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Exploiting Day- and Night-Time Metabolism of *Synechocystis* sp. PCC 6803 for Fitness-Coupled Fumarate Production around the Clock

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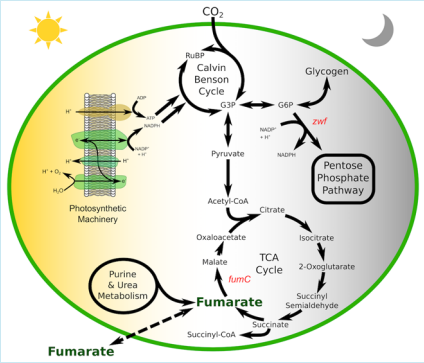
Supporting Information

ABSTRACT: Cyanobacterial cell factories are widely researched for the sustainable production of compounds directly from CO_2. Their application, however, has been limited for two reasons. First, traditional approaches have been shown to lead to unstable cell factories that lose their production capability when scaled to industrial levels. Second, the alternative approaches developed so far are mostly limited to growing conditions, which are not always the case in industry, where nongrowth periods tend to occur (e.g., darkness). We tackled both by generalizing the concept of growth-coupled production to fitness coupling. The feasibility of this new approach is demonstrated for the production of fumarate by constructing the first stable dual-strategy cell factory. We exploited circadian metabolism using both systems and synthetic biology tools, resulting in the obligatorily coupling of fumarate to either biomass or energy production. Resorting to laboratory evolution experiments, we show that this engineering approach is more stable than conventional methods.



KEYWORDS: *cyanobacteria, Synechocystis, fumarate, stable production, fitness-coupled, metabolic engineering*

CO_2 can be fixed directly into diverse (carbon) compounds using sunlight and genetically engineered cyanobacterial cell factories.[1] Large scale applications of these cell factories are limited due to their tendency to lose their production capability.[2] In essence, this production instability occurs because the commonly used heterologous metabolic pathways used in the engineering approaches directly compete with biomass formation, which imposes a high fitness burden on production strains. These strains thus become susceptible to suppressor mutations, such as insertions or deletions, that remove the fitness burden at the cost of product formation.[3] To ensure stable production in cyanobacterial cell factories, stable metabolic engineering approaches need to be developed and optimized.

This can be achieved with so-called "growth-coupled production strategies" (i.e., obligate coupling of the synthesis of a specific target product to the growth of that cell factory), which stabilizes production traits.[4,5] When the formation of product and biomass are aligned (i.e., obligatorily coupled), nonproducing mutants that emerge spontaneously are out-competed by the fitter producing strains according to Darwinian selection principles.[6] Although attempts have been made at engineering growth-coupled strategies in phototrophs,[7−10] implementation proved difficult. For instance, a common principle employed in the engineering of growth-coupled strategies is the linking of a product-forming pathway to the capacity of the cell to regenerate energy and/or redox cofactors. This principle has been proposed to hold for photoautotrophs[10] but it remains to be demonstrated in the laboratory. This might be caused by the plasticity conferred to cyanobacteria by all the alternative electron transfer pathways through photosystems I and II, which makes it very difficult to achieve the desired coupling.[9]

Recently we developed a method to design growth-coupled production strategies based on a new principle.[11] Instead of using energy or redox regeneration, our method is based on the direct stoichiometric coupling of pathways responsible for the formation of biomass precursors, to the production of target compounds. This concept has been developed into an algorithm that can be used to "find reactions usable in tapping side-products" (FRUITS). By analyzing existing genome-scale metabolic models, this open source pipeline identifies anabolic side-products that can be coupled to cell growth by the deletion of their reutilization pathway(s). We have previously validated FRUITS with the production of acetate in *Synechocystis* sp. PCC6803 (hereafter, *Synechocystis*) under photoautotrophic conditions.[11] Here, we have shifted the focus

