechococcus* sp. PCC 6301, a close relative of our working WT strain, *Synechococcus* sp. PCC 7942.  
276 Kinetic isotope effect ( $\epsilon_{\text{Rubisco}}$ , avg.  $\pm$  s.e.) was measured using the substrate depletion method (41–44);  
277 see Methods and Supplemental for more detail. Rate of carboxylation ( $V_C$ ), Michaelis-Menten constant for  
278  $\text{CO}_2$  in ambient air at modern levels of atmospheric  $\text{O}_2$  ( $K_C^{\text{Air}}$ ), rate of carboxylation normalized to the  
279 Michaelis-Menten constant ( $V_C/K_C^{\text{Air}}$ ), and specificity for  $\text{CO}_2$  vs.  $\text{O}_2$  ( $S_{\text{C/O}}$ ) are from (38); additional details  
280 on these values are within.  $S_{\text{C/O}}$  is a unitless measure of the relative preference for  $\text{CO}_2$  over  $\text{O}_2$ , and is  
281 calculated as  $(V_C/K_C)/(V_O/K_O)$  where  $K_C$  and  $K_O$  are the Michaelis-Menten constants for  $\text{CO}_2$  and  $\text{O}_2$   
282 concentrations respectively, and  $V_C$  and  $V_O$  are the carboxylation and oxygenation turnover rate under  
283 substrate-saturated conditions.  $V_C/K_C^{\text{Air}}$  measures the ability of rubisco to function at low  $\text{CO}_2$   
284 concentrations because it describes the initial response rate of carboxylation to the  $\text{CO}_2$  concentration.

### 285 ANC strain fractionates more than WT strain

286 Counter to expectations based on enzyme KIEs (Table 1), larger  $\epsilon_p$  values were  
287 observed for ANC than WT in all  $\text{CO}_2$  and light conditions tested (Figure 3). This was consistent  
288 with results from a similar ancestral analog, where larger  $\epsilon_p$  values exceeded WT at ambient  
289 and elevated  $\text{CO}_2$  levels (36). The highest ANC  $\epsilon_p$  values were observed for cultures grown at  
290 high light, where growth was comparatively slow (doubling time  $\approx$  50 hours, Figure 3 and Table  
291 S3). ANC  $\epsilon_p$  values were also modulated by light and  $\text{CO}_2$  differently than WT (Figure 3A).  
292 Compared to the reference condition, ANC  $\epsilon_p$  values did not increase in high  $\text{CO}_2$  and only  
293 increased in high light. In contrast, WT  $\epsilon_p$  values were indifferent to high light and only increased  
294 in high  $\text{CO}_2$ . This result contrasted with the ancestral strain in (36) where  $\epsilon_p$  values increased by  
295  $\approx 10\text{‰}$  at 2%  $\text{CO}_2$ .

296  
297





298  
299 **Figure 3: Whole cell carbon isotope fractionation by WT and ANC strains.**  $\epsilon_P$  (‰) values (avg.  $\pm$  s.e.)  
300 for A) WT, and B) ANC strains across growth conditions.. For each strain, the maximum  $\epsilon_P$  possible  
301 based on the traditional model ( $\epsilon_P = \epsilon_{\text{Rubisco}}$ ) is shown as a gray line (avg.  $\pm$  s.e.). Most measured ANC  
302 values exceed the theoretical limit ( $\epsilon_P > \epsilon_{\text{Rubisco}}$ ), while all WT  $\epsilon_P$  values do not ( $\epsilon_P < \epsilon_{\text{Rubisco}}$ ). WT  $\epsilon_P$  values  
303 increase in response to elevated CO<sub>2</sub> concentrations, while ANC  $\epsilon_P$  values increase in response to  
304 elevated light flux. See Supplemental for full results.

305  
306 In addition, the traditional box model described above cannot accommodate  $\epsilon_P$  values in  
307 excess of  $\epsilon_{\text{Rubisco}}$  (Figure 1). However, average ANC  $\epsilon_P$  values exceeded ANC  $\epsilon_{\text{Rubisco}}$  in all  
308 growth conditions (Figure 3), particularly under high light conditions where the largest difference  
309 was seen ( $\epsilon_P = 24.30 \pm 0.12\text{‰}$  VS  $\epsilon_{\text{Rubisco}} = 17.23 \pm 0.61\text{‰}$ ). The traditional box model also states  
310 that  $\epsilon_P$  values are solely modulated by changing external pCO<sub>2</sub> concentrations (Figure 1), which  
311 cannot accommodate the ANC  $\epsilon_P$  observations.

### 312 Proposed influence of a light-powered carbonic-anhydrase

313 The data showed two primary observations that could not be reconciled by the traditional  
314 box model: i) ANC  $\epsilon_P$  often exceeds  $\epsilon_{\text{Rubisco}}$ , and ii) ANC  $\epsilon_P$  varies primarily with light and not  
315 CO<sub>2</sub>. Based on independent knowledge of carbon acquisition strategies in Cyanobacteria, we  
316 augmented the traditional box model to include an additional isotope fractionation step, and a  
317 parameter affected by light. A good candidate that fulfills both these criteria would be a process  
318 that uses energy derived from light to catalyze the energy-coupled unidirectional hydration of  
319 CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup>—a “powered” carbonic anhydrase (CA).

