PHOTOSYNTHETIC MAGIC

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PRODUCE

A major requisite of cyano-cell factories, according to expert's opinion, is that they must be able to produce in a stable fashion under industrial conditions. A recent quantitative analysis of the various ways to convert the energy of photons to chemical bonds has revealed that the direct utilization of sunlight is the most efficient [1]. This however means that cells will be exposed to diurnal regimes in which they will inevitably be exposed to periods of darkness. Our goal here is to achieve the first photoautotrophic cell factories that are able to stably produce fumarate around the clock.

Overview

Synechocystis does not naturally produce fumarate. However, model guided engineering found that removing a single gene within *Synechocystis* leads to a stable cell factory that produces fumarate as it grows during the day. Nevertheless, at night our cells do not produce fumarate, since at night, they don't grow. To overcome this challenge, we have taken a systems biology approach which interweaves theory, modeling, and experimentation to implement stable nighttime production of fumarate. We theorized that we can redirect the nighttime flux towards fumarate production by removing a competing pathway via knockout of the *zwf* gene. Additionally, we also took inspiration from nature and speculated that the incorporation of the glyoxylate shunt would further increase our nighttime production of fumarate. Our models corroborate these predictions, however, they also suggest that the stability of the glyoxylate shunt is sensitive to the timing of when the shunt is turned on (i.e. expressed). We therefore took a robust approach to incorporate the glyoxylate shunt enzymes under ideal expression conditions.

Highlights

- Engineered a Δ*fumC*Δ*zwf Synechocystis* strain, that uses different fumarate production strategies during day and night.
- Developed a method to make fully segregated libraries in polyploid organisms
- Created the first fully segregated library representing the entire genome (99.9% confidence) of *Synechocystis* upstream of the glyoxylate shunt genes. This library is now ready to be tested to further increase nighttime fumarate production.
- Stable production of fumarate directly from CO_2 around the clock Qp_{night} of 18.4 μM $grDW^{-1}hour^{-1}$ Qp_{day} of 58.77 μM $grDW^{-1}hour^{-1}$

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Our cyano-cellfactory must be able to stably grow and produce under industrial conditions. At the industrial scale, *Synechocystis* and other cyanobacteria are often grown in large outdoor ponds or in greenhouses [2], where natural solar radiation is the primary source of light. This means that the cultures are subject to an oscillating light-dark cycle. Therefore, we aim to make a cyanobacterial cell factory that is able to produce fumarate in a stable fashion during ,not only the day, but also the night.

We mimicked industrial conditions in the lab by tailoring commercially available photobioreactors (MC1000-OD, PSI, Czech Republic) to be capable of simulating dynamic white light regimes. This involved developing new algorithms to incorporate the on-line measurements (e.g. OD_{730}) with the desired oscillatory light intensity patterns, which then had to be coded into the in-house software package that controls the photobioreactors. These relatively complex sinusoidal functions that we deduced may then also be optionally coupled with algorithms that generate stochasticity resembling the one cells encounter in production scenarios. In combination, these new developments allow us to use lab-scale photobioreactors to mimic industrial settings operating at high cell densities in which cells perceive fluctuating light intensities on top of the sinusoidal light regimes inherent to day-night cycles. This effort, albeit time consuming and with little application of synthetic biology methods, was crucial to ensure the connectivity of our metabolic engineering strategies to the "real-world" beyond the academic laboratorium.

Why is stability an issue?

Stable production at the industrial scale is a challenge of biotechnology. Production rates are often not sustained and will diminish throughout the cultivation. Furthermore, maximal production rates cannot be reached again by the same culture, even with the addition of fresh medium. This is due to the phenomena of strain instability [3].

This generalized phenomenon can be easily understood in the light of evolution theory, Darwinian selection and population dynamics. By introducing heterologous production pathways, cellular resources are forcibly diverted towards an extraneous product, and away from anabolic processes (i.e. growth). Cells which then lose the ability to produce the product are able to grow faster and eventually take over the population based on simple Darwinian selection, resulting in the irreversible loss of production [4].

Promising solutions to strain instability can involve the alignment of production of the desired compound with the fitness of the cell, i.e. the cell must produce in order to grow. One method for stable production is to knock-out genes whose proteins recycle anabolic byproducts [5].

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Experts in biotechnology have indicated to us at the onset of this project that the process unpredictability that emerges from the instability of engineered strains in production settings is one of the major technical hurdles of the field [6]. This occurs because most commonly used metabolic engineering approaches make products in direct competition with biomass formation, which imposes high fitness burdens on production strains. This ultimately leads to a rapid appearance of suppressor mutations, for instance in the form of insertions or deletions, that impair the culture's ability to form product [7][8]. So called ***growth-coupled production*** (i.e. obligate coupling of the synthesis of specific target products with bacterial growth) can help stabilize production traits. When the formation of product and biomass are aligned, non-producing mutants that emerge spontaneously are outcompeted by the fitter producing strains according to Darwinian selection principles [9]. The theoretical framework behind the engineering of growth-coupled strategies has thus far been underpinned by the same principle - linking a product-forming pathway to the capacity of the cell to regenerate energy and/or redox co-factors. This principle has been proposed for photoautotrophs but never successfully implemented in the laboratory [10]. The cause behind this has to do with the plasticity conferred by all the alternative electron flows surrounding the photosystems I and II, which makes it very difficult to achieve the desired strict coupling. However, recent advancements in the designing of metabolic engineering growth-coupled production strategies may bring the solution to this hurdle.

How to stably produce fumarate in *Synechocystis* during the day?

The 2015 Amsterdam iGEM team has pioneered the development of a method to design growth-coupled strategies based on a completely different principle. Instead of using energy or redox regeneration, this is now based on the direct stoichiometric coupling of pathways uniquely responsible for the formation of biomass precursors to the production of target compounds. This is achieved through the deletion of the native metabolic route(s) that cells have to reintroduce side-products of anabolism, leading to their accumulation, and hence, ensuring their growth-coupled production.

This concept has been developed into an algorithm to 'Find Reactions Usable In Tapping Side-products' - FRUITS. By analyzing existing genome-scale metabolic models, it identifies anabolic side-products that can be coupled to cell growth by the deletion of their re-utilization pathway(s). This pipeline is freely-available at https://gitlab.com/mmp-uva/fruits.git. When applied to *Synechocystis* growing under photoautotrophic conditions, FRUITS predicts that nine compounds can be coupled to growth, of which one is fumarate.

Prediction is that during growth, so in the light (or during the day in an industrial setting), fumarate is produced as a by-product of specific anabolic reactions within purine and urea metabolism and then re-assimilated through the TCA cycle via the activity of *fumC* (fumarase). If the only fumarate assimilation

very important for the economic feasibility of the technology here developed. The predictions of FRUITS regarding fumarate have been experimentally tested, leading to the first photoautotrophic cell factory that is able to stably produce fumarate directly from CO_2.

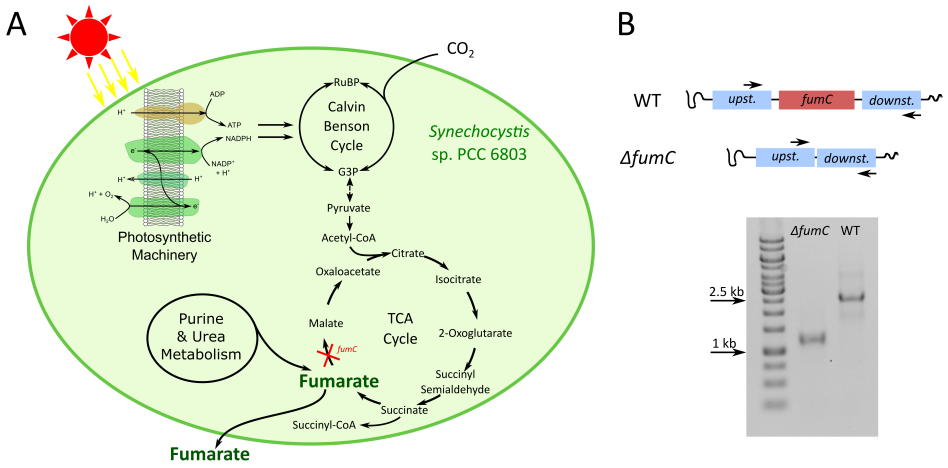

Figure 2.1 *Schematic representation of fumarate metabolism in Synechocystis sp. PCC6803. (a) A deletion of fumC gene (red X) in the TCA cycle blocks fumarate re-assimilation, thereby enabling its accumulation. (b) PCR confirmation of the Δ fumC mutant. With the primers indicated (black arrows), a clean knockout of fumC gives a single DNA band ~ 1.2 kb, while for wild type (WT) is ~ 2.5 kb. Abbreviations: RuBP, Ribulose-1,5-bisphosphate; G3P, Glyceraldehyde 3-phosphate.*

Results and discussion

Extracellular fumarate production by a markerless *fumC* deletion *Synechocystis* strain

We used a clean *Synechocystis fumC* deletion mutant (Δ*fumC*) to experimentally test its capacity to produce fumarate, using the wild type *Synechocystis* as a control. Under constant light conditions, *Synechocystis* wild type and the Δ*fumC* strain grew similarly during the exponential growth phase. Wild type reached a slightly higher optical density after entering stationary growth phase (fig.2.2A). Not surprisingly, there was no extracellular fumarate production in *Synechocystis* wild type. In contrast, the Δ*fumC* strain excreted significant amounts (> 1 mM) of fumarate throughout the cultivation (fig.2.2B). These results very nicely match the the in silico predictions that indicated that disrupting *fumC* would culminate in fumarate accumulation.