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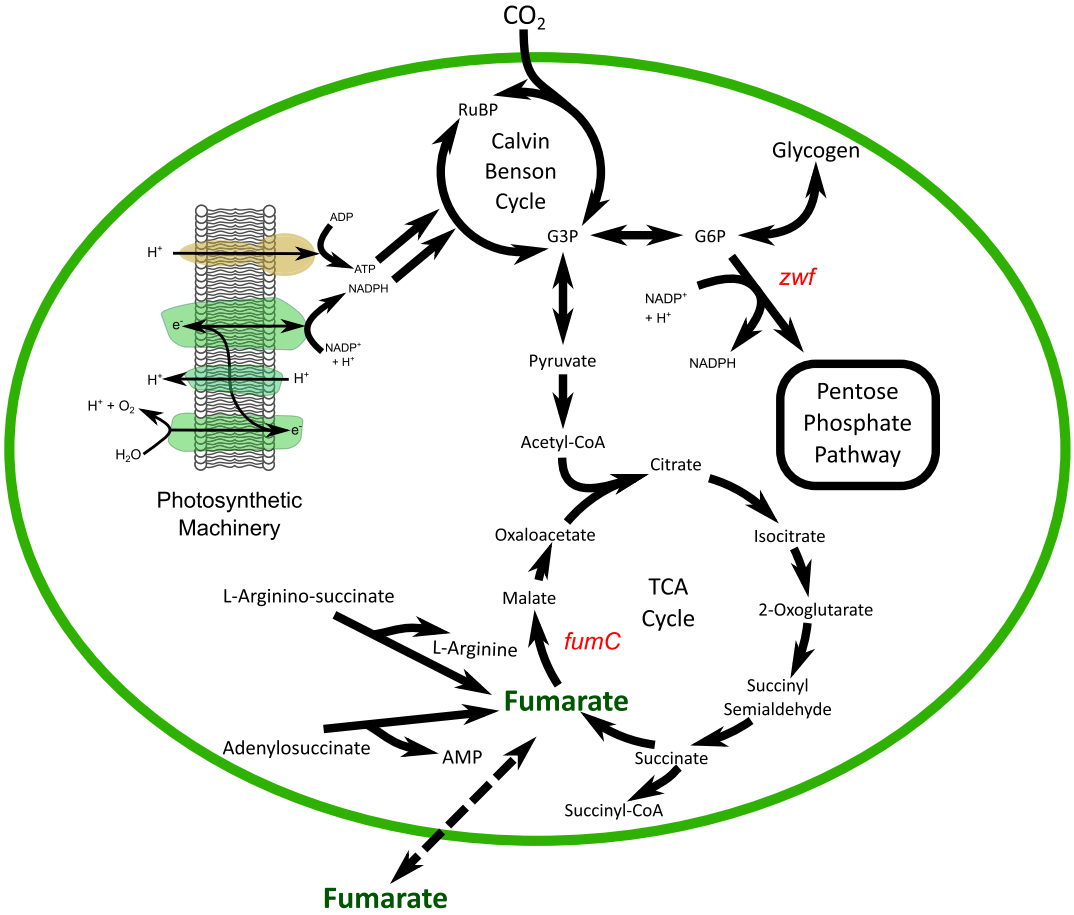

Figure 1. Schematic overview of metabolism in *Synechocystis* surrounding fumarate. During day-time fumarate is produced as part of the purine and urea metabolism. Removing the *FumC* blocks the reutilization of fumarate, causing it to accumulate and eventually leak out. At night-time *Synechocystis* uses the pentose phosphate pathway (PPP) to generate energy-carriers from glycogen. Removal of the *zwf* gene, disables the OPPP and forces the utilization of the disrupted TCA cycle.

to yet another product, fumarate, as simulations suggest that it will be produced in much larger amounts (>4-fold).[11,12] Furthermore, fumarate is a very interesting compound from a biotechnological point-of-view, with multiple applications across diverse fields, such as an acidity regulator in the food industry,[13] a suitable building block for polymer (e.g., biodegradable plastic) production,[14] etc. Thus far, the majority of fumarate is produced from refined fossilized carbon deposits by chemical synthesis. Given the aforementioned applications of fumarate, a sustainable and biobased route for its production would be highly relevant.

An evident inconvenience of this (or any) growth-coupled production strategy is that if cells do not grow, they do not produce. Particularly at the industrial scale, cyanobacteria are often dependent on natural solar radiation as their primary source of light and energy.[15] This means these cultures are subject to an oscillating light−dark cycle. Thus, during a significant amount of time, cyanobacteria will be in the dark, a condition where no growth may be expected, and by consequence, no product is formed. To overcome this problem, we propose to combine different strategies of aligning genetically engineered product formation with microbial fitness in a single host; that is to say, to couple product formation to the intrinsic abilities of cyanobacteria to adjust their metabolism to suit their surrounding conditions. Here, we will demonstrate the feasibility of engineering such a host, by coupling fumarate production to both day and night-time metabolism.