320 Cyanobacteria have been shown to have two modes of active C<sub>i</sub> uptake: uptake of  
321 hydrated C<sub>i</sub> (predominantly H<sub>2</sub>CO<sub>3</sub> and HCO<sub>3</sub><sup>-</sup>) and uptake of CO<sub>2</sub> (46). In order for the CCM to  
322 function, either mode would need to produce a high, non-equilibrium concentration of HCO<sub>3</sub><sup>-</sup> in  
323 the cytoplasm (9, 11). This is thought to be achieved by coupling CA to an energy source (e.g.  
324 light or an ion gradient) that drives the one-way hydration of CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup> in the cytoplasm (47).  
325 There is now excellent data supporting this hypothesis in Cyanobacteria, where accessory  
326 proteins that bind to the cyanobacterial homologue of Complex I NADH dehydrogenase (the  
327 inducible NDH-1MS or constitutive NDH-1MS') are known to mediate CO<sub>2</sub> uptake specifically  
328 (48–50). Additionally, one of these accessory proteins, CupA in NDH-1MS and CupB in NDH-

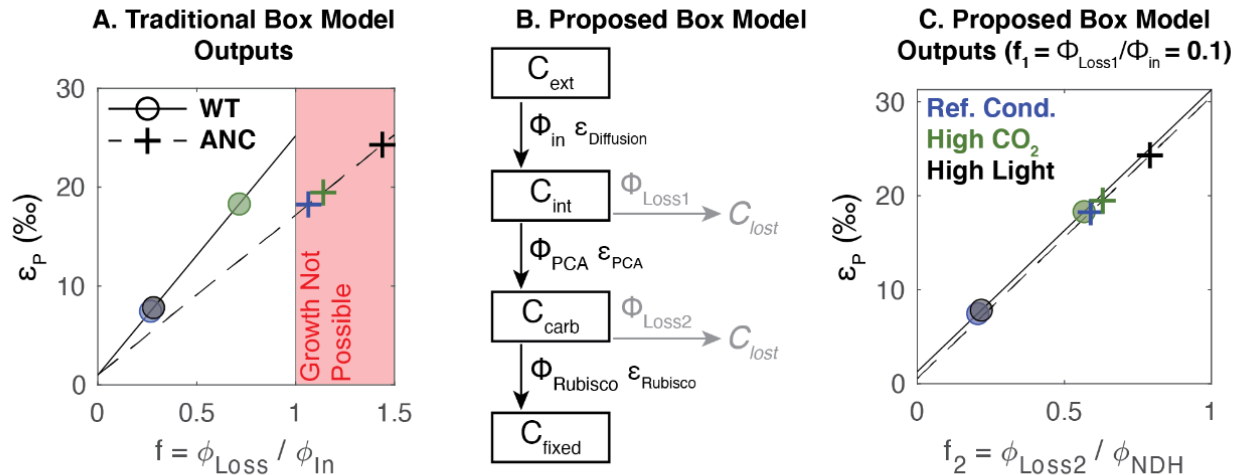
329 1MS', is homologous to a carbonic anhydrase and contains a telltale zinc active site situated  
330 near a proton channel in a membrane subunit (51). This powered carbonic anhydrase Complex  
331 I variant in Cyanobacteria therefore couples inorganic carbon uptake directly to the  
332 photochemical electron transport chain. Moreover, a similar protein complex has been  
333 described in proteobacterial chemoautotrophs, suggesting that energy-coupled CO<sub>2</sub> hydration is  
334 widespread (52).

335 A unidirectional CA would affect  $\epsilon_p$  for two reasons. First, CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> are isotopically  
336 distinct. At equilibrium in standard conditions, HCO<sub>3</sub><sup>-</sup> is  $\approx 8\%$  more enriched in <sup>13</sup>C than CO<sub>2</sub> (53,  
337 54). Therefore, if a cyanobacterium is predominantly taking up CO<sub>2</sub>, then the internal C<sub>i</sub> pool  
338 from which biomass is formed is isotopically lighter (<sup>13</sup>C-depleted) than if HCO<sub>3</sub><sup>-</sup> is the dominant  
339 source of C<sub>i</sub>. Second, unidirectional CO<sub>2</sub> hydration is expected to impart a substantial isotope  
340 effect, with calculated values ranging from  $\approx 19$  to 32‰ (53, 55–58). Therefore, there are two  
341 mechanistic reasons that  $\epsilon_p$  could exceed  $\epsilon_{\text{Rubisco}}$  in conditions where energized CO<sub>2</sub> uptake and  
342 hydration is active. Indeed, unidirectional CO<sub>2</sub> hydration has also been proposed to contribute to  
343 algal  $\epsilon_p$  values that exceed known algal  $\epsilon_{\text{Rubisco}}$  values (17).