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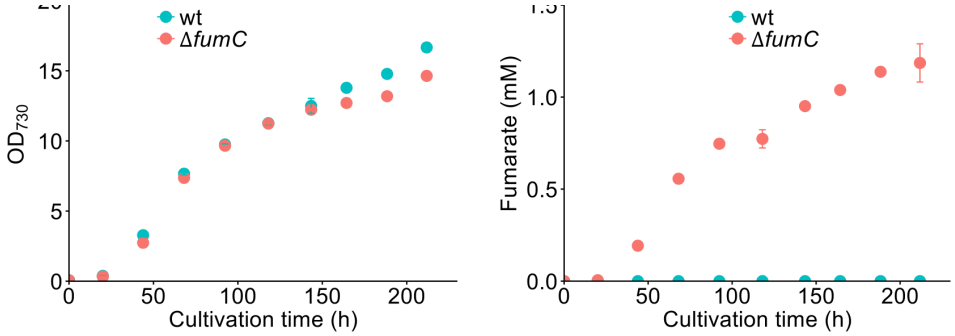

Figure 2.2 *Cell growth and extracellular fumarate production in different Synechocystis strains. (a) Cell growth of both wild type and Δ fumC in Multi-Cultivator under constant light illumination for over 200 hours. (b) Extracellular fumarate production for both strains. Error bars indicate the standard deviations.*

While undoubtedly promising (!) these initial growth experiments do not assure that biomass and fumarate formation are strictly aligned. The strong stoichiometric coupling which we are striving to engineer implies that at different growth rates one would expect a linearly proportional change in the biomass specific production rate. The latter remained to be tested with this initial set of experiments alone.

Growth-coupled production of fumarate in Δ*fumC* strain

We tested whether fumarate production and growth rate are aligned in the Δ*fumC* strain, by performing 12 independent photonfluxostat at different, yet constant, growth rates [6]. This was achieved by dosing the biomass specific light flux to intensities ranging from 30 to 100 µmol photons $m^{-2}s^{-1}OD^{-1}$. For each cultivation maintained at a different growth rate, samples were taken at different sampling times to quantify extracellular fumarate concentration. Fumarate productivities were subsequently calculated and plotted against the respective growth rate (fig. 3.A). The results obtained indicate that fumarate productivity is indeed proportional to cell growth rate, implying that both physiological traits are strongly coupled as here desired. Furthermore, we compared the linear fit between fumarate productivity and growth rate based on our experiment, with the outcome of the simulations using FBA on the metabolic network reconstruction of *Synechocystis*. It is important to highlight that we did not in anyway tweak the modeling parameters, which were taken directly from the original report [11]. Still, both fits match strikingly well, corroborating that indeed the hypothesis that fumarate production and growth rate are aligned in the Δ*fumC* strain seems to hold up to scrutiny.

We also calculated the carbon partitioning towards fumarate in the Δ*fumC* strain during the multiple cultivations carried out (Fig. 3B). We did not see any significant changes in carbon partitioning irrespective of the biomass specific light flux. This indicates that irrespective of the growth rate, as long as cells are illuminated, the fumarate yield on biomass is constant. This result also support theoretical predictions, which state that fumarate production is only affected by

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to growth.

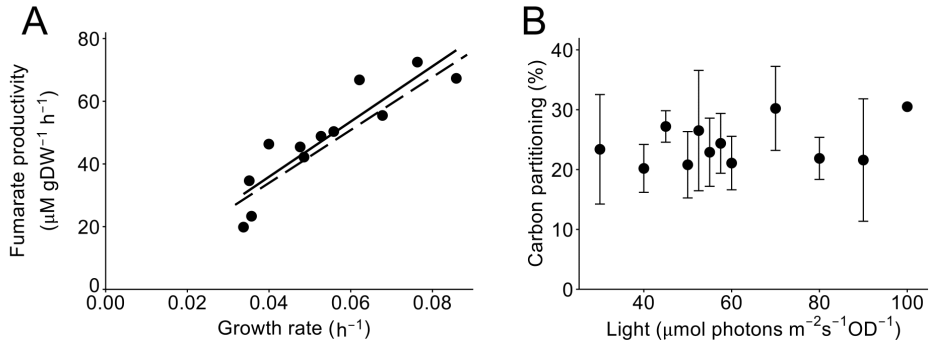

Figure 2.3 *Strict relationship between growth and product formation in the ΔfumC strain. (a) A linear relationship between growth rate and biomass specific fumarate productivity. Each point represents a single observation, and solid line is a linear fit of all experimental data points. Dash line is based on in silico FBA simulations of the genome-scale metabolic model of Synechocystis using biomass maximization as the objective function. (b) Carbon partitioning of fumarate production on biomass at different light regimes. Error bars indicate the standard deviation of carbon partitioning calculated at times throughout the cultivation (n>3).*

Stability of fumarate production by the Δ*fumC* strain

While we considered the body of evidence supporting fumarate growth-coupled production in the Δ*fumC* strain to be very convincing, whether this does indeed improve the phenotypic stability of the production trait remained to be tested. As explained above, the root of the instability comes from Darwinian selection for fitter strains. When using classical metabolic engineering strategies, the fitter strains are the non-producing ones; When using our new growth-coupled strategy, it should be the producing ones. Conditions in which cells are under a strong selection pressure for fastest growth and in which the propagation bottlenecks are smallest, are predicted to result in the fastest drops in productivity [12]. Such conditions, while maintaining the total population size relatively constant, are best met under turbidostat cultivation [13], and so these provide the harshest test ground to assess the stability of production strains.

We cultivated the Δ*fumC* strain under turbidostat regimes operated at non-light limiting conditions at maximal growth rate extending for over 3 weeks. During this period we did not observe any significant changes in production rate (fig.2.4A) - a true testament to the stability of our daytime fumarate producing strain. As a control, we compared how a *Synechocystis* strain that was engineered using classical approaches to produce lactate [14] fairs under the same conditions. Lactate production in this strain was achieved by the heterologous expression of lactate dehydrogenase from Lactococcus lactis, yielding an initial carbon partitioning as the one here reported for fumarate. As

selects for fitter cells, which when using our engineering method, means the producing ones.

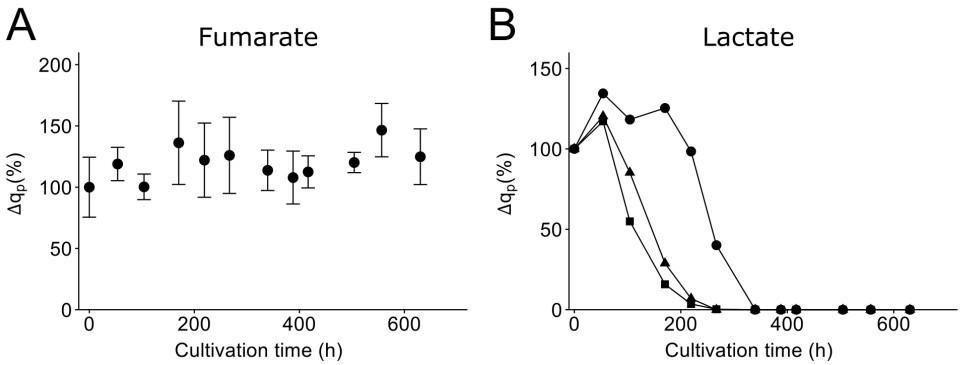

Figure 2.4 *Production stability of fumarate (a) and lactate (b) during prolonged turbidostat cultivation under continuous light.For fumarate, productivity in corresponding time points was normalized based on the average productivity at the first time point (set to be 100%). The error bars indicate the standard deviations of 4 replicates. For lactate, each symbol represents a single observation normalized by its first time point (set to be 100%). The initial burden of deviating carbon from biomass formation for both products is similar, highlighting the success of the engineering strategy deployed for fumarate.*

Conclusion

The model guided metabolic engineering strategy to achieve stable growth coupled production of fumarate during the day (i.e. while cells are growing) in the photosynthetic cyanobacterium *Synechocystis* has been successfully implemented and validated. We provide evidence that (i) the Δ*fumC* strain produces fumarate; (ii) does so in a growth-coupled fashion; and (iii) that this approach completely stabilizes the production trait. This is the first report of fumarate production directly from CO_2 using an engineered cyanobacterium. Thus far, albeit stable, this production is limited to the day since that is when cells grow. Further modeling and genetic engineering in this module of the project, will exploit how this can also be done during the night or exploiting the incorporation of pathways that are not native to *Synechocystis*.