■ RESULTS

Day-Time Production of Fumarate. The analysis of the *Synechocystis* genome-scale metabolic model (GSMM), constrained to simulate photoautotrophic growth,[9] reveals that fumarate is predicted to be produced as a byproduct of specific anabolic reactions within purine metabolism and urea metabolism and is reassimilated through the TCA cycle via the activity of FumC (fumarase) (Figure 1). If this single fumarate assimilation pathway is removed, by the construction of a *fumC* deletion strain, FRUITS predicts that fumarate will accumulate at a yield of 0.848 mmol fumarate per gDW, with a very low impact on the growth rate (0.001 h^{-1}). In this study, we have tested this prediction by the construction and validation of a Δ*fumC Synechocystis* mutant. Under constant illumination, *Synechocystis* wild-type and the Δ*fumC* strain grow similarly during the exponential growth phase, while, wild-type reached a slightly higher optical density after entering the stationary phase (Figure 2A). These observations indicate that any potential growth rate difference is too small for us to detect. Not surprisingly, there was no extracellular fumarate production in *Synechocystis* wild-type. In contrast, the Δ*fumC* strain excreted significant amounts of fumarate throughout the cultivation (>1 mM final concentration; see Figure 2A). These

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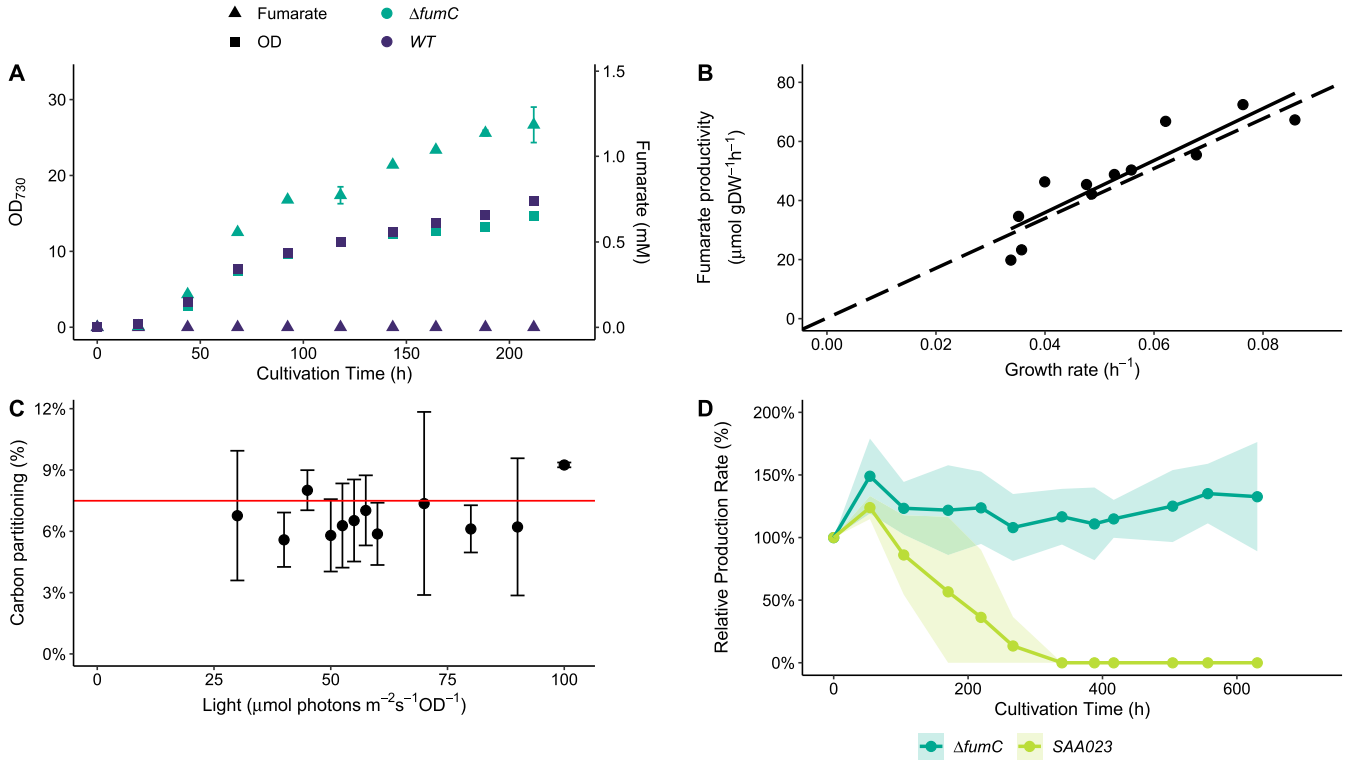