344 The activity of a powered CA could modulate  $\epsilon_p$  independent of external C<sub>i</sub>  
345 concentrations, counter to the traditional model which proposes  $\epsilon_p$  as a direct correlate of  
346 external pCO<sub>2</sub> (12, 13). Because his powered CO<sub>2</sub> uptake and hydration is driven by light  
347 energy, e.g. via cyclic electron flow around photosystem I (51), this may explain why the  
348 highest ANC  $\epsilon_p$  values, those exceeding ANC  $\epsilon_{\text{Rubisco}}$ , were observed under high light conditions.  
349 Furthermore, on short timescales ( $\approx$ minutes) cyanobacterial C<sub>i</sub> uptake can be modulated by light  
350 intensity alone, fully independent of external C<sub>i</sub> concentrations (59), and CO<sub>2</sub> uptake can occur  
351 in the absence of carbon fixation (60, 61). Based on these physiological and isotopic  
352 observations, a powered CA is likely responsible for  $\epsilon_p$  values in excess of  $\epsilon_{\text{Rubisco}}$  in ANC.

### 353 **Proposed model for carbon isotope fractionation**

354 As discussed above, the traditional box model cannot produce  $\epsilon_p > \epsilon_{\text{Rubisco}}$  (Figure 1).  
355 Based on our isotopic data and understanding of cyanobacterial physiology, we proposed a  
356 simple modification of the traditional model that can rationalize our measurements of growth and  
357  $\epsilon_p$  for both WT and ANC strains (Figure 4). The traditional box model was able to rationalize our  
358 data from WT in the sense that the values of the CO<sub>2</sub> leakage term,  $f$ , fit from  $\epsilon_p$  are compatible  
359 with growth (i.e.  $f < 1$ ). In this model,  $f = 1$  implies that all carbon uptake leaks out of the cell.  
360 However, using the traditional model on the ANC strain data yields values of  $f$  inconsistent with  
361 growth ( $f > 1$ ; Figure 4A), which clearly violates growth curve data that shows ANC was able to  
362 grow in all conditions (Figure 2).  
363



364  
 365 **Figure 4: Proposed box model based on experimental results.** A) Experimental results (circles and  
 366 crosses) plotted onto traditional box model outputs (dashed and solid lines) for WT and ANC respectively.  
 367 Uncertainties are smaller than data points. Colors indicate growth conditions: blue = reference condition  
 368 (0.05% pCO<sub>2</sub> (v/v), 120 μE); green = high CO<sub>2</sub> (5% pCO<sub>2</sub> (v/v), 120 μE); black = high light (0.05% pCO<sub>2</sub>  
 369 (v/v), 500 μE).  $f$  is as defined in Figure 1; region where  $f > 1$  is shaded in red. B) Proposed box model  
 370 architecture, with main carbon pools of interest in boxes. Subscripts indicate external (*ext*), internal (*int*),  
 371 carboxysome (*carb*), and fixed (*fixed*) carbon pools. Fluxes are denoted by  $\Phi$  where subscripts indicate  
 372 fluxes into the cell (*in*), out of the cell (*Loss1*, *Loss2*), into the carboxysome (*PCA* for *Powered Carbonic*  
 373 *Anhydrase*), and into fixed biomass (*Rubisco*), each with a corresponding isotopic fractionation denoted  
 374 with  $\epsilon$ . Loss fluxes were assumed to have no isotopic fractionation. In this proposed model,  $f_1$  is defined  
 375 as  $\Phi_{Loss1} / \Phi_{in}$ , and  $f_2$  is defined as  $\Phi_{Loss2} / \Phi_{PCA}$ . See text for model assumptions. C) Experimental results  
 376 plotted onto proposed box model outputs for  $f_1 = 0.1$ ; colors and symbols are the same as Panel A.  $\epsilon_P$   
 377 is defined as the difference in  $\delta^{13}C$  between  $C_{ext}$  and  $C_{fixed}$ . See Supplemental for full solution and results;  
 378 only results for  $f_1 = 0.1$  are shown. All analyses were performed using MATLAB and Statistics Toolbox  
 379 (vR2020b).

380  
 381 We therefore modified the traditional box model by making a distinction between carbon  
 382 in the cytosol ( $C_{int}$ ) and carbon in the carboxysome ( $C_{carb}$ ), and by adding an additional path  
 383 whereby carbon can be lost from the carboxysome ( $\Phi_{Loss2}$ , Figure 4B). In this modified model,  
 384 external  $C_i$  enters the cell (flux  $\Phi_{in}$ ) where it can either leak out ( $\Phi_{Loss1}$ ) or undergo active  
 385 hydration (flux  $\Phi_{PCA}$ , where *PCA* denotes *Powered Carbonic Anhydrase*). Intracellular  $C_i$  can  
 386 then enter the carboxysome, where it is either fixed (flux  $\Phi_{Rubisco}$ ) or ultimately leaks out of the  
 387 cell (flux  $\Phi_{Loss2}$ ). We made similar simplifying assumptions as the traditional box model: i) an  
 388 infinite supply of external carbon, ii) no isotopic fractionation for carbon lost from the cell, iii)  $\Phi_{in}$   
 389 has the isotopic fractionation associated with  $\epsilon_{Diffusion}$ , and iv) the system is at steady state. We  
 390 did not add an explicit term for light energy used to power  $C_i$  uptake. Instead, the model  
 391 included an energized CA (denoted *PCA*) and its associated isotopic fractionation as free  
 392 parameters. In modeling each strain, we used the appropriate  $\epsilon_{Rubisco}$  measurements (Table 1).  
 393 We do not know the true value for  $\epsilon_{PCA}$ , but used a value of 30‰ similar to recent models of a  
 394 one-way CA in eukaryotic algae (62). For comparison with the traditional model, we plotted  
 395 Figure 4C with  $f_1 = 0.1$  so that it could be represented in two dimensions. However, in this  
 396 updated model, each value of  $\epsilon_p$  corresponds to a set of feasible  $f_1$  and  $f_2$  values that fall along a  
 397 line (Figure S7 and S8 for WT and ANC respectively). Therefore, our model constrains but does

