Synthetic Photosynthetic Consortia Define Interactions Leading to Robustness and Photoproduction

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Running Head: Synthetic Photosynthetic Consortia Interact & Produce

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ABSTRACT

Microbial consortia composed of autotrophic and heterotrophic species abound in nature, yet examples of synthetic communities with mixed metabolism are limited in the laboratory. Herein, we construct artificial synthetic consortia consisting of sucrose-secreting cyanobacteria, *Synechococcus elongatus* PCC 7942, paired with three disparate heterotrophs: *Bacillus subtilis, Escherichia coli*, or *Saccharomyces cerevisiae*. Comparison of these different dyads highlights underlying interactions in co-culture. We witness both engineered and emergent interactions between cyanobacterium and heterotroph shared across heterotrophic species. Heterotrophs can consume carbon fixed photosynthetically by cyanobacteria while non-sucrose byproducts of photosynthesis can negatively impact heterotroph growth. Surprisingly, all tested heterotrophic species can positively impact cyanobacterial growth in co-culture in comparison to monoculture. Growth of co-cultures is witnessed in batch and continuous culture as well as on agar plates. Co-cultures persist long-term and can survive perturbation, particularly when heterotrophic sucrose uptake is enhanced and/or heterotrophic sensitivity to byproducts of photosynthetic metabolism is mitigated. This level of robustness is infrequently witnessed in synthetic microbial communities. Furthermore, by exchanging partner heterotrophs, we demonstrate flexible, phototrophic production of alpha-amylase and polyhydroxybutyrate in co-cultures containing specialized strains of *B. subtilis* and *E. coli,* respectively. Production can be improved via engineered intervention, i.e. increased efficiency growing on sucrose, showing promise for future tuning of these communities as production platforms. Altogether these synthetic microbial consortia provide a platform to study autotroph-heterotroph interactions, while demonstrating promising

flexibility and stability for potential photoproduction strategies that capitalize on multi-species interactions.

IMPORTANCE

We describe a series of synthetic communities in which engineered cyanobacteria fix and secrete carbon to support growth of a broad range of evolutionarily-unrelated model heterotrophs. Because of the unprecedented flexibility of this consortia design, we can begin to characterize engineered and emergent interactions shared across multiple autotroph/heterotroph pairings. These observations allow us to evaluate characteristics and design principles that influence consortia robustness in a species-independent manner. For example, cyanobacterial productivity is improved by cohabitation with a broad range of heterotrophic species; an important observation for microalgal bioproduction. We show that the modular nature of our communities also allows them to be readily "reprogrammed" for photoproduction of a variety of compounds by substitution of the heterotrophic partner species. The unusual robustness and flexibility exhibited by our engineered consortia demonstrate promise as a platform that could be developed for the study of nascent symbioses, or as a highly-versatile photoproduction strategy.

INTRODUCTION

Microbial communities dominate natural environments while, historically, axenic experiments monopolize laboratories. While much has been gleaned from isolated microbes, there is increasing emphasis on the study of complex natural consortia. This interest is driven by the ubiquity and diversity of microbiomes related to human health, ecology, and bioindustrial applications. Microbial consortia divide metabolic labor to capitalize on environmental resources, are resistant to invasion, and are robust when faced with perturbations, all characteristics that are interesting biologically as well as desirable in engineered systems (1–3). Yet natural consortia inherently contain many species that are highly integrated; disentangling the interactions and functions within a community can be difficult (4–7).

Designed microbial communities can be used to gain insight into microbial ecology or be applied to industry and remediation. *Artificial consortia* consist of wild-type microbes with naturally synergistic interactions, while *synthetic consortia* are microbial communities engineered to interact (e.g. via signaling molecules or traded metabolites) - where the introduced communication and/or species are not found in nature (3). Because artificial consortia interact based on endogenous pathways, often in genetically intractable strains, tuning interactions to explore different questions or to program new functions is difficult relative to synthetic communities. Indeed, tunable designs within the nascent field of synthetic consortium engineering have provided bottom-up insights into complex dynamics including population behavior, game theory, pattern formation, and cross-feeding (8–13).

84

85 Despite their promise, synthetic systems have often been limited in construction
86 to engineered signals (i.e. quorum sensing) or complementary auxotrophies within
87 highly-related organisms (13–17). The inflexibility of most consortia designs means that
88 engineered interactions are highly contextualized to a limited number of species,
89 confounding the identification of general trends in interaction rather than scenario-
90 specific observations. Additionally, species with different growth modes (i.e. autotrophs
91 and heterotrophs) are relatively rare in synthetic designs in contrast to natural consortia
92 (18–20). Finally, contrary to the robustness seen in natural microbial communities,
93 synthetic consortia are frequently fragile, functioning only for short time frames or
94 requiring artificially structured environments (21, 22). Because of these limitations in
95 scope, as well as the scarcity in number of synthetic communities, academic and
96 industrial application of such consortia is restricted.

97

98 A *Synechococcus elongatus* PCC 7942 strain previously engineered to secrete
99 sucrose is ideally suited as a phototroph for incorporation into consortia with heterotrophs
100 (23). When faced with osmotic pressure, *S. elongatus* accumulates the compatible solute
101 sucrose (24, 25) which can be exported through heterologous expression of the sucrose
102 transporter gene, *cscB*. CscB is a proton/sucrose symporter (26, 27) that functions to
103 secrete sucrose in cyanobacteria because of high environmental pH (pH 8). *CscB*$^{+}$ *S.*
104 *elongatus* can divert up to 80% of photosynthetically-fixed carbon to soluble sucrose,
105 generating 36 mg of sucrose per liter per hour of illumination. Furthermore, expression of

106 *cscB* in this *S. elongatus* strain can be regulated via induction with isopropyl-beta-D-1-
107 thiogalactopyranoside (IPTG) (23).
108
109 In this work, we pair *cscB*$^+$ *S. elongatus* with varied heterotrophs (*Escherichia*
110 *coli*, *Bacillus subtilis*, or *Saccharomyces cerevisiae*) to investigate general rules of
111 microbial interaction while maintaining consortia tunability. In these consortia, *S.*
112 *elongatus* provides the sole carbon source for consumption by the heterotrophic species.
113 We see growth of both heterotrophs and cyanobacteria within all consortia, detail
114 emergent interactions between microbes, and demonstrate capacity for long-term
115 community persistence. Flexibility in community composition allows consortia to be
116 functionalized for photoproduction of metabolites and proteins via co-culture with
117 specialized heterotrophic partners. Together these findings develop a versatile platform to
118 investigate interactions between phototrophs and heterotrophs while showing promise as
119 functionalized co-cultures for bioproduction.
120
121 **MATERIALS AND METHODS**
122 **Strains, media, and axenic characterization.** *S. elongatus* PCC7942 (obtained from
123 ATCC #33912) was engineered to secrete sucrose through the expression of the sucrose
124 symporter CscB (23). Axenic cyanobacteria were checked for contamination via plating
125 on rich media. *E. coli* W was obtained from ATCC (#9637) and the corresponding W
126 Δ*cscR* strain was generously provided by Dr. Claudia Vicker's laboratory (28). *B. subtilis*
127 168 was obtained from ATCC (#23857) and *B. subtilis* 3610 *ΔsinI* was generously
128 provided by the lab of Dr. Richard Losick (29). A *ΔsinI* mutant strain of 3610 was used