Figure 2. Stable fumarate production in *Synechocystis*. (A) Cell growth (squares) and extracellular fumarate production (triangles) of both the wild-type (purple) and the Δ*fumC* (dark green) strain in a Multi-Cultivator illuminated with constant light for over 200 h. (B) A linear relationship between growth rate and biomass specific fumarate productivity. Each point represents a single observation, and the solid line is a linear fit of all experimental data points. The dashed line is based on *in silico* FBA simulations of the GSMM of *Synechocystis*, using biomass maximization as the objective function. (C) Carbon partitioning of fumarate production versus biomass at different photonfluxostat light regimes. The red line is based on in silico FBA simulations. (D) Stability of production of fumarate (dark green) and lactate (light green, using strain SAA023[21]) during prolonged turbidostat cultivation under continuous illumination. SAA023 is a lactate producing *Synechocystis* mutant, expressing the lactate dehydrogenase from *Lactococcus lactis* at the *slr0168* genomic locus. Productivity at corresponding time points was normalized based on the average productivity at the first time point (set to be 100%). The shade area indicates one standard deviation from 4 biological replicates. Error bars indicate the standard deviations ($n \geq 2$). If an error bar is not visible, it is smaller than the size of the symbol.

results match very nicely the *in silico* prediction that disrupting *fumC* will result in fumarate accumulation.

While undoubtedly promising, these initial simple growth experiments do not ensure that biomass and fumarate formation are strictly coupled. The strict stoichiometric coupling, which we are striving to engineer, implies that at different growth rates a linearly proportional change in the biomass specific production rate of fumarate is obtained, and this, remained to be tested.

Fumarate Production Rate Is Proportional to Growth Rate. We tested whether fumarate production and growth rate is coupled in the Δ*fumC* strain, by performing 12 independent photonfluxostat experiments at different, yet constant, growth rates.[16] This was achieved by dosing the biomass specific light flux to intensities ranging from 30 to 100 μmol photons m^{-2} s^{-1} OD^{-1}. From all cultivations maintained at a specific growth rate, samples were taken at multiple times during the cultivation, to quantify extracellular fumarate concentration. Fumarate productivities were subsequently calculated and plotted against growth rate (Figure 2B). The results obtained indicate that fumarate productivity is indeed proportional to growth rate, fully consistent with the idea that both physiological traits are indeed coupled. Furthermore, we compared the linear fit between the rate of fumarate productivity and growth rate, based on our experiments, with the outcome of the simulations using flux balance analysis (FBA)[18] on the metabolic network reconstruction of *Synechocystis* (Figure 2B). It is important to highlight that we did not in any way tweak the modeling parameters, which were taken directly from the original report.[9] Still, both sets of data match strikingly well, corroborating that indeed the hypothesis that fumarate production and growth rate are coupled in the Δ*fumC* strain seems to hold up to scrutiny.

We also calculated the carbon partitioning toward fumarate in the Δ*fumC* strain during the multiple cultivations carried out (Figure 2C). This was calculated based on the average optical density and fumarate concentration, between each two subsequent sampling points. Optical density was converted to gram of dry biomass based on a previous report.[16] The plotted carbon partitioning is an average of individual values obtained for each time interval with standard deviation. We did not see any significant changes in carbon partitioning irrespective of the biomass specific light flux (Figure 2C). This indicates that irrespective of the growth rate, as long as cells are illuminated, the fumarate yield on biomass is constant. This result also supports theoretical predictions stating that fumarate production is only affected by environmental conditions to the extent that the latter affect growth rate. In other words, fumarate production is stoichiometrically and obligatorily coupled to growth. It is important to note that the carbon partitioning values reported here are comparable with most of those