398 not uniquely determine  $f_1$  and  $f_2$ , nor does it allow for estimation of external  $C_i$  levels precisely  
399 because many pairs of  $f_1$  and  $f_2$  values can produce the same  $\epsilon_p$  value.

400 With the addition of a powered CA and an additional loss term, the model was able to  
401 rationalize our experimental data of  $\epsilon_p > \epsilon_{\text{Rubisco}}$  with leakage values compatible with cell growth  
402 ( $f_2 < 1$ ) (Figure 4C). Our model results implied that, overall, ANC lost more carbon than WT at  
403 the branch point before rubisco ( $\Phi_{\text{Loss2}}$ ); i.e. even though carbon was present in the cell, it could  
404 not be fixed by the ancestral Form 1B rubisco because of its slower carboxylation rate. The  
405 excess amount of  $\text{CO}_2$  available then allowed rubisco's kinetic isotope effect ( $\epsilon_{\text{Rubisco}}$ ) to be  
406 expressed. In addition, the model indicated that this effect would be exacerbated at high light  
407 (Figure 4C), which is consistent with the low ANC doubling rates seen in this condition (Figure  
408 2). These results implied that at high light, the powered CA was delivering high amounts of  $\text{CO}_2$   
409 to both the WT and ANC rubisco. WT was able to keep up with this flux, which was reflected in  
410 its fast growth rate (Figure 2) and no change in  $\epsilon_p$  vs. the reference condition (Figure 3).  
411 However, ANC was not, which led to its slowest growth rate (Figure 2), and highest  $\epsilon_p$  values  
412 across all conditions.

413 These results showed that other processes relevant to the CCM, in addition to rubisco,  
414 can play an important role in  $\epsilon_p$  values. While our approach is highly idealized and relies on a  
415 minimum set of fractionating processes associated with carbon fixation in Cyanobacteria  
416 (adding only one additional fractionation factor, and one additional leakage point), the results  
417 demonstrated that a simple addition to the traditional model accounting for a known mode of  
418 energized  $\text{CO}_2$  uptake could explain our experimental results. Moreover, one useful implication  
419 of this model is that carbon isotope values may measure the efficiency of the CCM and carbon  
420 fixation in Cyanobacteria, as much as or more than it informs ambient environmental  $\text{CO}_2$   
421 concentrations.

## 422 **Consequences for understanding the evolution of carbon-fixing metabolism**

423 The traditional box model used to describe  $\epsilon_p$  values observed in the biomass of  
424 oxygenic photoautotrophs can produce  $\epsilon_p$  values ranging from  $\epsilon_{\text{diffusion}}$  to  $\epsilon_{\text{Rubisco}}$  (Figure 1).  
425 However, we and others have observed anomalous  $\epsilon_p$  values exceeding  $\epsilon_{\text{Rubisco}}$  in a variety of  
426 modern and synthetic organisms. Over Earth history,  $\epsilon_p$  values inferred from the carbon isotope  
427 record sometimes exceed the largest measured modern  $\epsilon_{\text{Rubisco}}$  (21, 34). Explaining these  
428 diverse observations has moved us and others to posit the presence of additional fractionating  
429 processes beyond rubisco carboxylation contributing to measured  $\epsilon_p$  values. One reasonable  
430 explanation is the presence of a one-way CA reaction, which carries a large kinetic isotope  
431 effect of  $\approx 30\text{‰}$  (53, 55–58).

432 This notion was first proposed more than 20 years ago based on measurements of  
433 cyanobacterial cultures with  $\epsilon_p$  values exceeding  $30\text{‰}$  (63). Recent measurements of  $\epsilon_p > \epsilon_{\text{Rubisco}}$   
434 motivated similar conclusions regarding multiple fractionating processes in eukaryotic algae  
435 (17). In these diverse experiments, anomalous  $\epsilon_p$  values were observed during relatively slow  
436 growth; in (63)  $\epsilon_p > \epsilon_{\text{Rubisco}}$  occurred early in the growth curve as cells were acclimating to fresh  
437 culture media, in (17)  $\epsilon_p > \epsilon_{\text{Rubisco}}$  occurred during nitrogen and phosphorus limitation, and in this  
438 study  $\epsilon_p > \epsilon_{\text{Rubisco}}$  was observed in a mutant strain growing slowly while expressing a

439 reconstructed ancestral rubisco. These observations indicated that growth physiology affects  
440 isotopic fractionation by photosynthetic algae and, in all cases, motivated a rethinking of the  
441 traditional box model (Figure 1) to include more physiological detail relating to the presence of a  
442 CO<sub>2</sub> concentrating mechanism.