129 to minimize chained growth making CFU counts of the strain reproducible (29). All
130 strains are listed in Table 1. *S. elongatus* was propagated in BG-11 (Sigma Aldrich) plus
131 1 g/L HEPES, pH 8 in constant light at 35 °C. *B. subtilis* and *E. coli* were propagated in
132 Luria Broth (LB) while *S. cerevisiae* was maintained in YEPD media. *E. coli*, *B. subtilis*,
133 and *S. cerevisiae* were struck from frozen stocks on rich media plates (LB for bacteria
134 and YEPD for yeast). Co-culture media were optimized for either prokaryotes (^{CoB}BG-11)
135 or *S. cerevisiae* (^{CoY}BG-11). ^{CoB}BG-11 consists of BG-11 supplemented with 106mM
136 NaCl, 4mM NH_4Cl and 25mM HEPPSO, pH 8.3-KOH. Indole (100 uM) was added to *B.*
137 *subtilis* 168 co-cultures as indicated and in alpha-amylase experiments. ^{CoY}BG-11 consists
138 of BG-11 supplemented with 0.36g/L Yeast Nitrogen Base without amino acids (Sigma
139 Aldrich), 106mM NaCl, 25mM HEPPSO, pH 8.3-KOH and 1mM KPO_3. Solid co-culture
140 plates were composed of ^{CoB}BG-11 media with 1% autoclaved noble agar (BD
141 Biosciences).
142
143 For characterization of *S. elongatus* growth and sucrose production, *S. elongatus*
144 was cultured axenically in baffled flasks of ^{CoB}BG-11 or ^{CoY}BG-11 and allowed to
145 acclimate for ≥ 12 hours. At time 0, 25 mL cultures were adjusted to OD_{750} = 0.5 and
146 1mM IPTG was added, as appropriate. Cultures were monitored at 24 hour intervals by
147 withdrawal of 1mL culture. $OD_{750}$was measured via photospectrometer (ThermoScientific
148 NonoDrop 2000c) and culture supernatant was analyzed for sucrose content via a
149 colorimetric Glucose-Sucrose Assay (Megazyme). To prepare heterotrophic strains,
150 single colonies were picked into their respective rich media and grown until turbid at
151 varying temperatures before co-culture (37 °C for *E. coli* and *B. subtilis*; 30 °C for *S.*

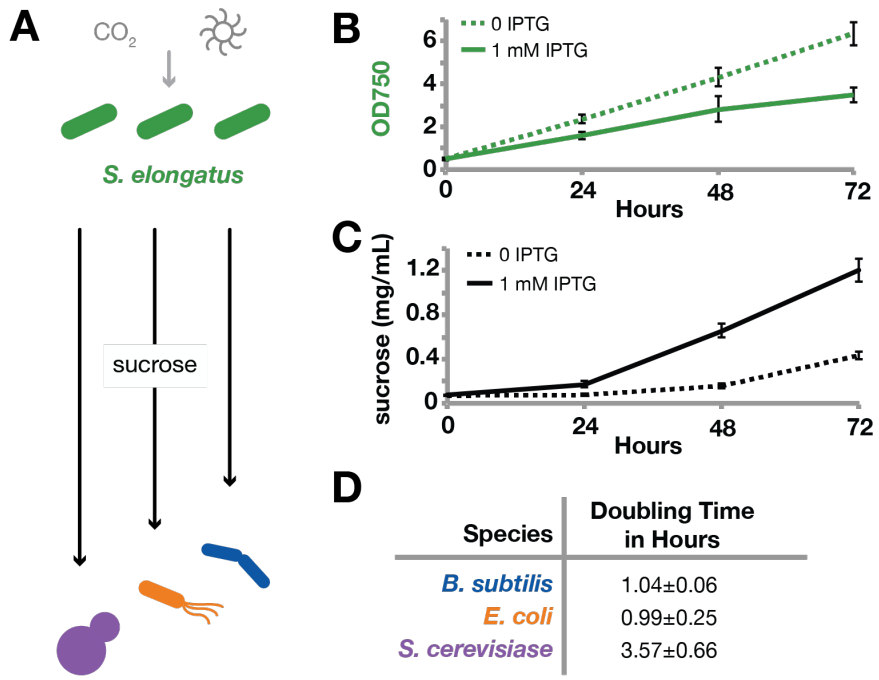
152 *cerevisiae*). Cells were diluted into the appropriate co-culture media +2% sucrose to
153 acclimate to co-culture media, and maintained within log phase growth (OD_{600} < 0.70)
154 before use in co-cultures. All acclimating cultures and co-cultures were grown at 35 °C,
155 150 rpm, 2% CO_2, in light (PAR = ~80μmol with 15W Gro-Lux Sylvania fluorescent
156 bulbs) within a Multitron Infors HT incubator. Heterotrophic growth was measured by
157 inoculating rinsed cells at 0.01 OD_{600} (bacteria) or 0.05 OD_{600} (yeast) into fresh co-culture
158 media at the indicated sucrose concentration. Data for growth rate was collected from 25
159 mL flask cultures while 96-well plates with 1 mL culture volumes were used to assay the
160 gradient of [sucrose] in Fig. 2C; plates were read on a BioTek Synergy Neo plate reader.
161
162 **Batch co-cultivation & quantification.** Flask co-cultures were completed in 25 mL
163 volumes in baffled flasks. Cyanobacteria and heterotrophs were acclimated to ^{CoB}BG-11
164 or ^{CoY}BG-11 media prior to inoculation into co-cultures. All co-cultures were grown at 35
165 °C, 150 rpm, 2% CO^2, in light (15W; Gro-Lux; Sylvania) within a Multitron Infors HT
166 incubator. 1 mM IPTG was added when indicated. Growth in co-cultures was monitored
167 every 12 hours: *S. elongatus* was measured by the count of gated red-fluorescent events
168 on a quantitative flow cytometer (BD Accuri); heterotrophs were assayed by plating
169 dilution series on rich media to count colony forming units (CFU). Estimates of
170 $W303^{Clump}$ cell number were derived by counting CFUs, but numbers were adjusted for
171 the ~6.6 cells/clump as previously reported (31), and as confirmed under our culture
172 conditions. For dilution experiments, co-cultures containing *E. coli* or *B. subtilis* were
173 grown for 24 hours before 10 or 100 fold dilutions.
174

175 **Heterotroph exposure to variable [cyanobacteria].** *B. subtilis* and *E. coli* were
176 recovered from rich media as above, washed in ^{CoB}BG-11 and inoculated at an OD_{600} of
177 .01 in ^{CoB}BG-11 media + 2% sucrose with cyanobacteria at different densities (OD_{750} 0,
178 0.5, 1, and 2). *S. cerevisiae* was treated identically except they were inoculated at ~$3x10^5$
179 cells/mL (OD_{750} = 0.03) and ^{CoY}BG-11 was used. These samples were split into two 36-
180 well plates and incubated and exposed to either constant light or dark conditions while
181 maintaining the other growth parameters. Heterotroph counts were determined by plating
182 on rich media for colony counts as above after initial setup (time 0) and after 12 hours of
183 culture. Ratios of the viable cell counts from the light vs. dark cultures or log_{10} of these
184 ratios after 12 hours are reported.
185
186 **Structured growth perturbation.** To test the ability of co-cultures to withstand
187 environmental perturbation, flask co-cultures were inoculated and grown as previously
188 described for 24 hours before plating on 100 uL of co-cultures were plated on solid co-
189 culture Petri dishes. After five days, uneven lawns of heterotrophs and cyanobacteria
190 arose. Cells were picked from these plates into 96-well plates and allowed to grow for 2-5
191 additional days. Any well that demonstrated cyanobacterial growth (as judged visually by
192 green appearance) by the end of 48 hours was spotted on rich media to determine the
193 presence or absence of heterotrophic symbionts. Solid culture and 96-well plate growth
194 was completed at 35 °C, 0 rpm, 2% CO^2, in constant light (15W; Gro-Lux; Sylvania)
195 within a Multitron Infors HT incubator.
196