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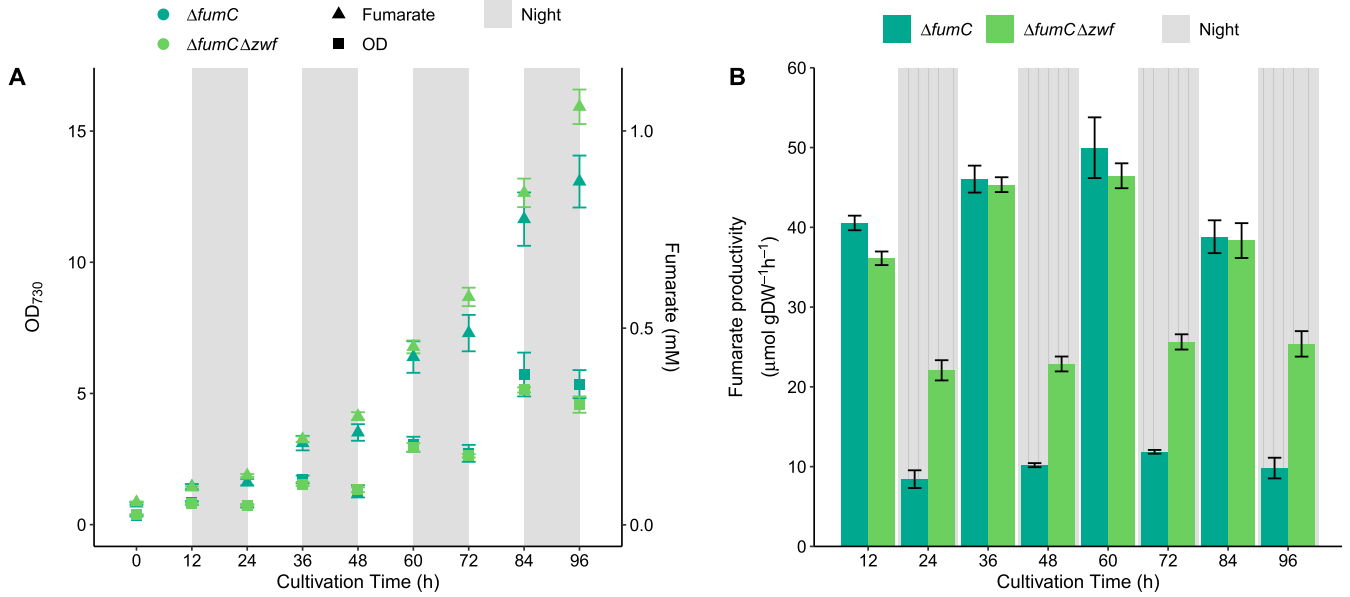

Figure 3. Stable fumarate production around the circadian clock. (A) Cell growth (squares), extracellular fumarate production (triangles), and (B) fumarate productivity of both the Δ*fumC* (dark green) and the Δ*fumC*Δ*zwf* (green) strain in a batch Multi-Cultivator under the 12-h-day/12-h-night cycle. OD_{730} and fumarate concentrations were measured at every perceived dawn and dusk. Fumarate productivity was calculated separately for each day and night period, using the fumarate concentration differences, while both dividing by the average OD_{730} and the time interval between dawn and dusk. Cell dry weight was calculated based on a conversion of OD_{730} into 148 mg L^{-1} OD_{730}^{-1}. The error bars indicate the standard deviations of four replicates for the Δ*fumC* and three replicates for the Δ*fumC*Δ*zwf* strain. If an error bar is not visible, it is smaller than the size of the symbol.

obtained using more conventional metabolic engineering strategies for a wide variety of products.[1]

Genetic Stability of Fumarate Production by the Δ*fumC* Strain. Although we show that the Δ*fumC* strain produces fumarate in a growth-coupled fashion, this is no evidence of its (improved) genotypic stability, compared to traditional metabolic engineering approaches. As explained before, the root of the instability comes from Darwinian selection for fitter (i.e., faster growing under the selected conditions) strains and the occurrence of random mutations in the genome. For cyanobacterial cell factories, production pathways compete directly with cellular fitness, thus mutations resulting in the suppression or elimination of the production pathway(s) are positively selected for. Our strain on the other hand integrates fumarate production with biomass production, using an evolutionary stable approach. This approach is more stable for two main reasons. First, fumarate production is enabled via the removal of the *fumC* gene, a strategy which is stable as long cells do not re-evolve a new fumarate assimilation pathway—an event which we expect to be highly unlikely even on the longer time-scales potentially deployed in industrial settings. Second, detrimental mutations impacting the production rate can only occur in pathways that will also lower fitness, hence evolutionary pressure will counter-select nonproducing mutants. To test the stability of our Δ*fumC* strain and compare its improved stability to conventional cell factories, we performed a short-term evolution experiment. We chose conditions in which cells are under strong selection pressure for faster growth and in which the propagation bottlenecks are small, as these are predicted to result in the fastest drop in productivity.[19] Such conditions are best met under turbidostat cultivation,[20] and so these cultivations provide the harshest test ground to assess the stability of our production strains.