Methods

Strains

The *Synechocystis* Δ*fumC* strain was obtained from the collection of the Molecular Microbial Physiology Group from the University of Amsterdam. This strain has the *fumC* gene which encodes for the TCA cycle enzyme fumarase

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Batch and photonfluxostat cultivation

The batch cultivation was performed in Multi-Cultivator (MC1000-OD, PSI, Czech Republic), with light intensity controlled through a "cool-white" LED panel (PSI, CZ). BG11 supplied with 10 mM TES-NaOH (pH = 8.0) was used for *Synechocystis* cultivation at 30 °C and bubbled by a mix (v/v) of 99 % N_2 and 1% CO_2 at a flow rate of ~150 ml min^{-1}. The pre-cultures (OD_{730} ≈ 2) from the shake flask were used for inoculation in the Multi-Cultivator, with an initial OD_{730} of 0.05 and working volume of 60 ml. Continuous light was given at fixed light intensity of 30 µmol photons m^{-2} s^{-1} after inoculation, and 120 µmol photons m^{-2} s^{-1} when OD_{730} reached 0.5. Samples were taken daily, where OD was recorded and supernatant was prepared. Regarding the photonfluxostats[16], all the cultivation conditions are the same as for batch cultivation except for the light intensity settings. Light intensity was 30 µmol photons m^{-2} s^{-1} after inoculation. When OD_{720} (measured through the build-in OD sensor of the Multi-Cultivator at 720 nm calibrated to the external spectrophotometer at 730 nm) was above 0.6, light intensity was automatically adjusted every 5 min to ensure light intensity per OD_{720} was constant. This light regime was maintained until maximum capacity of the LED panel was reached. Under photonfluxostat mode, a "steady-state" would be achieved and constant growth rate can be reliably obtained. Samples were taken every a few hours during this phase, where OD_{730} was measured and fumarate concentration was quantified.

Turbidostat cultivation

We studied the genetic stability of our strains in populations maintained under turbidostat model [13]. In this continuous cultivation method, microbial populations are kept at a fixed biomass density by diluting the culture with fresh medium at the same rate as the populations grows. This feedback loop applies a strong selection pressure on cells to grow at the maximal specific growth rate. The turbidostat setup used in this experiment is based on a modified Multi-Cultivator, with additional pumps (Reglo ICC, ISMATEC, Germany) transferring fresh medium to the cultures, and subsequently, to a waste container (i.e. as in a classical chemostat). The "pycultivator" software package that controls the Multi-Cultivator and adjunct hardware, additionally sets the pumps to dilute the cultures if the selected OD_{720} threshold is reached. Cells from pre-cultures in shake flasks were inoculated at OD_{720} ~0.05 in 4 independent cylindrical vessels of the Multi-Cultivator, using the same conditions as specified before, except for the incident light intensity, which was fixed at 100 µmol photons m^{-2} s^{-1}. The OD_{720} was recorded every 5 min. When the threshold of OD_{720} > 0.6 was reached, cultures were diluted by 8% (v/v) with fresh BG11. Strain stability was assessed by monitoring growth rate and fumarate and lactate production in time. Growth rate was calculated by fitting a linear function through the natural logarithm of the OD_{720} during each cell "growth-dilution" cycle. Samples for exometabolite production were collected periodically throughout the cultivation period. The variation in production rate, expressed in percentage, was calculated relative to the one observed at the beginning of the cultivation

concentration of exometabolites, at least 500 µL aliquot was taken. Cells were removed through centrifugation for 10 min at 15,000 rpm at 4 °C. The resulting supernatant was then filtered (Sartorius Stedin Biotech, minisart SRP 4, 0.22 µm) for sample preparation. Exometabolite concentrations were measured by HPLC-UV/VIS (LC-20AT, Prominence, Shimadzu), with ion exclusion Rezex ROA-Organic Acid column (250x4.6 mm; Phenomenex) and UV detector (SPD-20A, Prominence, Shimadzu) at 210 nm wavelength. 10 µL of the HPLC samples were injected through an autosampler (SIL-20AC, Prominence, Shimadzu), with 5 mMH_2SO_4 as eluent at a flow rate of 0.15 ml min^{-1} and column temperature of 45 °C. The retention time for fumarate and lactate are 18.26 min and 16.20, respectively on the system used.

▼ Stable nighttime production - Δ*fumC*Δ*zwf*

Introduction

How to stably produce fumarate during the night?

Experts in the field of biotechnology explained to us that production with photoautotrophs is to the best of their knowledge not been explored. After all the claim to fame of these organisms is precisely their ability to use the energy of light. Nonetheless, a significant part of the energy from light captured during the day is used by these organisms to make storage compounds such as glycogen [24] or poly-hydroxybutyrate[25]. If we explore the production possibilities of fumarate in *Synechocystis* during the night, we face a challenge. As *Synechocystis* does not grow without sunlight, the growth coupled production strategy of fumarate is not possible during the night. Thus, we expect no growth coupled fumarate production. But is there another strategy in which we can exploit the night to produce fumarate?

In order to survive the night, *Synechocystis* produces energy by catabolizing its glycogen storage [24]. The TCA cycle is often associated with this process as it provides the electron carriers for ATP production via respiration. As fumarate is an intermediate metabolite in the TCA cycle, increasing the carbon flux through the TCA cycle could be exploited to increase fumarate production during the night.

An unexpected challenge, probably quite unique to photoautotrophs, that emerged is that flux measurements experimentally determined that *Synechocystis* does not use the TCA cycle at night as anticipated, but as rather evolved to prefer the Pentose Phosphate Pathway (PPP) [15]. During the night, the PPP can also act as an additional electron carrier producing pathway, which is preferred over the TCA cycle in *Synechocystis* for this function [15], potentially because it does not release so much of the energy already stored in the carbon bonds. End-product of PPP will be a pentose (5 carbon skeleton), while the TCA cycle will produce CO_2 which was so costly to fix in the first place!

To increase the flux through the TCA cycle, we took a modeling approach, which predicted that we could increase flux towards the TCA cycle by deletion of the *zwf* gene, which codes for Glucose-6-phosphate 1-dehydrogenase. It catalyzes the first step in the PPP. By removing the PPP, our model predicts that the cell is then forced to have a higher flux towards the TCA cycle to reconcile the loss in

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Extracellular fumarate production by a markerless *Synechocystis* Δ*fumC*Δ*zwf strain*

We constructed the Δ*zwf* and Δ*fumC*Δ*zwf* knockouts and confirmed complete absence of antibiotic resistant cassettes for both strains (fig.2.5). On our quest to a diurnal production system, we have characterised both strains in a batch experimentand compared the production capacity of these strains to Wild Type and to the Δ*fumC* strain, both during the day and night phases. We earlier showed that the Δ*fumC* strain is able to produce fumarate under continuous light. Therefore, we expect the Δ*fumC* to be able to produce fumarate during the day in our diurnal regime as well. From our systems biology approach, we predicted that the Δ*fumC*Δ*zwf* strain is able to produce fumarate in a similar fashion as the Δ*fumC* during the day, also using growth coupled production. On top of the strictly growth-coupled production, our model also predicted that the Δ*fumC*Δ*zwf* strain produces fumarate during the night from the flux that is redirected from the PPP towards the TCA. This implies that the Δ*fumC*Δ*zwf* should have an increased total daily fumarate production over the course of 24 hours.

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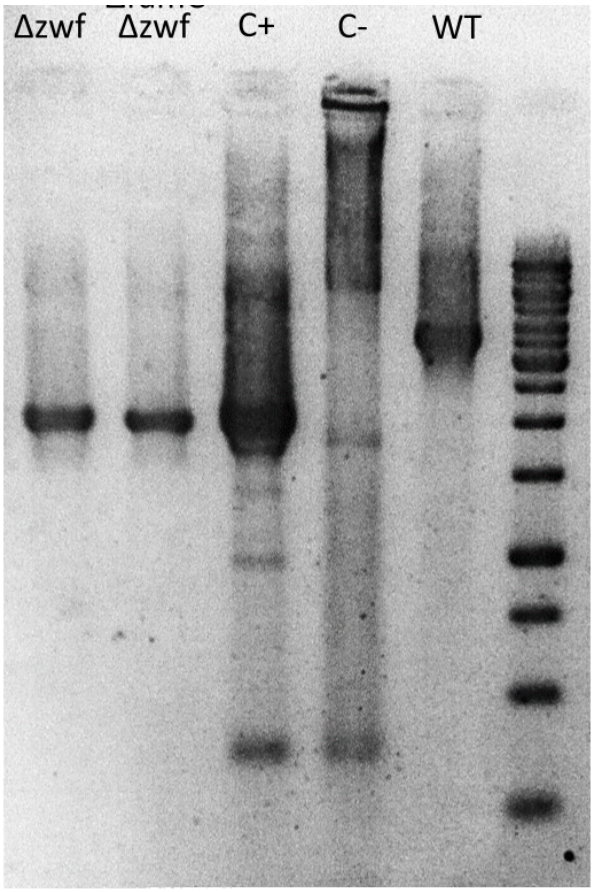

Figure 2.5 *PCR confirmation of gene deletions on Wild Type and ΔfumC background of Δzwf and ΔfumCΔzwf. A clean knockout gives a single DNA band at 2202 bp, while for wild type (WT) this is 3.430 bp. As a positive control (C+) the knockout plasmid was used and as negative control (C-) the mazF plasmid was used. Most importantly the Wild Type (WT) gene is not present anymore in our strains, which implies that the strains are fully segregated knock outs.*

Daytime production

From the diurnal batch culture, we were able to calculate the daytime production for the 4 strains. After 4 day night cycles at an OD_{720} of ~2, which is realistic for industrial settings, both the Δ*fumC* and the Δ*fumC*Δ*zwf* strain produce fumarate with a maximum Qp_{day} of 58.77 μM $grDW^{-1}hour^{-1}$ for the Δ*fumC* and 53.55 μM $grDW^{-1}hour^{-1}$ for the Δ*fumC*Δ*zwf* over the course of one day fig.2.6. This confirms that (i) the Δ*fumC* is able to produce during the day, when mimicking industrial settings with a diurnal and sinusoidal light regime, and (ii) the Δ*fumC*Δ*zwf* produces a similar amount of fumarate during the day as the Δ*fumC*. As expected both the WT and the Δ*zwf* strain did not produce fumarate during the day.