197 **Heterotroph spotting on cyanobacterial lawns.** Lawns of *cscB*$^+$ cyanobacteria were
198 achieved via spreading of 250 uL of *cscB*$^+$ cyanobacteria (OD_{750} 0.5) on solid co-culture
199 plates with or without 1 mM IPTG. After the cyanobacteria had absorbed on to the plate
200 (>3 hours in the dark), 3 uL drops of heterotrophs were spotted on to the lawns.
201 Heterotrophs had been previously grown up in rich media and washed three times to
202 remove any media components before spotting. Media blanks and boiled cells were
203 spotted as negative controls. Plates were then grown in 35 °C, 2% CO^2, in constant light
204 (15W; Gro-Lux; Sylvania) within a Multitron Infors HT incubator.
205
206 **Long-term continuous co-cultivation.** Long-term co-cultures were incubated in
207 Phenometrics Environmental Photo-Bioreactors (33) with 150mL liquid volumes of a
208 mix of *cscB*$^+$ *S. elongatus* with either *S. cerevisiae* W303^{Clump} or *E. coli* W Δ*cscR* in the
209 appropriate co-culture BG-11 media + 1mM IPTG. Reactors were seeded with ~1x10^8
210 cells/mL of *S. elongatus* (OD_{750} = 0.5) and a final concentration of heterotroph equivalent
211 to ~1x10^6 W303^{Clump} cells/mL (final OD_{600} ~0.1) or ~5x10^7 W Δ*cscR* cells/mL (OD_{600}
212 ~0.05). Light was provided by onboard white, high-power LEDs (400μmol m^2 s^2)
213 continuously for *E. coli* W Δ*cscR* cultures, and with a 16:8 light:dark photoperiod for
214 W303^{Clump} co-cultures. The total density of co-cultures were monitored by onboard
215 infrared diodes, following a brief (3-12 hour) acclimation period where the time-averaged
216 optical density was allowed to settle to a fixed point following culture initiation, this
217 measurement was used to control attached peristaltic pumps that eject fresh media to
218 maintain the set target OD as previously described (33). Co-culture temperature was
219 maintained at 30°C by a heated jacket; cells were agitated continuously by a stirbar.

220 Daily, ~2 mL of co-culture volume was withdrawn and cyanobacterial and heterotrophic
221 cell counts determined by flow cytometry and plating, respectively (as described
222 above).
223
224 **Alpha-amylase production and quantification.** For production of alpha-amylase from
225 *B. subtilis* strain 168 was used in 8 mL cultures of ^{CoB}BG-11 with 100 uM indole in 6 well
226 dishes. Alpha-amylase production was measured after 24 hours of culture. Alpha-amylase
227 activity in supernatants was measured immediately after pelleting of cultures with the
228 EnzChek Ultra Amylase Assay Kit, Molecular Probes Life Technologies using the
229 manufacturer's protocol. Western blots confirmed presence of alpha-amylase in
230 supernatants after addition of NuPAGE LDS sample buffer (Invitrogen) followed by 10
231 minutes at 100 °C. Protein (10 uL) was run on NuPage 4-12% Bis-Tris gels (Life
232 Technologies) for in MES SDS running buffer for 50 minutes at 185V. The iBlot 2 Dry
233 Blot System (ThermoScientific) was used to transfer protein to nitrocellulose membranes
234 (iBlot 2 NC Regular Transfer Stacks). Anti-alpha amylase antibodies (polyclonal rabbit;
235 LS-C147316; LifeSpan BioSciences; 1:3,000 dilution) were used as the primary antibody
236 followed by peroxidase-conjugated donkey anti-rabbit antibodies (AffiniPure 711-035-
237 152 lot 92319; Jackson ImmunoResearch; 1:5,000 dilution) as the secondary antibody.
238 The western blot was visualized via Western Lightning® Plus-ECL, Enhanced
239 Chemiluminescence Substrate (PerkinElmer, ProteinSimple FluorChem M). Purified
240 alpha-amylase (Sigma Aldrich) was used as a control in all assays.
241

242 **PHB Production & quantification.** *E. coli* strains were transformed with pAET41
243 (Table 1; 32) before use in co-cultures for production. Co-cultures were set up as
244 described in 25 mL flasks. After one week of growth, the entire culture was spun down,
245 frozen, and stored at -80 until PHB content was quantified. PHB content was quantified
246 by standard methods (34, 35). Briefly: cell pellets were digested with concentrated H_2SO_4
247 at 90 °C for 60 min. The digestion solution was diluted with H_2O by 500 times and
248 passed through 0.2 μm filter. The solutions were subsequently analyzed by a high
249 performance liquid chromatography (HPLC, Agilent HPLC 1200) equipped with Aminex
250 HPX-87H column and UV absorption detector (35). The volume of each sample injection
251 was 100 μL. The mobile phase was 2.5 mM H_2SO_4 aqueous solution, with a flow rate of
252 0.5 mL/min for 60 min. 5 mM sodium acetate (Sigma Aldrich) was added as an internal
253 standard. The concentrations of PHB were determined by comparing the peak area with
254 that in standard curves from 0.1 ~ 30 mM.
255
256 **RESULTS**
257 **Cyanobacteria consortia with heterotrophs**
258 Systems where heterotrophs depend on a photosynthetic partner for growth were
259 developed. Engineered cyanobacteria, *cscB*$^+$ *S. elongatus,* forms the photosynthetic
260 component – carbon dioxide is fixed and made accessible to various heterotrophs for
261 growth (23). Model heterotrophic species with well-developed molecular tools were
262 selected, and include Gram-positive and Gram-negative bacteria as well as yeast (Fig.
263 1A).