443 Prior studies also worked to account for issues related to growth physiology—  
444 specifically growth rate, cell shape and size—to adapt the C3 plant model to unicellular algae  
445 (25). However, that landmark study measured  $\epsilon_p$  in wild-type Cyanobacteria—the same wild-  
446 type strain studied here—and, unlike this study and (39), found  $\epsilon_p$  to be roughly constant  
447 independent of environmental pCO<sub>2</sub> and growth rate. Popp and coauthors hypothesized that  
448 this observed independence stems from the large surface area to volume ratio (SA/V) of  
449 Cyanobacteria, which was taken to imply much faster passive CO<sub>2</sub> uptake (scaling with SA)  
450 than fixation (scaling with V). Because cyanobacterial  $\epsilon_p$  was constant  $\approx 17\text{‰}$  and less than  
451 known cyanobacterial  $\epsilon_{\text{Rubisco}}$  values, additional fractionating factors were not needed to explain  
452  $\epsilon_p$ , even though some active transport processes in Cyanobacteria were known at the time; the  
453 simple linear relationship between pCO<sub>2</sub> and  $\epsilon_p$  in C3 plants appeared to hold up in algae and  
454 Cyanobacteria as well.

455 This linear relation between pCO<sub>2</sub> and  $\epsilon_p$  does not hold for our ANC strain (Figure 3). We  
456 emphasize that ANC is not a true ancestral Cyanobacteria; rather it is a chimeric construct—a  
457 modern strain saddled with a Precambrian enzyme for its carbon fixation. This reconstructed  
458 ancestral rubisco is characterized by slower carboxylation kinetics (38) and a much lower  $\epsilon_{\text{Rubisco}}$   
459 (Table 1). In ANC, we observed anomalous  $\epsilon_p$  values exceeding  $\epsilon_{\text{Rubisco}}$  in all growth conditions,  
460 but especially in high light (Figure 3). As high light consistently slowed growth, induced chlorosis  
461 (yellowing of cultures, Figure S9) and increased  $\epsilon_p$ , we were motivated to consider the effects of  
462 light-related physiology on  $\epsilon_p$ . The yellowing of ANC cultures in high light was consistent with the  
463 well-described phycobilisome degradation pathway, which is typically induced in nutrient  
464 starvation conditions and taken to indicate that light levels exceeded the downstream capacity  
465 for CO<sub>2</sub> fixation (40, 64). We interpreted these observations as indicating that the replacement  
466 of the native rubisco with a reconstructed ancestor decreased the cellular capacity for CO<sub>2</sub>  
467 fixation, potentially due to (i) the inferior kinetics of the ancestral enzyme (Table 1) and (ii)  
468 potentially a partial incompatibility with the modern CCM, e.g. sub-optimal recruitment to the  
469 carboxysome (65).

470 Low CO<sub>2</sub> fixation capacity would not, on its own, explain anomalously high  $\epsilon_p$  values,  
471 however. An additional fractionating process is required to explain  $\epsilon_p$  values in excess of  $\epsilon_{\text{Rubisco}}$ ,  
472 which we assumed is due to light-coupled one-way hydration of CO<sub>2</sub>, which has a large  
473 calculated isotope effect (53, 55–58). Cyanobacteria have been shown to take up CO<sub>2</sub>  
474 independently of HCO<sub>3</sub><sup>-</sup> (46). In model Cyanobacteria, this activity is due to the Cup proteins  
475 (CupAS/B, also known as Chp proteins), which bind to the NADH-dehydrogenase-like complex  
476 (NDH-1) of Cyanobacteria (51, 66). The NDH-1 complex is involved in light energy capture via  
477 photosynthetic electron transport and cyclic electron flow around photosystem I (51) and,  
478 moreover, CO<sub>2</sub> uptake is stimulated by light alone and abrogated by inhibitors of photochemical  
479 electron transport (59). Not only has CupA been shown to carry a key Zn<sup>2+</sup> in a domain  
480 resembling a carbonic anhydrase (51), but the *cupA* gene is induced under low CO<sub>2</sub> conditions  
481 (66). In order for CO<sub>2</sub> uptake to drive the CCM and promote CO<sub>2</sub> fixation, it would need to

482 produce a high, non-equilibrium  $\text{HCO}_3^-$  concentration in the cytoplasm (9, 11). We and others  
483 therefore assumed that the complex of NDH-1 and CupAS/B (termed NDH-1<sub>3/4</sub> in the literature)  
484 couples light energy to the one-way hydration of  $\text{CO}_2$  to  $\text{HCO}_3^-$  at a carbonic anhydrase-like  
485 active site (51).