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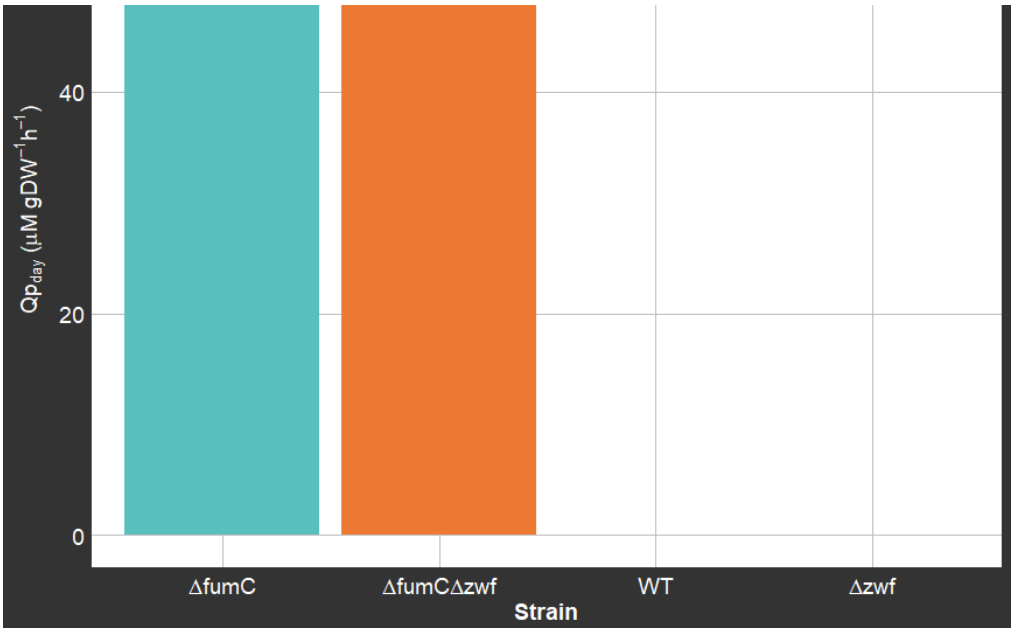

Figure 2.6 *Qp_{day} of the different strains. of the four different strains during the fourth 24h period. This experiment has been carried out with similar results 5 times independently for ΔfumCΔzwf and 6 times for the ΔfumC. ΔfumCΔzwf has a higher Qp_{day} than the ΔfumC. WT and Δzwf do not produce fumarate during the night.*

Night time production in conditions mimicking industrial settings

Based on our modeling results we expected the Δ*fumC*Δ*zwf* to have increased fumarate production during the night compared to the Δ*fumC* strain. We can see that already during the fourth 24h period, the Δ*fumC*Δ*zwf* has a Qp_{night} of 18.4 µM $grDW^{-1}hour^{-1}$, while the Δ*fumC* has a Qp_{night} of 6.69 µM $grDW^{-1}hour^{-1}$ (fig.2.7). This beautifully confirms the predicted results obtained from modeling.

It must be noted however, that nighttime fumarate production can be perceived as finite since it relies on the glycogen storages that were built up during the day. The reported QP is calculated by looking at the night as whole. Although

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Even if we do not correct for OD, there is an increase in fumarate concentration during the night (fig. 2.8), further supporting that the Δ*fumC* indeed (unexpectedly) produces fumarate during the night. This can be easily explained by one of the pitfalls of constraint based analysis techniques such as the ones we used - they assume optimality. The dynamic flux balance analysis predicts that using the PPP at night is preferred over the TCA, and hence, the corresponding flux distributions deduced do not lead to fumarate production. The thing is that Life does not always have to conform to the optimality principles that underlie the modeling, as there might be other factors which are not captured in the model at play. For instance, one could speculate that cells adopt an anticipatory behavior during the night and want to make some amino acids that are derived from TCA intermediates. That could explain why a residual flux towards the TCA could still be beneficial. One thing remains clear, though - deletion of the *zwf* gene combined with the *fumC* drastically increases fumarate production in the dark - nearly 3 fold! This also opens up possibilities for the production of other TCA cycle intermediates at night with *Synechocystis*

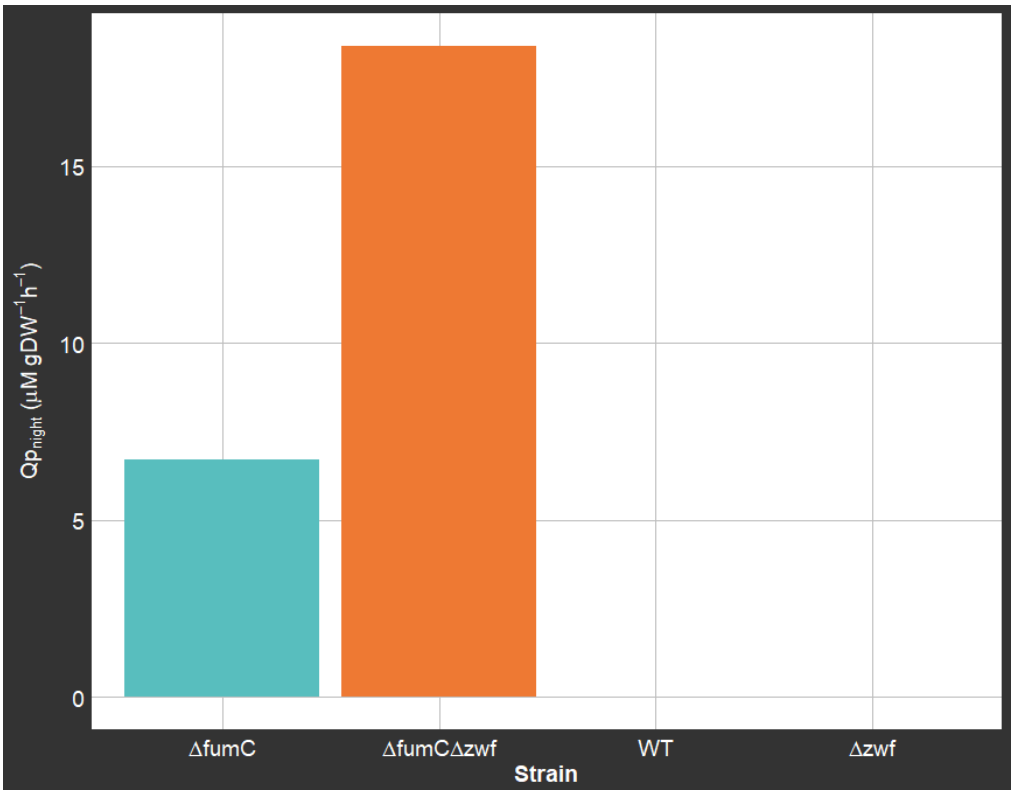

Figure 2.7 *Qp_{night} of the four different strains during the fourth 24h period. This experiment has been carried out with similar results 5 times independently for ΔfumCΔzwf and 6 times for the ΔfumC. ΔfumCΔzwf has a higher Qp_{night} than the ΔfumC. WT and Δzwf do not produce fumarate during the night.*