Species	Doubling Time in Hours
B. subtilis	1.04±0.06
E. coli	0.99±0.25
S. cerevisiase	3.57±0.66

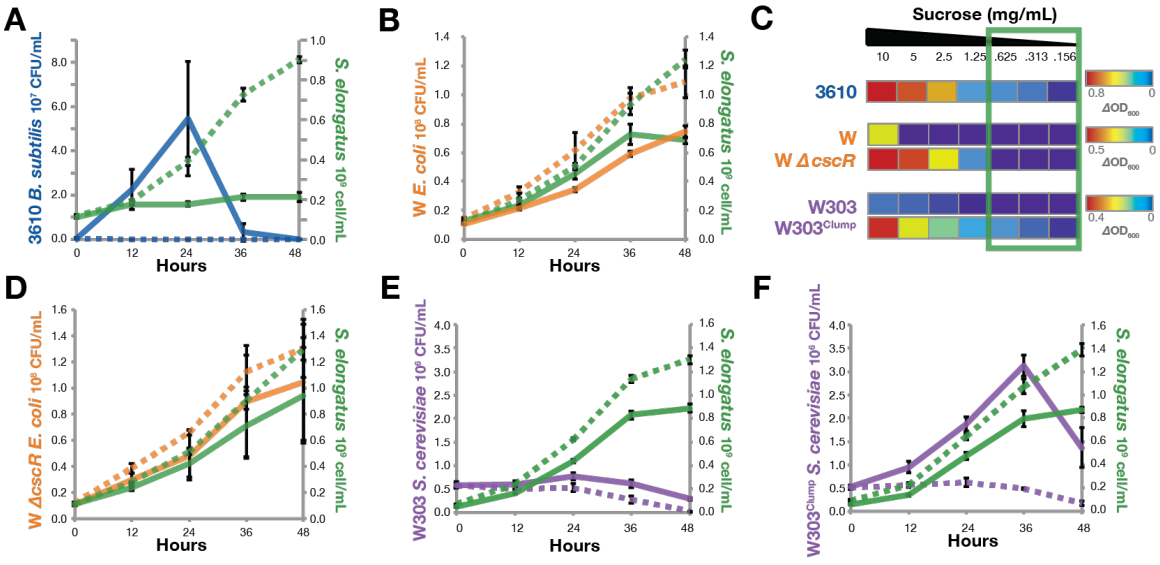
Fig. 1 Axenic characterizations of candidate strains.

(**A**) This schematic shows the engineered microbial community design - *cscB*$^+$ *S. elongatus* (green) capture light and CO_2 via photosynthesis. Fixed carbon is then secreted as sucrose (black arrows) to support the growth of *B. subtilis* (blue), *E. coli* (orange), or *S. cerevisiae* (purple). Axenic *cscB*$^+$ *S. elongatus* was grown in ^{CoB}BG-11 with (solid line) and without IPTG (dashed line) to induce sucrose secretion. Cell density (**B**) and sucrose levels in culture supernatants (**C**) were measured. Error bars are standard deviation of 8 biological replicates. For characterization of cyanobacteria in ^{CoY}BG-11 see Fig. S1. (**D**) Heterotroph growth in isolation was characterized via growth rate in co-culture buffer supplemented with 2% sucrose Error is standard deviation of ≥ 3 replicates.

Growth of consortia was designed to rely upon sucrose secretion by *cscB*$^+$ *S. elongatus* in response to osmotic pressure and IPTG induction followed by sucrose consumption by heterotrophs. We optimized media with compositions of nitrogen, salt, and buffer – these are termed ^{CoB}BG-11 for use in cyanobacteria/bacteria consortia and ^{CoY}BG-11 for the cyanobacteria/yeast consortium (see Materials and Methods). We

verified that *S. elongatus* grows and produces sucrose in these media (Fig. 1B, Fig. S1). When *cscB* expression is induced, growth of the cyanobacteria is slightly impaired due to the efflux of carbon (Fig. 1B,1C, Fig. S1A; Ducat et al. 2012). Consequently, increased amounts of sucrose are present when *S. elongatus* is induced to express *cscB* (Fig. 1C, Fig. S1B). Separately, we verified that all heterotrophs are capable of growth in these media when exogenous sucrose (2%) is provided as the sole carbon source (Fig. 1D).

Sucrose-producing cyanobacteria, *cscB*$^{+}$ *S. elongatus,* directly support heterotroph growth in co-cultures that contain no external carbon sources (Fig. 2, Fig. S2). In all consortia, *S. elongatus* grows with a single heterotrophic microbe in the appropriate co-culture media (see Materials and Methods) with or without 1mM IPTG to induce *cscB* expression and sucrose export. Growth of cyanobacteria and viable heterotrophs in co-cultures were tracked over 48 hours in constant light via flow cytometry and analysis of colony forming units (CFUs), respectively.



296 **Fig. 2 *S. elongatus* supports microbial communities in batch culture.** Batch cultures of *cscB*$^+$ *S.*
297 *elongatus* in co-culture with *B. subtilis* (blue), *E. coli* (orange), or *S. cerevisiae* (purple) were grown in
298 constant light. *cscB*$^+$ *S. elongatus* counts/mL were determined by flow cytometry every 12 hours for co-
299 cultures containing *B. subtilis* (**A**; green), *E. coli* (**B**, **D**; green), and *S. cerevisiae* (**E**, **F**; green). Co-cultures
300 with uninduced (dashed lines) or induced CscB expression (solid lines) were tested. Heterotroph viability
301 was monitored by colony forming unit (CFU) for all *B. subtilis* (**A**; blue), *E. coli* (**B** strain W, **D** strain W
302 Δ*cscR*; orange) and *S. cerevisiae* (**E** strain W303, **F** strain W303^{Clump}; purple) co-cultures. Data for A, B, D,
303 E, and F, are representative, same-day experiments where error bars are the standard error in 3 biological
304 replicates. Additional replicates in Fig. S2. (**C**) Axenic heterotroph growth was tested in defined media
305 with varying concentrations; the range of sucrose that *cscB*$^+$ *S. elongatus* can secrete in 48 hours is denoted
306 by a green box. Average OD_{600} is shown as a metric of growth for ≥ 6 biological replicates). OD_{600} was
307 correlated to viable colony forming units (CFU) in Fig. S3. No contaminants/heterotrophic colonies grew
308 from axenic controls.
309
310 *B. subtilis* growth in co-culture is dependent on IPTG-induced sucrose secretion
311 (Fig. 2A, Fig. S2A). Without induction of *cscB* to enable sucrose secretion, *B. subtilis*
312 fails to grow showing sensitivity to sucrose availability. When IPTG is present in co-
313 cultures, the growth of *B. subtilis* in co-culture is not monotonic; viability decreases after
314 48 hours of induction.
315
316 *E. coli*, in contrast, grows monotonically independent of induction of sucrose
317 secretion (Figure 2B, Figure S2B & S2C). In axenic culture, *E. coli* W only exhibits
318 growth when supplemented with > 5 mg/mL sucrose (Fig. 2C), yet *cscB*$^+$ *S. elongatus*
319 secretes ~1 mg/mL of sucrose by in 48 hours of growth (Fig. 1C). We also tested the
320 growth of *E. coli* engineered for increased growth in low sucrose (hereafter referred to as