486 It is readily apparent that  $\epsilon_{\text{Rubisco}}$  does not set an upper bound on  $\epsilon_p$ , nor does it predict  
487 which strains will have larger  $\epsilon_p$  values *in vivo* (Figure 3). This inference was only possible  
488 because we measured the isotope fractionation due to the ancestral rubisco ( $\epsilon_{\text{Rubisco}}$ ) and  
489 compared it to ANC strain biomass ( $\epsilon_p$ ), in contrast with the study of (39), which only measured  
490  $\epsilon_p$ . While our ANC  $\epsilon_p$  values ( $\approx 18\text{--}24\text{‰}$ ) fell within the range of  $\epsilon_p$  values derived from the carbon  
491 isotope record (22), they exceeded the measured  $\epsilon_{\text{Rubisco}}$  (Figure 3). As such, the relative  
492 consistency of ANC  $\epsilon_p$  values does not indicate that the traditional box model is applicable  
493 across geologic time as claimed in (39). Rather, a model including some additional fractionating  
494 process is required to explain our observation that  $\epsilon_p > \epsilon_{\text{Rubisco}}$  in ANC. Attention has been paid  
495 to outliers where  $\epsilon_p$  exceeds  $\epsilon_{\text{Rubisco}}$  precisely because they violate the assumptions underlying  
496 the dominant model used to interpret the carbon isotope record (17). In addition, ANC  $\epsilon_{\text{Rubisco}}$   
497 ( $17.23 \pm 0.61\text{‰}$ ) is anomalously low; not only is it  $\approx 8\text{‰}$  less than WT  $\epsilon_{\text{Rubisco}}$  ( $25.18 \pm 0.31\text{‰}$ ) but  
498 it is among the lowest measured rubisco KIEs. However, only thirteen unique rubisco KIEs have  
499 been measured thus far (for recent review see (18)) while  $\approx 300$  distinct rubiscos have been  
500 kinetically characterized (7, 67).

501 The carbon isotope record has been used to reconstruct  $p\text{CO}_2$  measurements for the  
502 vast majority of Earth's history (32, 33) because more direct observations of the past  
503 atmosphere from ice only extend back  $\approx 1$  million years (31). The large carbon isotope  
504 fractionation between  $C_i$  and  $C_o$  (imparted by rubisco) observed in modern environments  
505 roughly matches the carbon isotope differences between carbonates and kerogen-rich rocks in  
506 the geological record. Because of this correlation and prior work, it has been assumed that  
507 proxies calibrated on modern oxygenic photoautotrophs over short timescales can largely be  
508 applied to ancient samples to infer paleo- $p\text{CO}_2$  concentrations. However, our study suggests  
509 that the carbon isotopic fractionations observed in both modern environments and throughout  
510 the geological record reflect not just the environmental abundance of  $\text{CO}_2$  and/or the rubisco  
511 present, but also the operation of  $C_i$  uptake processes like the NDH-1 complex discussed  
512 above.

513 As shown in the companion paper by Avi Flamholz and coauthors, a CCM is not  
514 required in the early atmosphere, where a variety of proxies generally agree that  $p\text{CO}_2$  was very  
515 high (up to  $\approx 0.8$  bar) (68). However, Flamholz et al. found that expression of carbonic  
516 anhydrases or  $C_i$  uptake systems greatly improved autotrophic growth in intermediate  $\text{CO}_2$   
517 levels ( $\approx 1\%$  partial pressure)—levels that are thought to have been important for perhaps much  
518 of Precambrian time (69). These results indicated that oxygenic photoautotrophs expressing  
519 energized  $\text{CO}_2$  uptake may have arisen relatively early in Earth's history, and rubisco has likely  
520 operated in concert with some sort of CCM over Proterozoic time (9). Therefore, a uniformitarian

521 framework is unjustified: a model that omits all aspects of the CCM (e.g. the traditional box  
522 model) might apply early in Earth history if  $p\text{CO}_2$  was sufficiently high, but would not necessarily  
523 apply to ancient or modern Cyanobacteria they all express complexes catalyzing light-driven,  
524 energized  $\text{CO}_2$  uptake (or any other processes associated with large carbon isotope fraction).

525 A carbon isotope model that engages more fully with photosynthetic physiology, i.e. one  
526 which includes some representation of the CCM (Figure 4), is required to describe  $\epsilon_p$  values and  
527 more accurately constrain environmental  $\text{CO}_2$  concentrations from environmental context (e.g.  
528 light and nutrient levels) and physiological parameters (e.g.  $\epsilon_{\text{Rubisco}}$ , photosynthetic capacity,  
529 growth rate). Notably, the model proposed here represents only a first step in this direction as it  
530 substantially simplifies the bacterial CCM (11); a similar statement applies to box models of  
531 Eukaryotic algae, which also express complex CCMs (17, 70). Future work on carbon isotope  
532 fractionation by cyanobacteria should grapple in more detail with photosynthetic physiology,  
533 including the separate uptake of  $\text{C}_i$  from external  $\text{CO}_2$  and  $\text{HCO}_3^-$  pools, integration of both light  
534 and dark reactions, and effects of nutrient limitation. As mechanistic biochemical understanding  
535 of cyanobacterial  $\text{C}_i$  uptake improves (51), it may also become feasible to directly measure or  
536 better constrain the isotopic fractionation associated with these processes. Coupling such a  
537 model with experiments in natural and engineered organisms will help validate the model and  
538 improve our ability to understand environmental and evolutionary changes to the carbon cycle  
539 across Earth history.