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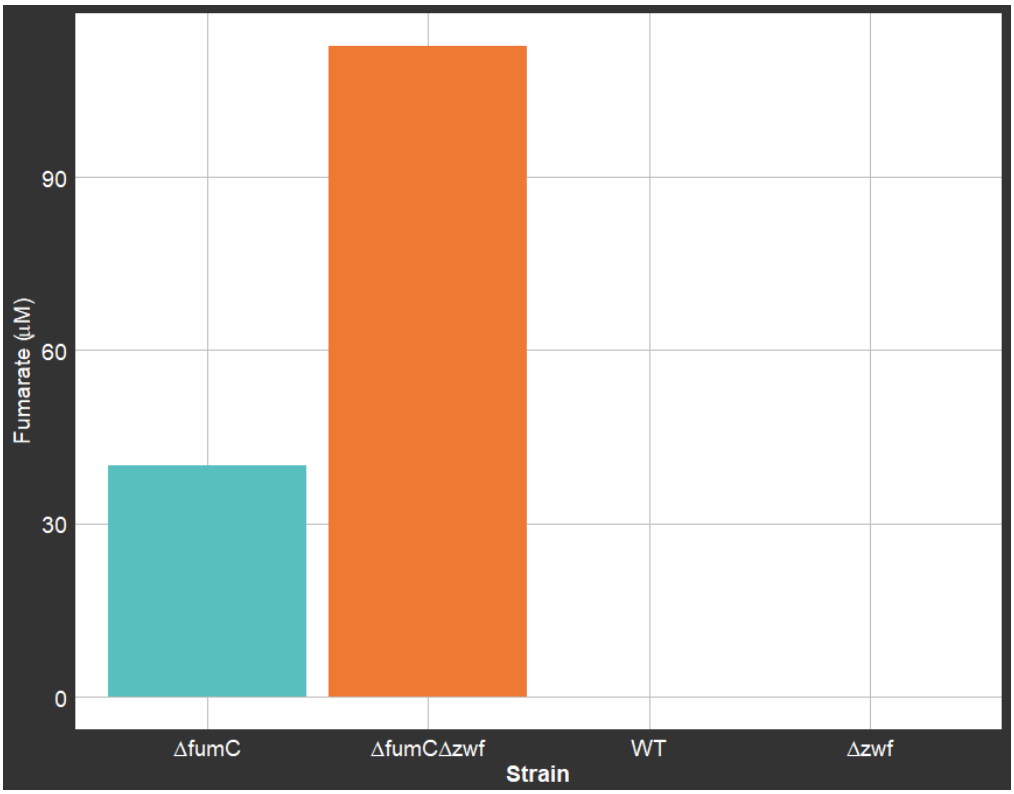

Figure 2.8 *Fumarate production of the four different strains during the night. The fumarate production value is not corrected by OD. This confirms that the ΔfumCΔzwf and the ΔfumC strain produce fumarate at night.*

Overall production in conditions mimicking industrial settings

The productivity of our strains in an industrial setting is the combined production of day and night. We calculated the Qp_{daily} over the course of a 24h period (figure 2.9). We find that the Δ*fumC*Δ*zwf* has a Qp_{daily} of 32.83 µM $grDW^{-1}hour^{-1}$, while the Δ*fumC* has a Qp_{daily} of 23.00 µM $grDW^{-1}hour^{-1}$. The titers of the strains are 27.39 mg L^{-1} and 48.48 mf L^{-1} after four 24h periods. We can thus conclude that the Δ*fumC*Δ*zwf* produces more fumarate over the course of a natural day. This clearly shows the benefit of having a day/night production system (and it is extremely gratifying to see that all our modeling and experimental efforts were not in vain!).

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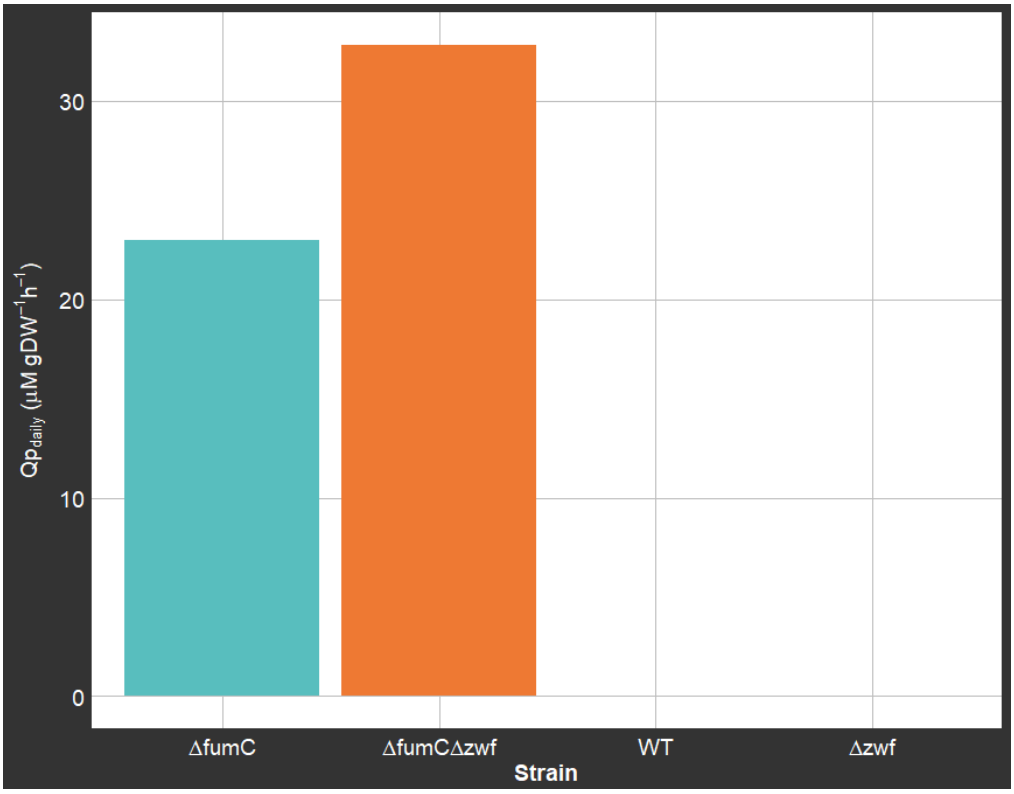

Figure 2.9 *Qp_{daily} of the four different strains during the fourth 24h period. This experiment has been carried out with similar results 5 times independently for ΔfumCΔzwf and 6 times for the ΔfumC. ΔfumCΔzwf has a higher Qp_{daily} than the ΔfumC. WT and Δzwf do not produce fumarate during the night.*

	Qp_{day}	Qp_{night}	Qp_{daily}
ΔfumC	53.6	6.70	23.0

Table 2.1 *Fumarate production parameters for the four different strains. Qp values are in mM grDW^{-1}hour^{-1} measured after the fourth day/night cycle ,*

Conclusion

We showed that the Δ*fumC* and the Δ*fumC*Δ*zwf* are both able to produce fumarate during the daytime using the growth coupled strategy. During the night, the Δ*fumC*Δ*zwf* produces more fumarate than the Δ*fumC*, which confirms the extensive modeling we did for this part of the project. We can confirm that at night the Δ*fumC*Δ*zwf* is forced to direct carbon from glycogen catabolism towards the TCA cycle to form fumarate. We thus engineered a *Synechocystis* cell factory that is able to produce fumarate around the clock, using two different production strategies - both stable - one for day and another for night. On top of this, since we used only knock-outs and did not resort to the cloning of heterologous genes, the Δ*fumC*Δ*zwf* will be a stable production strain for many generations to come. As a bonus, the higher nighttime production of the Δ*fumC*Δ*zwf* compared to the Δ*fumC* does imply that by knocking out the Δ*zwf*, we force flux to the TCA cycle. This is an important finding, as it opens up opportunities for the nighttime production of valuable TCA cycle intermediates in *Synechocystis*. To our knowledge such a diurnal, dual strategy, photoautotrophic cell factory has never been reported before.

Methods

Strain construction: Δ*zwf* and Δ*fumC*Δ*zwf* and segregation

The *zwf* gene encodes glucose-6-phosphate 1-dehydrogenase, which catalyses the first step in the Pentose Phosphate Pathway. We knocked out the *zwf* gene in the Wild Type and the Δ*fumC* background, to construct the Δ*zwf* and the Δ*fumC* Δ*zwf* mutants. We used the Markerless knock out method. The homologous regions of the *zwf* gene were amplified from the *Synechocystis* genomic DNA, with Herculase polymerase using primers BP1, BP2, BP3 and BP4. The biobrick T vector used was the pFL-AN. Resulting in plasmid in *zwf* knockout plasmids, which were used for the first and second round of transformation.

Characterising Δ*zwf*, Δ*fumC* and Δ*fumC*Δ*zwf*

In order to characterise the different *Synechocystis* strains, we performed different cultivation experiments. We simultaneously performed a batch and a turbidostat experiment in a modified Multi-Cultivator under a photonfluxostat

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turbidostat set up, we cultivated four strains i) Wild Type, ii) Δ*zwf* , iii) Δ*fumC* Δ*zwf*, and iv) Δ*fumC* all in duplicates. The light intensity per OD followed a sinusoidal regime to simulate day/night cycles yielding 16 hours of darkness (0 $\mu E\ s^{-2}\ OD^{-1}$) and 8 hours of light (peaking at 120 $\mu E\ s^{-2}\ OD^{-1}$), calculated by equation 2.1, where t is the time in hours.

$$2.1\ \frac{\mu E}{s^2} = 240 \sin(2\pi \cdot (\frac{t}{24} + \frac{1}{4})) - 120$$

This equation returns negative values during the period, so they are clamped at a minimum value of 0. All cultures are inoculated at an initial OD_{720} of 0.05 and were grown at a constant light intensity of 20 µE until all vessels reached an OD_{720} of 0.6. At this point, we switched the light output to the designated light regime.