321 the *ΔcscR E. coli* strain; Arifin et al. 2011). Although *ΔcscR E. coli* grow on lower
322 concentrations of sucrose axenically (≥1.25 mg/mL; Fig. 2C), we observed the same
323 monotonic growth pattern with decreased growth in the presence of IPTG (Fig. 2A & 2C,
324 Fig. S2B & S2C). This suggests that in the first days of co-culture, while exported
325 sucrose concentrations are low (≤1 mg/mL), *E. coli* strains cannot utilize sucrose
326 effectively and dominantly depend on other metabolites from *S. elongatus*, such as
327 extracellular polymeric substances (EPS; Villa et al. 2015; Rossi & De Philippis 2015).
328
329 *S. cerevisiae* growth in co-culture requires genetic mutations to enhance sucrose
330 utilization. Wild type *S. cerevisiae* W303 did not grow in co-culture regardless of IPTG
331 induction. This is likely due to poor growth in low sucrose concentrations (Fig. 2C). To
332 overcome *S. cerevisiae* W303's limited growth, an engineered strain, hereafter referred to
333 as $W303^{Clump}$, was examined. $W303^{Clump}$ was derived from previous directed evolution
334 experiments of *S. cerevisiae* W303 in low sucrose media, which revealed mutations that
335 enhance fitness in dilute sucrose (31). $W303^{Clump}$ (originally called Recreated02;
336 Koschwanez et al. 2013), contains a nonsense mutation in ACE2 as well as other
337 causative fitness-enhancing mutations that arose (in genes CSE2, IRA1, MTH1, and
338 UBR1). Mutations to ACE2 compromise the degradation of the mother-daughter septum
339 resulting in clumps of cells (~6.6 cells/clump on average). These aggregates grow in low
340 sucrose due to increased local cell concentration and increased hexose availability after
341 extracellular cleavage of sucrose by an invertase (38). Unlike the parental strain,
342 $W303^{Clump}$ exhibited growth in low concentrations of sucrose when grown in isolation
343 (Fig. 2C). As a result, $W303^{Clump}$ grows in co-culture with *cscB*$^{+}$ *S. elongatus,* but only

344 when sucrose secretion was induced (Fig. 2D). Similar to *B. subtilis*, W303^{Clump} *S.*
345 *cerevisiae* demonstrate growth followed by decreased viability in co-culture over 48
346 hours.
347
348 Cyanobacterial light driven metabolism is the source of heterotroph growth
349 inhibition when sucrose is not limiting (Fig. 3A). The lack of monotonic growth in *B.*
350 *subtilis* and *S. cerevisiae* co-cultures indicate interactions beyond sucrose feeding
351 between heterotrophs and cyanobacteria (Fig. 2A, 2F). To focus on products other than
352 fixed carbon that influence heterotrophic viability and eliminate the confounding variable
353 that cyanobacteria only generate sucrose in the light (23), co-cultures were supplemented
354 with exogenous sucrose and cultivated in the light or dark. After 12 hours of co-
355 cultivation, heterotroph viability of each of the three species was determined, revealing
356 decreased growth or death correlated with increasing concentrations of cyanobacteria
357 solely in illuminated cultures (Fig. 3B-D, Fig. S4). This effect is most apparent in strains
358 of *B. subtilis* (Fig. 3B, Fig. S4A) where the viability of heterotrophic species decreases by
359 orders of magnitude when co-cultured in the light with high concentrations of *S.*
360 *elongatus*.

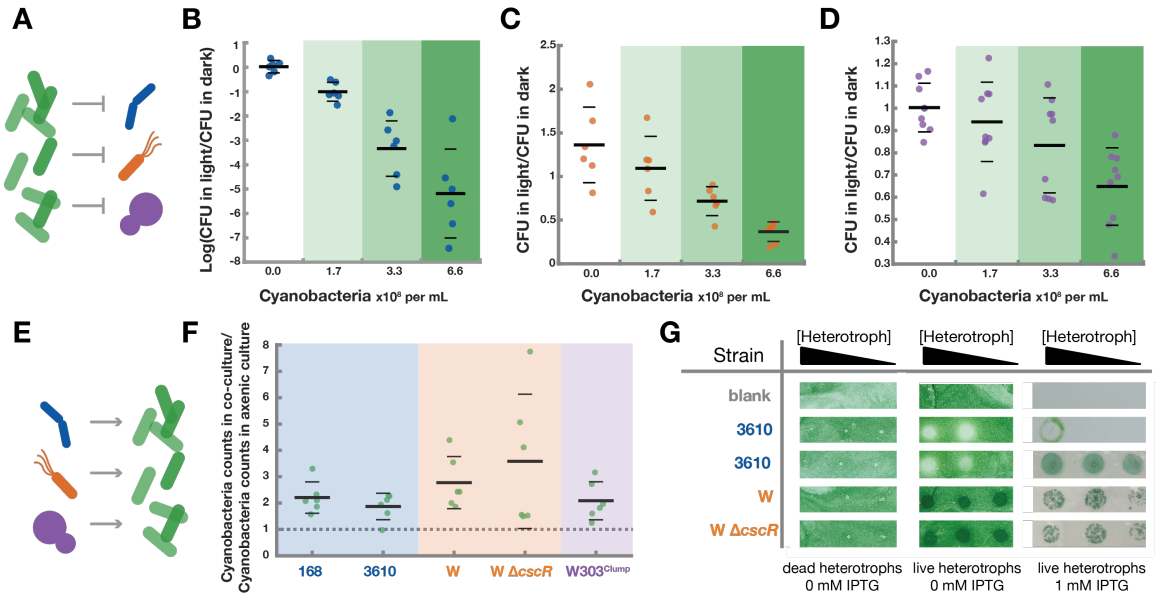
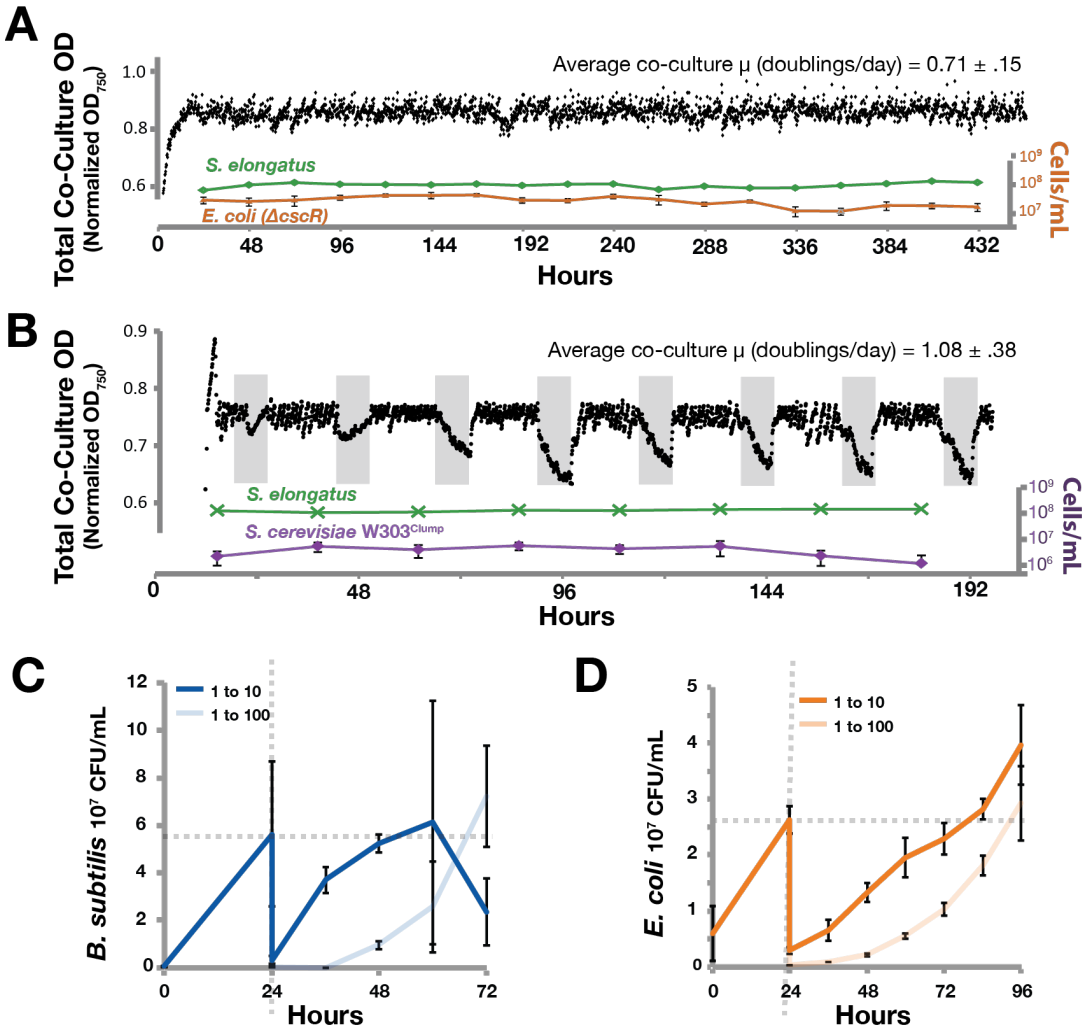