We cultivated the Δ*fumC* strain under turbidostat conditions at its maximal growth rate (i.e., without light limitation), for a period of over 600 h. During this period, we did not observe any significant changes in production rate (Figure 2D), a true testament to the genetic stability of our fumarate producing strain. As a control, we tested a similar cell factory, engineered using classical approaches, for the production of lactate,[21] under the same conditions. Lactate production in SAA023 was achieved by the heterologous expression of lactate dehydrogenase from *Lactococcus lactis*, yielding an initial carbon partitioning comparable to the one here reported for fumarate. As expected, lactate production was lost within 5 to 10 days for a culture of this strain (Figure 2D). This result reinforces the stringency with which this regime selects for fitter cells, which—when using our novel engineering method—means the producing ones.

Night-Time Production of Fumarate. To address night-time fumarate production, we once again turned to the *Synechocystis* GSMM. We approximated night-time metabolism by simulating chemoheterotrophic conditions with constrained glycogen utilization in the absence of light.[22] As for the objective function, we initially chose to maximize ATP consumption, as *Synechocystis* does not grow during the night but is still metabolically active, presumably to cover cellular maintenance costs.[23,24] However, these initial simulations predicted that there would be no fumarate production for a Δ*fumC* strain under night-time conditions. This prediction is in contrast to our experimentally measured night-time fumarate production rates from our Δ*fumC* strain (Figure 3A). As mentioned previously, fumarate is produced as a byproduct of anabolic reactions. Our data then suggests that although *Synechocystis* does not grow at night, it still has residual anabolic activity. We therefore decided to use the maximization of a heterotrophic growth equation as our

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objective function, which also accounts for ATP consumption, for the sake of a qualitative proxy for night-time metabolism.

In the interest of further increasing the night-time production of fumarate, we used the algorithm OptKnock[25] to identify gene deletions that would redirect night-time metabolic flux toward fumarate production (Table S1). The gene of which deletion was predicted to be most significant is *slr1843*, or more commonly known as *zwf*. The product of the *zwf* gene catalyzes the first reaction in the oxidative pentose phosphate pathway (OPPP). FBA predicts for the Δ*fumC* strain under these night-time conditions, the OPPP to be the primary mode for the production of energy in the form of electron carriers.[1,26,27] Additionally, the OPPP is predicted to provide precursors for glycine production. Therefore, simulations of a Δ*fumC*Δ*zwf* double mutant predict that night-time fumarate production will increase, relative to the Δ*fumC* strain, for potentially two reasons: (i) fumarate is produced as an end-product of the TCA cycle, which provides energy carriers and (ii) fumarate is produced as a byproduct of L-proline degradation as an alternative pathway for the production of glycine precursors. Of these two sources, the TCA cycle is predicted to be the predominant contributor, supplying 73% of flux associated with the increase in night-time fumarate production.

We tested the production of fumarate by the Δ*fumC* Δ*zwf* strain in a 12 h−12 h sinusoidal day−night rhythm and compared its performance with the Δ*fumC* strain. During this cultivation we used the photonfluxostat to adjust light intensity to biomass density, in order to extend the range of stable exponential growth. This allowed us to collect multiple samples from similar conditions, without the complications of a turbidostat. The biomass density and fumarate concentration were measured daily at the beginning and end of each day period, so we could calculate the production rate of fumarate for day and night separately.

After about 100 h of cultivation, we found the Δ*fumC* strain to reach a slightly higher biomass density (Figure 3A), while the Δ*fumC*Δ*zwf* strain produced more fumarate (Figure 3A), suggesting more carbon was diverted into fumarate by Δ*fumC*Δ*zwf*. After calculating the specific production rate of fumarate for both strains in day and night separately (Figure 3B), we found both strains to produce fumarate at similar rates during the day (Two-sided *t* test, H0: Δ*fumC* = Δ*fumC*Δ*zwf*, $p = 0.2377$). During the night, however, Δ*fumC*Δ*zwf* turned out to produce fumarate at more than twice the rate of Δ*fumC* (Figure 3B). The Δ*fumC* is designed to only produce fumarate in a growth coupled fashion as mentioned before. However, although no significant growth was observed during the night, we did observe fumarate production in Δ*fumC* during that period (Figure 3B). As discussed before, this finding supports the idea that anabolic reactions are still active during the night.[28] With the Δ*fumC*Δ*zwf* strain producing as predicted by our model, we have further evidence for anabolic reaction being active during the night. One explanation may be found in *Synechocystis* preparing for the day. A more detailed study of the night-time behavior of these strains may reveal how these reactions are regulated.