540 Carbon fixation was a fundamental challenge that autotrophs overcame early in the  
541 history of Earth's biosphere (8). These early processes were recorded in some fashion in the  
542 carbon isotope record, but robust interpretation of this record must take into account that the  
543 carbon cycle is an amalgam of both environmental changes and evolutionary processes,  
544 mediated by physiology. As a starting place, Earth scientists often apply uniformitarian  
545 assumptions (i.e. assuming that physical and chemical processes behave the same now as they  
546 did billions of years ago) in order to reason about the past. Such an approach is powerful but  
547 these assumptions are challenged by biological processes that undergo substantial evolution on  
548 geologic timescales. Recent work has used statistical inference to reconstruct ancestral gene  
549 sequences and resurrect ancient proteins in order to study biological evolution over geologic  
550 timescales; and was used to reconstruct the ancestral rubisco studied here (38). Here, and in  
551 the companion paper by Flamholz et al., we took a "synthetic biological" approach, constructing  
552 modern organisms with ancestral components so that specific aspects of ancient organisms can  
553 be isolated and tested. These "ancestral-like" organisms helped sharpen our understanding of  
554 the physiological and environmental factors determining growth (Flamholz et al.) and isotopic  
555 fractionation (this work) in both ancient and modern autotrophs, and showed that models rigidly  
556 based on modern taxa are likely not universally applicable across geologic timescales.  
557 However, we now have synthetic biological approaches that offer a way to probe these long  
558 timescale co-evolutionary problems by producing ancient process analogs of carbon fixation in  
559 the laboratory.

560

## 561 Materials and Methods

### 562 **Ancestral enzyme reconstruction**

563 Ancestral Rubisco enzyme sequences were previously reported and characterized by Shih et al.  
564 (2016) (38). Briefly, for both the large subunit (LSU) and small subunit (SSU) of Rubisco,  
565 encoded by *rbcL* and *rbcS* respectively, the most recent common ancestor (MRCA) for Form 1A  
566 ( $\alpha$ ), 1B ( $\beta$ ), and 1A/B ( $\alpha/\beta$ ) clades were predicted from independently derived phylogenetic  
567 trees for RbcL and RbcS containing a broad diversity of Form 1A and 1B Rubisco (>100  
568 sequences). Maximum-likelihood algorithms were used to reconstruct the most probable  
569 ancestral sequence for each clade. Ancestral sequences were then expressed in *Escherichia*  
570 *coli* and purified, and enzyme kinetics were measured.

571

### 572 **ANC strain generation**

573 The 'ANC' strain studied here was generated by replacing the native large and small Rubisco  
574 subunits (*cbbL* and *cbbS* respectively) of the parent strain (*Synechococcus elongatus* PCC  
575 7942) with the reconstructed  $\beta$  ancestral *cbbL* and *cbbS* sequences. The NS2-KanR ('WT'  
576 strain) was generated by inserting a KanR cassette into neutral site 2 (NS2) (GenBank:  
577 U44761.1). *Synechococcus elongatus* PCC 7942 were transformed using the approach of  
578 Golden and Sherman (1984) (71). Briefly, cultures were grown to OD750nm = 0.5. Cultures  
579 were centrifuged at 18,000 x *g* for 2 minutes. Pellets were washed with 100 mM CaCl<sub>2</sub> and spun  
580 again at 18,000 x *g* for 2 minutes. Pellets were resuspended in BG-11 media followed by  
581 addition of plasmid and grown for 16 hours in the dark at 30°C. Transformants were then plated  
582 onto BG-11 + KAN100 agar plates and placed under 100  $\mu$ E of light at 30°C. Single colonies  
583 were then genotyped by PCR amplification of the Rubisco locus followed by sequencing. Table  
584 S1 lists plasmids and primers used in this study.

585

### 586 **Growth conditions**

587 For ambient CO<sub>2</sub> growth, NS2-KanR and  $\beta$  Ancestral Rubisco-KanR strains were grown in  
588 quadruplicate in a photobioreactor (Photon Systems Instruments - MC 1000) at the University of  
589 California, Berkeley (UC Berkeley) for four biological replicates total. Cultures were grown in  
590 buffered BG-11 media with 50mM HEPES at pH 8. Cultures were inoculated at a starting  
591 OD720nm = 0.015 and cultivated at 120  $\mu$ E, 30°C, and bubbled with ambient air. High CO<sub>2</sub>  
592 growth was performed using the same conditions as ambient growth with the exception of  
593 placing the photobioreactor in a 5% CO<sub>2</sub> chamber (Percival AR22L) and bubbling in air from the  
594 chamber. High light growth was performed using the ambient conditions above with the  
595 exception of using 500  $\mu$ E for light intensity. Cells were harvested by centrifugation at 6000 x *g*  
596 for 20 minutes at 4°C. Decanted pellets were then flash frozen with liquid N<sub>2</sub> and lyophilized  
597 overnight with the Millrock Technology Model BT85A freeze dryer. Doubling time was calculated  
598 by fitting the exponential phase of growth (*k*) using a Markov Chain Monte Carlo (MCMC)  
599 approach, using the generic model  $y = a \cdot \text{EXP}(k \cdot x) + b$ . Growth curves displayed in Figure 2 were  
600 smoothed with a rolling median (*n* = 12) to remove errant readings caused by bubbles advected  
601 in front of the detector. See Supplemental for more information.