Fig. 3 Emergent microbial interactions. Engineered consortia demonstrate emergent interactions that can be classified into two categories: negative effects that cyanobacteria have on heterotrophs (**A**) and positive effects heterotrophs have on cyanobacteria (**E**). *B. subtilis* 3610 (**B**), *E. coli* W Δ*cscR* (**C**), and W303^{Clump} *S. cerevisiae* (**B**) were co-cultured with various concentrations of *S. elongatus* and heterotroph CFUs were determined after 12 hours of cultivation in either light or dark. Ratios of CFU in light compared to CFU in dark are reported (**B-D**). Additional strains were tested in Fig. S4. Positive effects of heterotrophs on cyanobacteria (**E**) were observed in liquid (**F**), evidenced by the ratio of cyanobacteria cells measured in co-cultures relative to axenic controls after 48 hours in constant light. These co-cultures were inoculated with two orders of magnitude fewer *cscB*$^{+}$ *S. elongatus* (~1.7x10^{6} cells/mL) than the co-cultures depicted in Fig. 2 (~1.7x10^{8} cells/mL), and 1 mM IPTG was added to all cultures to induce sucrose export. Thick horizontal lines represent the average measurement for each condition while thin horizontal lines represent one standard deviation from the mean. Positive effects of heterotrophs on cyanobacteria in previous liquid batch experiments is summarized in Fig. S5. The influence of heterotrophs on cyanobacterial growth on solid media (**G**) was determined by plating a dilute lawn of *cscB*$^{+}$ *S. elongatus* on ^{CoB}BG-11 agar plates. The cyanobacterial lawn was overlaid with the specified strain in ten-fold serial dilutions of heterotroph and in constant light with or without IPTG.

379 Conversely, co-culture with heterotrophs can stimulate growth of *S. elongatus*
380 (Fig. 3E, Fig. S5). This was observed during batch cultures when cyanobacteria counts
381 were higher in co-cultures then in control axenic cultures at various time points (Fig. S5).
382 Because batch cultures of relatively dense *S. elongatus* can negatively impact
383 heterotrophic viability (Fig. 2A & 2E, 3A-D), and also lead to significant self-shading,
384 we inoculated low concentrations of cyanobacteria induced with IPTG to secrete sugar in
385 co-culture with heterotrophs. After 48 hours of co-culture, cyanobacteria numbers in co-
386 culture were normalized to axenic controls. We observe significant increases in
387 cyanobacterial growth in the presence of heterotrophic microbes, with total cell counts
388 increasing by between 80 and 250% on average (Fig. 3F). This beneficial effect of
389 heterotrophs can persist on solid media. On a lawn of dilute cyanobacteria induced with
390 or without IPTG, we spotted dilutions of *B. subtilis* or *E. coli* (Fig. 3G). Areas containing
391 *E. coli* and cyanobacteria grew up more quickly than the surrounding lawn of *S.*
392 *elongatus* alone. The effect of *B. subtilis* on cyanobacteria was more variable and
393 dependent upon induced sucrose export. Without IPTG, spots of *B. subtilis* inhibited
394 cyanobacterial growth however in the presence of IPTG, *B. subtilis* could stimulate
395 growth on or in the vicinity of the spot it was plated (Fig. 3G). *S. cerevisiae* was not
396 assayed in this manner because of poor growth of cyanobacteria on ^{CoY}BG-11 solid agar
397 plates. Collectively, these experiments indicate that all three evolutionarily unrelated
398 heterotrophs can significantly increase cyanobacterial growth under a range of growth
399 conditions.
400
401 **Robustness in designed photosynthetic consortia**

402 Co-cultures persist through environmental perturbations. To determine the
403 robustness of each species to varied culture conditions, co-cultures were switched from
404 batch to continuous culture, exposed to alternating light/dark cycles, subjected to dilution
405 or alteration of environment (Fig. 4).
406

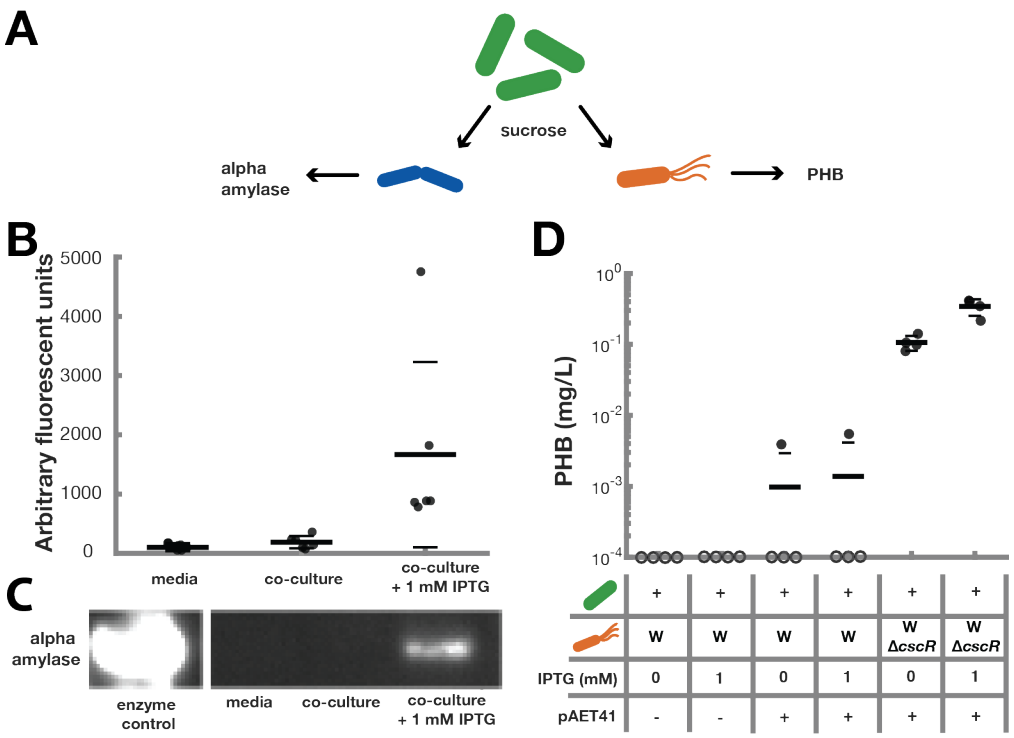


407

408 **Fig. 4 Co-cultures persist through time and perturbation.** Representative continuous co-cultures of *E.*
409 *coli* W Δ*cscR* /*cscB*$^{+}$ *S. elongatus* (**A**) and W303^{Clump} *S. cerevisiae*/*cscB*$^{+}$ *S. elongatus* (**B**) were cultured in
410 photobioreactors with 1mM IPTG. *E. coli*-containing consortia were kept in constant light while *S.*
411 *cerevisiae* communities were exposed to 16:8 hour light/dark photo period (grey spaces represent
412 darkness). Optical density of the entire culture (black scatter plots) as well as counts for the individual cell