We have no indication of phenotypic instability in the Δ*fumC*Δ*zwf* fumarate producing strain, and expect it to be as stable as the Δ*fumC* strain since both were created using the same metabolic engineering design principles, that is, relying solely on gene deletions. The evolution of a new Zwf-like protein, as argued for the FumC, is something that is likely to take a much larger evolutionary time than the ones typically used in industrial settings.

■ CONCLUSIONS

We engineered a new dual strategy cyanobacterial cell factory for the production of fumarate, by exploiting day- and night-time metabolism. Using a published GSSM, and the FRUITS and OptKnock algorithms, we were able to couple fumarate to growth, during the day, and to fitness, during the night, by introducing only two gene deletions. This is, to the best of our knowledge, the first cyanobacterial cell factory exploiting day- and night-time metabolism separately, in the same host. When compared to other cyanobacterial production hosts constructed using conventional metabolic engineering strategies, our day-time specific productivity of fumarate comes close to the one published for the production of butanol.[17] This ranks our strain in the middle among all the cyanobacterial production hosts[1] with two very important advantages: (i) it has been proven to be stable and (ii) it also displays night-time production. We also showed our approach to be more stable over time when compared to a cell factory constructed using conventional metabolic engineering strategies. This achievement demonstrates it is possible to obtain obligatory coupled production of carbon-rich compounds to fitness, even in conditions without net growth. Additional insight into day- and night-time metabolism will help to identify other pathways that can be exploited with similar approaches. Although the contribution of night-time production is relatively small, in the industrial settings where these cell factories are to be implemented, similar environmental dynamics are likely to be expected.[29] Our proposed metabolic engineering strategy demonstrates avenues to exploit these dynamics. Finally, not only do we report here the first truly stable method to produce fumarate directly from CO_2 but also the final production parameters obtained are the highest for a fitness-coupled method using a photoautotrophic cell factory. Moreover, since the coupling achieved is obligatory, evolutionary pressure can be exploited to obtain further improvements to fitness, and consequently, also to fumarate production. The approach taken here is applicable to other production systems. This is of particular relevance to production processes that, while striving for maximum production, also need to be robust against genetic drift and need to cope with environmental fluctuations, such as the ones exploited here in the form of day and night.

■ METHODS

Strains. Molecular cloning was performed in *E. coli* DH5α growing either in liquid Luria−Bertani (LB) broth at 37 °C in a shaking incubator with a shaking speed of 200 rpm, or on solidified LB plates containing 1.5% (w/v) agar. Antibiotics were added, when appropriate, in the medium for propagation of a specific plasmid. Concentrations of antibiotics used, alone or in combination, were 100 μg mL^{-1} for ampicillin and 50 μg mL^{-1} for kanamycin.

Glucose-tolerant *Synechocystis* was obtained from D. Bhaya, University of Stanford, Stanford, CA. Basic cultivation was performed in BG-11 medium at 30 °C in an shaking incubator shaking set at 120 rpm (Innova 43, New Brunswick Scientific), under constant moderate white-light illumination (∼30 μmol photons m^{-2} s^{-1}, measured with a LI-250 light meter). For *Synechocystis* mutant construction, we added kanamycin or nickel sulfate to the medium with a final concentration of 50

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μg mL^{-1} or 20 μM, respectively. Plates with *Synechocystis* cells were incubated at 30 °C in an incubator with white light (~30 μmol photons m^{-2} s^{-1}) and 1% CO_2.