Instead of the more commonly adopted 12h day/12h night, we chose a 8h day/ 16h night as indicated above. While longer days would have probably allowed us to reach higher levels of production in the lab, after visiting an actual production facility, we were convinced that this would not be representative of a real-world scenario. The structures surrounding the greenhouses in many production plans provide shading during dawn and dusk. This makes the sun rise somewhat later, and set somewhat sooner, for production photoautotrophs. Our light regime in the lab mimics this, and is yet another factor that confers credibility to the actual production numbers that we report..

Sampling and fumarate measurements

During the course of the experiment, we took samples at every perceived dawn and dusk. After sampling we had to determine the concentration of fumarate. 1 ml of sample was centrifuged at 15.000 rpm for 10 min. Then 500 µl supernatant was taken and filtered (Sartorius Stedin Biotech, minisart SRP 4, 0.22 µm) for sample preparation. Fumarate concentration was measured by HPLC-UV/VIS (LC-20AT, Prominence, Shimadzu), with ion exclusion Rezex ROA-Organic Acid column (250x4.6 mm; Phenomenex) and UV detector (SPD-20A, Prominence, Shimadzu) at 210 nm wavelength. 50 µL of the HPLC samples were injected through an autosampler (SIL-20AC, Prominence, Shimadzu), with 5 mM H_2SO_4 as eluent at a flow rate of 0.15 ml min^{-1} and column temperature of 45 °C. Fumarate retention time was determined as 18.16 and 18.36 min and fumarate samples were normalised by a correction factor composed of 10 mM divided by the measured TES concentration.

Production calculations

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coupled. We calculated the fumarate yield during the day, $yield_{day}$ by Δfumarate/ ΔOD in mmol OD^{-1} over the course of 1 day in the batch culture. By dividing this number by 8 hours, we could calculate Qp_{day} in mmol OD^{-1} h^{-1} for the day. During the night, no cell growth was assumed, therefore we expected no change in OD, however as *Synechocystis* physiology changes at night, this can influence the scattering of the light and thereby the OD measurement can change during the night. To account for this effect, we determined the night time fumarate yield, $yield_{night}$ as Δfumarate/mean OD. By dividing this number by 16 hours we could calculate Qp_{night} in mmol OD^{-1} h^{-1}. The overall 24h fumarate production could be determined by knitting together the nighttime production and the daytime production. we determined the $yield_{daily}$ as $yield_{day}$ plus $yield_{night}$. Dividing this number by 24 hours, we could determine Qp_{daily} in mmol OD^{-1} $hour^{-1}$. To transform these QPs to a more familiar unit, we multiplied all QP's by a conversion factor that converts OD_{720} to gram dry weight (148 mg $L^{-1}OD^{-1}$ [16]) . We then receive fumarate QPs in mM $gDW^{-1}hour^{-1}$.

2.2

$$yield_{day} = \frac{\triangle[fumarate]}{\triangle OD}$$

2.3

$$yield_{night} = \frac{\triangle[fumarate]}{meanOD}$$

2.4

$$Qp_{day} = \frac{yield_{day}}{8\ Hours} \cdot 148\ mg \cdot L^{-1} \cdot OD^{-1}$$

2.5

$$Qp_{night} = \frac{yield_{night}}{16\ Hours} \cdot 148\ mg \cdot L^{-1} \cdot OD^{-1}$$

2.6

$$Qp_{daily} = \frac{yield_{day} + yield_{night}}{24\ Hours} \cdot 148\ mg \cdot L^{-1} \cdot OD^{-1}$$

▼ Beyond the native metabolic network of *Synechocystis*

Introduction

Being able to force the cell to direct carbon flux towards the reactions of the native TCA cycle in the Δ*fumC*Δ*zwf* strain increases the fumarate production during the night. However, by knocking out fumarase, we disrupted the cyclic nature of the TCA, which may no longer operate as a cycle. This construct yields one fumarate per glycogen catabolized. We started wondering if there could be a way to use synthetic biology to improve the nighttime production efficiency in terms of carbon usage. We turned to nature for inspiration…

Many microorganisms express a glyoxylate shunt. The glyoxylate shunt consists of two enzymes which are not natively present in *Synechocystis*: isocitrate lyase (ICL) and malate synthase (MS). The first enzyme, ICL, catalyzes the reaction of isocitrate into glyoxylate and succinate, where succinate can then be consumed by succinate dehydrogenase to produce fumarate and FADPH2. As for glyoxylate, this compound is then consumed by MS, along with acetyl-CoA, to make malate. Malate is then converted to oxaloacetate by malate dehydrogenase, yielding NADPH (fig. 2.11).

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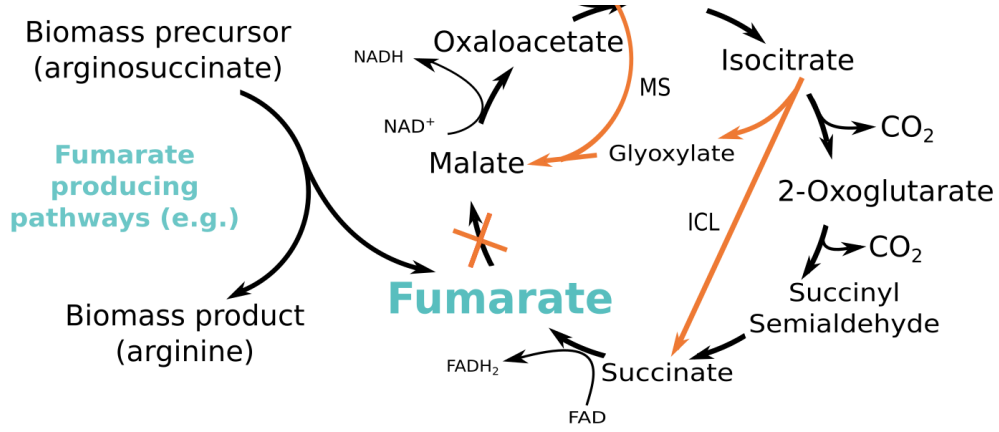

Figure 2.11 *The glyoxylate shunt with ΔfumC. The glyoxylate shunt is comprised of two enzymes: isocitrate lyase (ICL) and malate synthase (MS). The orange X signifies the knockout of the fumC gene.*

We hypothesized that if a synthetic glyoxylate shunt was introduced into *Synechocystis*, this would not only reconnect the stunted ΔfumC TCA cycle, but also increase the flux towards fumarate production, by feeding into reactions which produce electron carriers - one of the main roles of the TCA cycle [17]. This would potentially align fumarate production with an increase in fitness during the night, providing the type of positive selection pressure, which could be used to stabilize the expression of this heterologous pathway in a production strain as advised by experts.

We have explored this idea by introducing the glyoxylate shunt into the genome-scale metabolic model that had been guiding our metabolic engineering strategies thus far. We found that our hypothesis is corroborated by model simulations. However, timing the activation of this pathway turned out to be of the essence when engineering a stable strain. This led us to the construction of the first fully-segregated (i.e all copies of the chromosome have the same allele) promoter library in a polyploid organism such as the cyanobacterium we work with (*Synechocystis*), but not without first developing a method to do so.

Results and discussion

Modeling the shunt

We explored the possibilities for the introduction of the glyoxylate shunt in terms of fitness-gain and fumarate production, by running simulations in which the glyoxylate shunt had been "cloned" into our ur metabolic engineering strategies thus far. We found that our hypothesis is corroborated by model (Oh! If only it was that easy in the lab as well…)

We found that the glyoxylate shunt must be well timed: it cannot be active during the day, as the glyoxylate shunt will draw carbon from more prefered pathways. We are thus faced with another challenge: how do we time the

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through its promoter. That is why we aim to look for a promoter with the right expression pattern for the shunt genes to be activate only in the night. Photoreceptor-based transcriptional circuits could at a first glance be suitable control systems. However, the delays between transcriptional activation (or repression) and the actual increase (or drop) in enzyme levels can be many hours depending for instance on protein stability. Such delay could be sufficient to remove the competitive edge of harboring the genes encoding the shunt, and therefore, rendering it phenotypically unstable.

Synechocystis harbors circadian clocks with periods close to 24 h [18]. Chances are, that there already is a promoter present in its chromosome with the right expression levels at the right time for our glyoxylate shunt enzymes. These cellular clocks allow organisms to anticipate the environmental cycles of day and night by synchronizing circadian rhythms with the rising and setting of the sun. The rhythms originate from the oscillator components of circadian clocks and control global gene expression and various cellular processes, hinting at the presence of an appropriate promoter for our shunt genes. To express the glyoxylate shunt only during the night and with the right expression levels, we aim to use what nature has to offer and make a glyoxylate shunt promoter library.