413 types were tracked (green *S. elongatus,* orange *E. coli* W Δ*cscR*, purple W303^{Clump} *S. cerevisiae*).
414 Additional photobioreactor cultures for *E. coli* W Δ*cscR* and W303^{Clump} *S. cerevisiae* are presented in Fig.
415 S6 and S7, respectively. Extended W303^{Clump} *S. cerevisiae*/*cscB*$^{+}$ *S. elongatus* co-cultures are presented in
416 Fig. S8. Recovery of heterotrophs in batch co-cultures of *B subtilis* 3610/*cscB*$^{+}$ *S. elongatus* (**C**) or *E. coli*
417 W Δ*cscR* /*cscB*$^{+}$ *S. elongatus* (**D**) following dilution (vertical dashed grey line) was monitored by viable
418 colony counts. Perturbations on to solid media are presented in Fig. S9.
419
420 *E. coli*/*S. elongatus* co-cultures persist through time. Co-cultures of induced *cscB*$^{+}$
421 *S. elongatus* and W *ΔcscR E. coli* were grown continuously in photobioreactors to
422 control cyanobacterial density under constant light (Fig. 4A). Cultures maintain stable
423 ratios for more than two weeks (Fig. 4A, Fig. S6).
424
425 Co-cultures of *S. cerevisiae* W303^{Clump} and *cscB*$^{+}$ *S. elongatus* are robust over time
426 and through variable light conditions. To further alter culture conditions, *cscB*$^{+}$ *S.*
427 *elongatus* induced to secrete sucrose was cultured with *S. cerevisiae* W303^{Clump} with an
428 alternating diurnal illumination regime (16 light:8 dark, Fig. 4B). Sustained growth in
429 these continuous cultures indicates that yeast persist through periods of darkness when
430 cyanobacteria are unable to supply sucrose or other photosynthates (Fig. 4D, Fig. S7;
431 Ducat et al. 2012). In similar experiments extended over longer time periods, *S.*
432 *cerevisiae* maintains viability in continuous culture with sucrose-secreting *S. elongatus*
433 for more than two months (Fig. S8).
434
435 Prokaryotic co-cultures with cyanobacteria persist through population bottlenecks
436 and changes in environmental structure. Co-cultures of *B. subtilis*/*S. elongatus* and *E.*

437 *coli*/*S. elongatus* were subjected to single dilutions (1 to 10 or 1 to 100) to simulate
438 population bottlenecks. Visible cyanobacterial growth occurred throughout these
439 experiments, while heterotroph growth was measured via CFU. In perturbed cultures,
440 heterotrophs can return to pre-dilution levels within three days (Fig. 4C & 4D). To further
441 change environment structure, co-cultures containing *cscB*$^+$ *S. elongatus* and *B. subtilis*
442 3610 or *ΔcscR E. coli* were moved from liquid to solid environments and back again.
443 This transfer disrupts ratios of different species within co-culture and alters any
444 interactions dependent on the co-culture constituents being in well-mixed environments.
445 After growth of liquid culture on agar, green areas of the agar plate were picked into
446 liquid cultures again disrupting the community and seeding a culture with variable
447 numbers of phototrophs and heterotrophs. Cyanobacterial growth was recovered in
448 constant light followed by the qualitative determination of heterotroph presence. In the
449 majority of cultures both cyanobacteria and the corresponding heterotroph persisted
450 through these perturbations, although *B. subtilis* was lost from the co-culture somewhat
451 more frequently than *E. coli* (Fig. S9).
452
453 **Bioproduction from functionalized co-cultures.**
454 As multiple species can be co-cultured with *cscB*$^+$ *S. elongatus*, it is possible to
455 exchange heterotrophs to functionalize consortia for desired activity. In this design, the
456 heterotrophic species of the consortia acts as a "conversion module" to metabolize the
457 products of photosynthesis into target bioproducts in a one-pot reaction (Fig. 5A). We
458 tested two heterotrophic strains capable of producing distinct products: enzymes (Fig. 5B,
459 5C) and chemical precursors (Fig. 5D).



460

461 **Fig. 5 Photoproduction of enzymes and metabolites from co-culture.** Flexible functionalization of co-
462 cultures was accomplished via the addition of heterotrophs capable of producing target compounds (**A**).
463 Alpha-amylase is naturally produced and secreted by *B. subtilis* strain 168. Supernatants from 24-hour
464 cultures of *B. subtilis* 168 alone (blue background) or in co-culture with *cscB*$^+$ *S. elongatus* (blue and green
465 background) were tested for enzymatic activity (**B**). Western blots also reveal the presence of alpha-
466 amylase in co-cultures containing IPTG (**C**). *E. coli* is capable of making PHB when carrying the pAET41
467 plasmid. Batch co-cultures of *E. coli* (with or without pAET41 to enable PHB production) and *cscB*$^+$ *S.*
468 *elongatus* were cultivated for one week with or without IPTG to induce sugar and PHB content of the total
469 culture was analyzed (**D**). Filled circles represent measured values; hollow circles placed on the x-axis
470 represent cultures in which no PHB was formed or was produced at levels below the detection limit. Thick
471 horizontal lines represent the average measurement for each condition while thin horizontal lines represent
472 one standard deviation from the mean.

473

474 Alpha-amylase is produced in co-cultures of *B. subtilis* strain 168 and *cscB*$^+$ *S.*
475 *elongatus*. *B. subtilis* is a chassis for enzyme production (39) and strain 168 naturally

476 produces active alpha-amylase (40). In consortia with *S. elongatus, B. subtilis* 168
477 produces alpha-amylase after 24 hours in constant light (Fig. 5A, 5B). The resulting
478 alpha-amylase is functional as determined by enzymatic assay, and accumulates at
479 significantly higher levels in co-cultures with *cscB*$^+$ *S. elongatus* induced to secrete
480 sucrose (Fig. 5A).
481
482 Engineered *E. coli*/*S. elongatus* communities produce PHB. We co-cultured *E.*
483 *coli* strains harboring a previously described PHB production plasmid, pAET41 (32) with
484 *cscB*$^+$ *S. elongatus* for one week in constant light and measured PHB present in the total
485 biomass. As with alpha-amylase production for *B. subtilis, E. coli* produces PHB in co-
486 culture with *S. elongatus* (Fig. 5D). While production from the *E. coli* W strain is similar
487 with and without IPTG, the *ΔcscR E. coli* W mutants that utilize sucrose more effectively
488 produce significantly more PHB (Fig. 2C, Fig. 5D, Arifin et al. 2011). Furthermore, upon
489 the addition of IPTG, the *ΔcscR E. coli* W strain can produce 3 times as much PHB in co-
490 culture than uninduced consortia. Taken together, these results demonstrate that consortia
491 can be flexibly programmed for photoproduction of different bioproducts by employing
492 different heterotrophic organisms.
493
494 **Discussion**
495 Our engineered communities demonstrate interactions that cause the flux of fixed
496 carbon to flow from an engineered phototroph to disparate heterotrophs. These consortia
497 divide metabolic labor between partner species: cyanobacteria are responsible for
498 photosynthesis while the heterotrophs consume fixed carbon via heterotrophic