Plasmid and Strain Construction. All plasmids, strains, and primers are listed in Table S2. For the marker-less deletion of a gene in the *Synechocystis* chromosome, two plasmids were required (Figure S1). Plasmid one contains up- and downstream homologous regions (~1 kb each) of the target gene, while the second plasmid has an extra selection cassette flanked between both homologous regions. The selection cassette consists of a kanamycin resistance fragment and a nickel-inducible *mazF* expression fragment. As an endoribonuclease that cleaves mRNA at any ACA triplet sequence, MazF acts as a general inhibitor for the synthesis of all cellular proteins.[30] For *fumC* gene knock out plasmids, each homologous region was amplified by PCR, using genomic DNA of *Synechocystis* as a template. The two fragments were fused together and completely amplified with *Pfu* DNA Polymerase (Thermo Scientific). The resulting blunt DNA fragment was purified with a gel extraction kit (Thermo Scientific), and provided with one extra 3′-adenosine ("A") by *Taq* DNA Polymerase (Thermo Scientific). Through TA cloning, the obtained DNA fragments were ligated to the BioBrick "T" vector pFL-AN,[31] resulting in pWD060. Due to the extra XbaI restriction site introduced through primers between the two homology regions, a selection cassette with an XbaI on both sides (from pWD42) can be easily inserted into pWD60. The resulting plasmid is named pWD061. The *zwf* gene knock out plasmids were constructed following the same method, with the plasmid name pWD84 (with only *zwf* gene homologous regions) and pWD085 (with an extra selection cassette flanked between both *zwf* gene homologous regions). All the fragments amplified in this study were confirmed by Sanger sequencing at Macrogen Europe (The Netherlands).

The *fumC* and *zwf* gene deletion in *Synechocystis* was achieved using two rounds of transformation as previously reported (Figure S1).[11] In the first round the selection cassette is integrated into the chromosome. The second round is to remove the selection cassette. Briefly, for both rounds of transformation, corresponding *Synechocystis* cells were collected either directly from plate or from liquid culture. After being washed twice with fresh BG-11 medium through centrifugation at 5 000 rpm for 5 min, cells were further concentrated to a total liquid volume of 200 μL ($OD_{730} \approx 2$). Either pWD61 for *fumC* and pWD086 for *zwf* (first round) or pWD060 for *fumC* and pWD085 for *zwf* (second round) was mixed with cells to a final concentration of 10 μg mL^{-1}, and then the mixture was illuminated under moderate light (~30 μmol photons m^{-2} s^{-1}) for 4 to 6 h. Next, the mixture was spread on a commercial membrane (Pall Corporation), resting on a BG-11 plate, with neither kanamycin nor nickel sulfate, and incubated at 30 °C. After further illumination for 16−24 h, the membrane with the mixture of cells was transferred to another BG-11 plate with kanamycin (first round) or nickel sulfate (second round). After about 1 week, the colonies that appeared were picked and scratched sequentially on a new BG-11 plate with kanamycin or nickel sulfate, respectively. Colonies which grew on a BG-11 plate with kanamycin but not with nickel sulfate (first round), or on BG-11 plate with nickel sulfate but not kanamycin (second round), are candidates for PCR confirmation (with 35 cycles). Further segregation by serial dilution in liquid culture was applied when necessary.

Strains Cultivated in Multi-cultivator. Precultures from the glass flasks at OD_{730} around 1 to 1.5 were used for inoculation to the Muti-Cultivator MC1000 (PSI, Czech Republic), at a starting OD_{730} of approximately 0.05. Continuous low light (30 μmol photons m^{-2} s^{-1}) was given overnight, and then light was switched to 120 μmol photons m^{-2} s^{-1} until the end of the cultivation. During the experiment, 1% CO_2 and 99% N_2 was provided to the cultures through a gas mixing system GMS 150 (PSI, Czech Republic). The growth curves were monitored by measuring OD_{730} (Spectrophotometer Lightwave II, Biochrom), and the supernatant was further processed for either fumarate or lactate quantification.

Batch Cultivation, Day−Night Batch Cultivation, Prolonged Cultivation, Fumarate and Lactate Quantification. See Supporting Information for details.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00289.

> Methods for batch cultivation, day-night batch cultivation, prolonged cultivation, and fumarate and lactate quantification along with (i) the schematic drawing of the markerless construction method used here for *Synechocystis* in Figure S1; (ii) the fumarate quantification in Figure S2; (iii) the model simulation results by OptKnock in Table S1; and (iv) the plasmids, strains and primers used in Table S2 (PDF)

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Author Contributions

W.D. and B.v.d.P. carried out all strain engineering. M.G. modeled the night-time production. W.D. designed and carried out the day-time production experiments while J.A.J. carried out the night-time production experiments. F.B.S., J.A.J., W.D., and M.G. wrote the first draft of the manuscript. B.B. and K.J.H. critically reviewed the manuscript.

Author Contributions

#W.D. and J.A.J. contributed equally to this work.

Notes

The authors declare the following competing financial interest(s): Klaas J. Hellingwerf is the scientific advisor of Photanol B.V., a University of Amsterdam spin-off company aiming at commercializing sustainable applications with cyanobacteria. The other co-authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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