602

### 603 **Carbon isotope analysis**

604 Carbon isotope data is reported using delta notation ( $\delta^{13}\text{C}$ ) in units of per mille (‰) where  $\delta^{13}\text{C}$   
605 =  $[(^{13}\text{C}/^{12}\text{C})_{\text{sa}} / (^{13}\text{C}/^{12}\text{C})_{\text{ref}} - 1] \cdot 1000$ , where the subscripts 'sa' and 'ref' denote sample and  
606 reference respectively. The reference used is the Vienna Pee Dee Belemnite (VPDB).  $\delta^{13}\text{C}$



607 values of cyanobacterial cells were measured on an EA-IRMS (Elemental Analyzer Isotope  
608 Ratio Mass Spectrometer; Costech Thermo Delta-V) at the California Institute of Technology  
609 (Caltech) in Pasadena, CA. Each biological replicate was run four times with two different  
610 isotope standards – urea (-27.8‰) and sucrose (-10.45‰). A suite of urea and sucrose  
611 standards were run at the beginning, middle, and end of run for sample bracketing and to  
612 assess drift throughout the run. An average  $\delta^{13}\text{C}$  and standard error were calculated and  
613 reported for each biological replicate (see Supplemental for more information). The  $\delta^{13}\text{C}$  of the  
614 starting  $\text{CO}_2$  gas was measured on the Thermo Mat 253 Ultra at Caltech. The CALT-2049C  
615 standard was used, which has a  $\delta^{13}\text{C}_{\text{VPDB}}$  value of -3.62‰.  $\text{CO}_2$  gas from high  $\text{pCO}_2$   
616 experiments was sourced from a  $\text{CO}_2$  tank, while the  $\text{CO}_2$  gas in ambient  $\text{pCO}_2$  experiments  
617 was distilled from ambient lab air through cryogenic distillation at Caltech.  $\epsilon_p$ , the carbon isotope  
618 fractionation between  $\text{CO}_2$  gas and bulk cyanobacterial cells, was calculated as  $(\alpha_{\text{CO}_2/\text{bio}} -$   
619  $1) \times 1000$ , where  $\alpha_{\text{CO}_2/\text{bio}} = {}^{13}\text{R}_{\text{CO}_2}/{}^{13}\text{R}_{\text{bio}}$ , where  ${}^{13}\text{R}$  is the ratio of  ${}^{13}\text{C}$  to  ${}^{12}\text{C}$  in the analyte. We note  
620 this in contrast to other isotope literature where  $\epsilon_p$  is calculated as  $\alpha_{\text{bio}/\text{CO}_2} - 1) \times 1000$ , which would  
621 cause the positive values in this study to be negative. In this study, more positive  $\epsilon_p$  values  
622 indicate more C-13 depleted; see Supplemental for more detail.

623

#### 624 **Rubisco KIE assay**

625 *Syn6301* and  $\beta$ -MRCA Rubisco were purified according to previous methodologies (72, 73) at  
626 University of California, Davis and then shipped on dry ice to Caltech. Clarified lysate from a  
627 BL21 DE3 Star *E. coli* culture expressing Rubisco was subjected to ammonium sulfate  
628 precipitation, at the 30-40% cut for *Syn6301* and at the 40-50% cut for  $\beta$ -MRCA, followed by  
629 anion exchange chromatography and size exclusion chromatography. We then used the  
630 substrate depletion method to measure the KIE of the *Syn6301* and  $\beta$ -MRCA Rubiscos ( $\epsilon_{\text{Rubisco}}$ ),  
631 as used previously in similar studies (41–44). Briefly, an assay mix of  $\text{HCO}_3^-$ , bovine carbonic  
632 anhydrase, Rubisco, ribulose 1,5-bisphosphate (RuBP),  $\text{MgCl}_2$ , bicine, and dithiothreitol (DTT)  
633 was prepared. As the reaction progressed to completion, aliquots of that assay mix were  
634 injected into pre-filled exetainers containing phosphoric acid that both stopped the reaction and  
635 converted all inorganic carbon species to gaseous  $\text{CO}_2$ . The  $\delta^{13}\text{C}$  of these  $\text{CO}_2$  aliquots was  
636 then measured on a Delta-V Advantage with Gas Bench and Costech elemental analyzer at  
637 Caltech. Here, instead of RuBP being given in excess,  $\text{CO}_2$  was given in excess. In addition,  
638 instead of determining the fraction of  $\text{CO}_2$  ( $f$ ) consumed independently to create a Rayleigh plot,  
639 we fit the curvature of the  $\delta^{13}\text{C}$  results to find  $f$  before converting to a Rayleigh plot to calculate  
640  $\epsilon_{\text{Rubisco}}$ , similar to previous studies (42). See Supplemental for more information.

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