499 metabolism, making the design inherently modular. In constructing these consortia, we
500 observed unforeseen interactions with common features shared across different
501 heterotrophic species. Namely, we observe that cyanobacterial growth can have negative
502 impacts on heterotrophs, while, conversely, growth of the cyanobacteria is stimulated in
503 the presence of heterotrophs. The significance and implications of these findings are
504 discussed.
505
506 Co-cultures containing *B. subtilis* or *S. cerevisiae* rely on induction of sucrose
507 excretion (Fig. 2A, 2E), indicating sucrose availability limits heterotrophic growth. In
508 contrast, *E. coli* receives other forms of fixed carbon from *S. elongatus,* as evidenced by
509 growth in co-cultures with no external carbon source (Fig. 2, Fig. S2). Presumably, *E.*
510 *coli* must catabolize other metabolites (e.g. secreted fixed carbon or EPS) and growth is
511 independent of IPTG induction because sucrose levels stay too low for *E. coli* utilization
512 during short-term co-culture (Fig. 2C).
513
514 Engineering improved sucrose utilization is one general strategy to improve
515 consortia design. In the case of *S. cerevisiae,* limited growth of the wild type W303 yeast
516 was seen in co-culture with *cscB*$^{+}$ *S. elongatus* induced to secrete sucrose (Fig. 2E). *S.*
517 *cerevisiae* metabolizes sucrose by secreting invertase that hydrolyzes sucrose to glucose
518 and fructose; the resulting hexoses are imported, although they can also diffuse away
519 which limits growth at low cell densities in low sucrose environments (31, 41). The
520 engineered W303^{Clump} strain has improved sucrose uptake due to increased likelihood that
521 hexoses will be imported instead of diffusing away, and we observed this evolved strain

522 grow more successfully in co-culture than the wild type W303 (Fig. 2F). Similarly, we
523 tested W *E. coli* strains with improved sucrose utilization due to a deletion in the
524 repressor of the chromosomally encoded sucrose catabolism, *cscR* (27). While this
525 alteration did not lead to immediate gains in 48 hour batch cultures where sucrose levels
526 remained ≤1 mg/mL (Fig. 2A, Fig. S2), the mutation greatly increased PHB production in
527 longer experiments when co-cultures were functionalized for bioproduction (Fig. 5D).
528 Taken together, selecting or engineering heterotrophic partners to utilize very low
529 concentrations of sucrose would likely enhance consortia productivity and robustness.
530
531 Within these engineered communities we observe emergent interactions between
532 the cyanobacteria and heterotrophs that are consistent across consortia. All three
533 heterotrophs demonstrated decreased growth and viability when exposed to high densities
534 of cyanobacteria in the light (Fig. 3). While the effect is likely multifaceted, a product
535 that is only secreted in the light, including unavoidable byproducts of photosynthesis (e.g.
536 oxygenation and/or reactive oxygen species; Villa et al. 2015), may be key in this
537 interaction. Consistent with this, many evolved consortia containing oxygenic
538 phototrophs contain partners with strategies to mitigate reactive oxygen species (36, 42,
539 43); similar strategies may be employed to further improve the stability of synthetic
540 consortia. It is possible that growth suppression may be caused by competition for media
541 components, however heterotrophs can grow in media conditioned by *S. elongatus*
542 indicating only a minor role for this phenomena (20, 23).
543

544 A second emergent interaction between the microbes within our photosynthetic
545 consortia is the stimulation of phototroph growth in the presence of heterotrophs. It is not
546 known what limitation on *S. elongatus* is being alleviated by the presence of a living
547 partner heterotroph - although these observations are analogous to other natural
548 examples, such as interactions between *Prochlorococcus* and "helper heterotrophs" (42),
549 or experiments where microalgae accumulate more biomass in the presence of other
550 microbes than in isolation (44). However, the generality of the positive effect we observe
551 is somewhat surprising, as the complements of secreted bioproducts from each of the
552 heterotroph species is likely different. Broadly, heterotrophic metabolism is expected to
553 provide additional CO_2, however our consortia are cultivated in an enriched CO_2
554 environment (2%) well above atmospheric levels. Instead, we suspect cross-feeding of
555 other metabolite(s). It is possible that many distinct partner heterotrophs could boost
556 yields of *S. elongatus*, a prospect with significant implications for cyanobacterial
557 cultivation. Autotroph/heterotroph co-cultures might also be less susceptible to invasion
558 by contaminating species, a frequent problem of scaled and laboratory experiments (25).
559
560 The robustness exhibited by the synthetic communities presented here is a useful
561 feature that may facilitate future studies and applications. For example, we observe that
562 continuous culture enables co-culture conditions that are stable from weeks to months,
563 likely because the density of *S. elongatus* remains low (Fig. 4, Fig. S6-S8) in comparison
564 to batch co-cultures (Fig. 2, Fig. S2). Similarly, co-cultures persist through dilutions of 1
565 to 10 or 1 to100 (Fig. 4C & 4D) and can transition between differently structured growth
566 environments (Fig. S9). This stability is a promising feature of the consortia for

567 academic and applied experiments; for example long-term culture could be used to
568 examine fitness enhancing mutations in one or both species potentially leading to
569 heterotrophs with decreased sensitivity to cyanobacteria in the light or select for physical
570 attachment between phototroph and heterotroph.
571
572 Because our engineered system supports the growth of three distinct "workhorse"
573 model microbial organisms, there is flexibility within this platform for industrial and
574 academic applications. As demonstrated, we can take advantage of prior efforts to
575 engineer other microbes and capitalize on the strengths of each species by swapping
576 heterotrophs that produce different compounds. This work has shown that co-culture with
577 cyanobacteria can drive the production of alpha-amylase from *B. subtilis* and PHB from
578 *E. coli* – both commercially relevant products. Exploration of fundamental consortia
579 dynamics through this approach can also take advantage of flexibility within the platform
580 design. For example, target heterotrophic species may be selected based on the general
581 interactions observed here while additional 'omics level characterization can be pursued
582 in order to illuminate the molecular mechanisms underpinning emergent interactions.
583 With a more complete understanding of the full complement of inter-organismal
584 interactions, it may be possible to install additional control elements to further improve
585 consortial design for academic and bioindustrial purposes.
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587
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718 **Table 1. Strain and plasmid collection.**

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Strain	**Origin**
Synechococcus elongatus PCC7942	ATCC 33912
Synechococcus elongatus trc-lac/cscB	(23)
Bacillus subtilis 168	ATCC 23857
Bacillus subtilis 3610 *ΔsinI*	(29)
Escherichia coli K-12 BW25113	(30)
Escherichia coli W	ATCC 9637
Escherichia coli W Δ*cscR*	(28)
Saccharomyces cerevisiae W303	Ancestor strain; (31)
Saccharomyces cerevisiae W303^{Clump}	Recreated02 strain; (31)
Plasmid	
pAET41	(32)

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