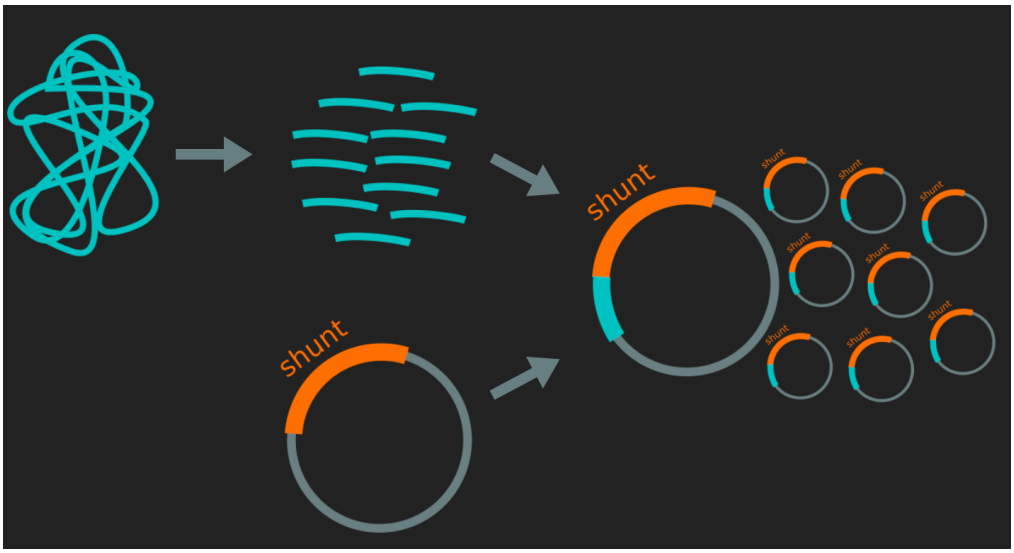


Figure 2.12 *The promoter library DNA is prepared by cutting up the genome of Synechocystis into fragments that have on average the size of a promoter (100-1000bp). Each of these fragments will be introduced upstream of the glyoxylate shunt genes, resulting in thousands of unique plasmids.*

Making the library

The glyoxylate shunt promoter library was constructed by cutting up the genome of *Synechocystis* into fragments that have, on average, the size of a promoter (between 100-1000 bp). Subsequently, each of these fragments was

library at least once, the DNA was extracted from *E. coli*. We required our final library to be integrated into the the genome at the neutral site *slr0168* of *Synechocystis* to ensure that the difference in observed phenotype can be attributed to the difference in promoter, rather than something else (e.g. changes in plasmid copy number). However, the introduction of the promoter library into *Synechocystis* was easier said than done.

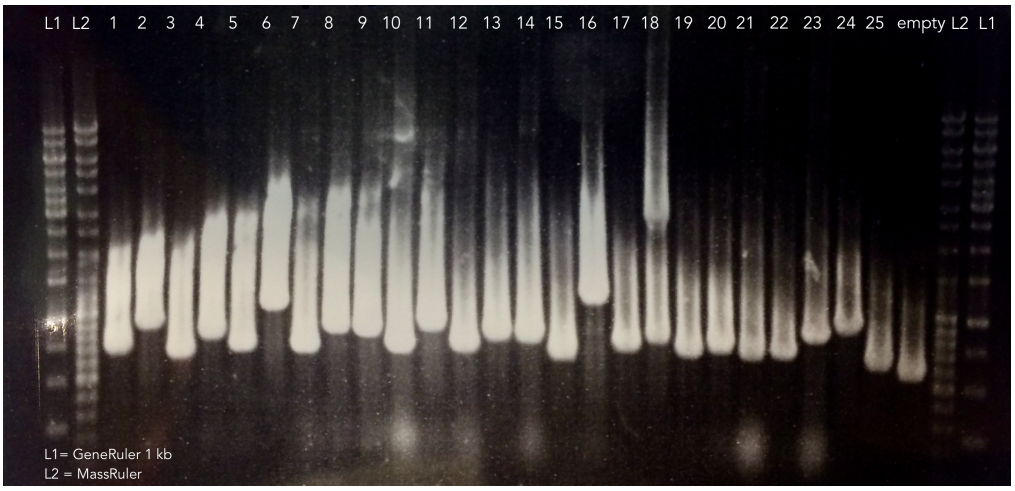


Figure 2.13 *After transforming the library DNA into E. coli, a colony PCR with primers flanking the promoter region confirmed that every insert had a different length.*

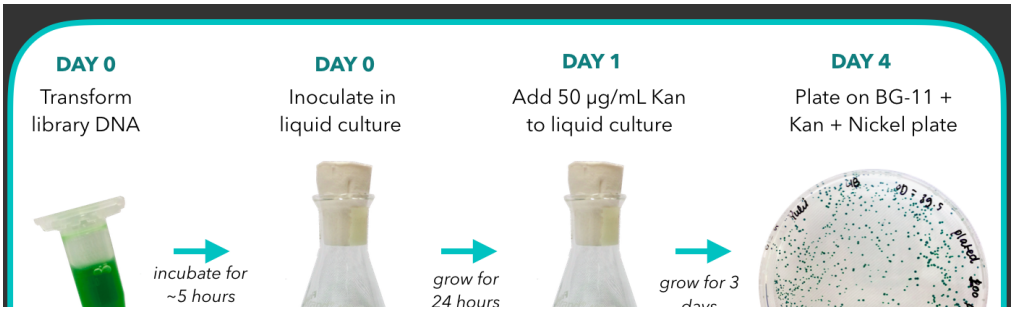
Synechocystis is a polyploid, each cell containing 4 to 20 copies of the genome depending on the physiological state and environmental conditions [19]. Polyploidy poses a challenge in creating a stable mutant strain, since the newly introduced genes have to be 'fully segregated' (i.e. present in all chromosome copies) to avoid that they revert to wild type in future generations. However, a fully segregated promoter library in *Synechocystis* is something that has not yet been reported and poses a major challenge - How does one guarantee that all members of the library are fully segregated? - Our supervisor, told us that we had three options:

- Quit here;
- Colony PCR tens of thousands of colonies;
- Or come up with a new method to do it.

We chose the latter and decided to develop a method to create a fully

endoribonuclease that is regulated by a nickel-induced element (NIE) [21], killing the cells upon exposure to elevated concentrations of nickel. The *mazF* cassette needs to be introduced into the neutral site *slr0168* of the *Synechocystis* genome, enabling the introduction of the glyoxylate shunt into the neutral site at later time points, replacing the *mazF* cassette itself. With this selection method, complete segregation of the glyoxylate shunt in the genome can be tested simply by adding nickel - if the colony is not fully segregated, even if there is only one copy of the *mazF*-cassette in the genome, the cell will be killed upon exposure to nickel! However, when a colony is fully segregated, and all the *mazF* cassettes are replaced by glyoxylate shunt genes, the colony remains unharmed. Furthermore, we wanted the glyoxylate shunt promoter library to represent the entire genome (i.e. as many colonies as possible to reach a probability of having all the positions in the chromosome in the library at least once approaching 100%). To expand the library, we allowed the cells time to segregate before exposing them to nickel, as cells often shift towards full segregation over time [22]. A positive selection marker was used to push cells towards full segregation. However, high amounts of antibiotics might hamper cell growth and thereby slow down segregation. Studying the segregation dynamics, we found the sweet-spot between antibiotic concentration and the timing before exposing cells to nickel: after transformation (day 0), cells were supplemented with kanamycin (50 µg/ml) on day 1 and nickel 20 µM on day 4 (fig. 2.14).

Using our newly developed method, we created the first fully segregated library representing the entire genome (99.9% confidence) of *Synechocystis* upstream of the glyoxylate shunt genes. What is more; the library is now ready to be tested. The beauty of the glyoxylate shunt promoter library approach lies in the fact that expressing the shunt at both the appropriate time and level increases fitness. Therefore, we can now easily select the ideal promoter using long term cultivations and letting Darwinian selection do the rest. The promoter that expresses the shunt the most optimally will be the one that grows the fastest, outcompeting the rest of the population. This will also be the best fumarate producer, which will eventually be used to further increase the nighttime fumarate production.



Conclusion

Using the genome-scale metabolic model, we found that the glyoxylate shunt can increase the growth rate of our fumarate producing strain, but only under specific conditions: (i) it cannot be active during the day, as this will result in a decrease in fitness, and (ii) the expression of glyoxylate shunt enzymes need to be at the right levels. In order to find a promoter with the right expression characteristics for the glyoxylate shunt, we developed a method to create a fully segregated promoter library in polyploid organisms, such as our own cyanobacterium *Synechocystis*. Using our own developed method, we created the first fully segregated library representing the entire genome (99.9% confidence) of *Synechocystis* upstream of the glyoxylate shunt genes. This library is now ready to be tested to further increase nighttime fumarate production.

Methods

Construction of the glyoxylate shunt genes in a plasmid
The glyoxylate shunt genes are derived from *Chlorogloeopsis fritschii* PCC 9212 and are obtained from the Bryant Lab in a plasmid called pAQ1Ex_cpc_MS-ICL_Sp [23]. To get both genes expressed under the same promoter, the glyoxylate shunt will be placed in a plasmid (pFLXN-h1h2-rbs-yfp-0168; obtained from MMP) that contains a ribosomal binding site (rbs) in front of each gene and a BglII restriction site where the genomic DNA fragments will be inserted. To push segregation and to select in*E. coli*, a kanamycin (Kan) resistance cassette was introduced in the plasmid.

Both plasmids were digested with NcoI and BamHI (Thermo Scientific FastDigest), placed on a 1% agarose gel, cut out and purified (Qiagen PCR/gel purification kit). Subsequently, the insert (MS-ICL) was ligated in the vector (pFLXN-h1h2-rbs), replacing the YFP fragment using T4 ligase and transformed to DH5alpha competent cells. Colonies were confirmed with colony PCR using primers h2_down_rv and h1_up_fw. The confirmed colonies were inoculated in LB (+50 µg/mL Kan), after which the plasmid, now called pFLXN-h1h2-rbs-MS-ICL (fig. 2.15), was extracted and sequence confirmed by Macrogen Europe (the Netherlands).

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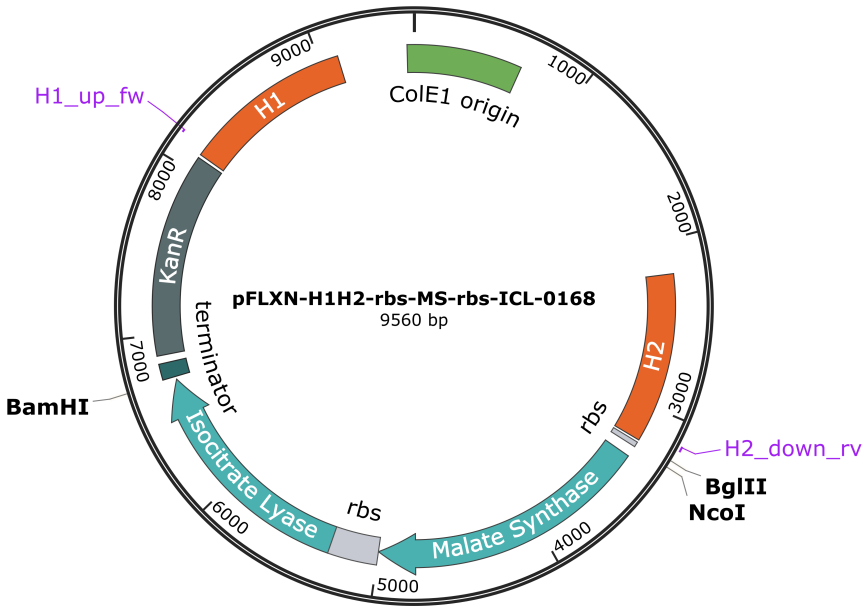

Figure 2.15 *Plasmid map of pFLXN-H1H2-rbs-MS-rbs-ICL containing the glyoxylate shunt genes with homologous regions to be integrated into the neutral site slr0168. Genomic DNA fragments were inserted at the BglII site.*

Method to create a promoter library in polyploid organisms

Construction of the glyoxylate shunt promoter library DNA using E. coli

The construction of a promoter library consists of multiple steps. First, the genomic DNA of *Synechocystis* was extracted on a large scale (20 mL, OD_{730}~5) and purified using RNAse. Then, the purified genomic DNA was digested with Sau3AI (Thermo Scientific FastDigest), yielding fragments ranging from 100-1000 bp. These fragments will be referred to as the inserts.

To open pFLXN-h1h2-rbs-MS-ICL, enabling it to integrate the inserts, the plasmid was digested with BglII which is compatible with Sau3AI (Thermo Scientific FastDigest; incubated for 3 hours, added Phosphatase after 1.5 hour to prevent self ligation).

Next, the insert was ligated in the vector using a ligation ratio of 1:7 (v:i) and transformed into Dh5-Alpha Competent E. coli cells (MCLAB) multiple times, yielding it total over 100,000 positive colonies. This led to a probability of 99.9%

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$$N = \frac{ln(1-p)}{ln(1-f)} = \frac{ln(1-p)}{ln(1-\frac{i}{g})}$$

N = number of recombinants needed
p = desired probability that any fragment in the genome will occur at least once in the library
f = i/g = fractional proportion of the genome in a single recombinant
i = average insert size
g = genome size

In our case
i = 256 bp
g = 3.6 E+6 bp
p = 0.999 (99.9% confidence)

$$N = \frac{ln(1-p)}{ln(1-f)} = \frac{ln(1-0.999)}{ln(1-\frac{256}{3.6E+6})} = 97136 \text{ clones needed}$$

Box 2.1

The colonies were washed of the plates, stored as glycerol stocks (15% glycerol; -80°C) and inoculated overnight. The plasmids were extracted using a MiniPrep (Qiagen) and stored as the promoter library DNA.

Segregation Dynamics in Synechocystis

In order to obtain a fully segregated library that represents the entire genome of *Synechocystis*, the segregation dynamics was studied, aiming to find the sweet-spot between kanamycin concentration and timing before exposing the library to Nickel.

Transformation
A pre-culture (fully segregated Δ*fumC-mazF*Ω) of 100 mL (20 mL per transformation) was used (OD_{730} = 1). The culture was spun down (3900 rpm; 10 min), washed twice with fresh BG-11 to remove possible antibiotics, and concentrated 100 times to a volume of 1 mL (200 µL per transformation) (OD_{730} = 100). Next, the library DNA was added (30 µg DNA/mL culture) to 800 µL culture. As a negative control, 200 µL of culture without DNA was used. The cultures were incubated for 5 hours (30 °C; ~30 µmol/m2/s of constant white light), after which the 200 µL per transformed culture was diluted in fresh BG-11(20 mL) without antibiotics and incubated (~22 hours)(day 0). Subsequently, kanamycin was added in the following concentrations to push segregation (day 1):

- negative control: 40 µg/mL Kan
- Kan40: 40 µg/mL Kan
- Kan50: 50 µg/mL Kan
- Kan75: 75 µg/mL Kan
- Kan100: 100 µg/mL Kan

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	BG-11	BG-11 + Kan	BG-11 + Kan + Nickel
Clones that did not integrate fragment	x		
Clones that integrated fragment	x	x	
Clones that are fully segregated	x	x	x

Table 2.2 *The ability of clones to grow (x) on different BG-11 plates. Concentrations: Kanamycin: 50 µg/mL; Nickel: 20 mM.*

Every day, the cells were plated on the three different BG-11 plates using a dilution series droplet design (fig. 2.16).

The OD_{730} of the cultures were measured, after which the dilution series was prepared using a 96 well-plate and a multichannel pipet. First, all the BG-11 was added in appropriate amounts to the wells following the pipetting scheme, after which the inoculum from the different cultures was added to row A. After mixing properly, the dilution series were made by pipetting 10 µL from row A to B. After mixing, 10 µL was pipetted from row B to C, and so on, resulting in a dilution series that contained two rows per culture:

10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}.
$10^{-0.5}$, $10^{-1.5}$, $10^{-2.5}$, $10^{-3.5}$, $10^{-4.5}$, $10^{-5.5}$, $10^{-6.5}$, $10^{-7.5}$

From each well, 5 µL will be plated in droplets on the three types of BG-11 plates.

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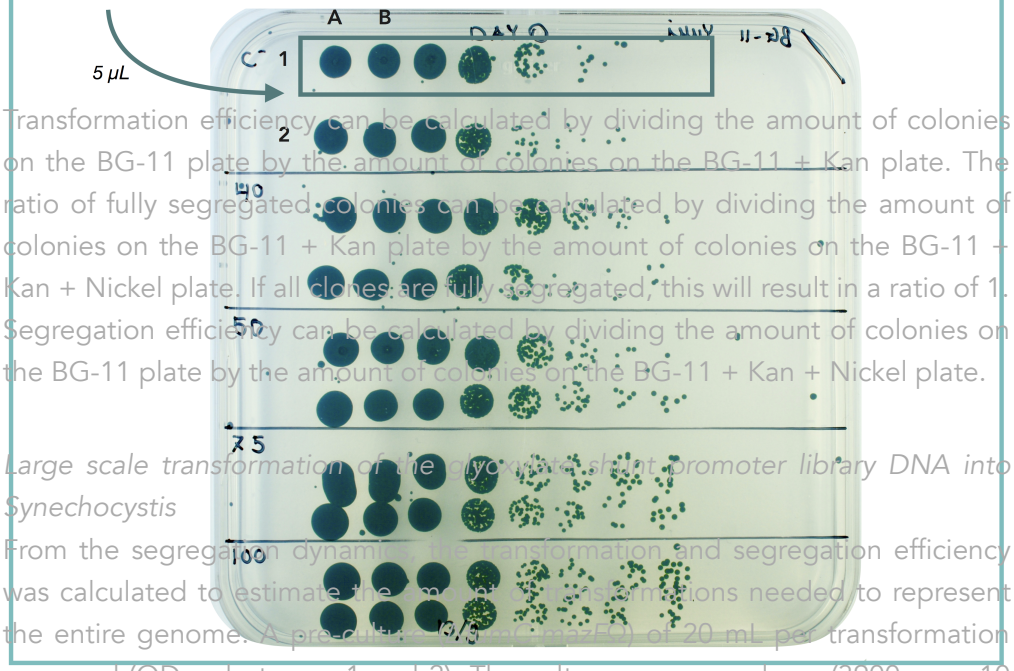
	Control				Kan40				Kan50				Kan75				Kan100			
	1		2		3		4		5		6		7		8		9		10	
	BG11	Inoc	BG11	Inoc	BG11	Inoc	BG11	Inoc	BG11	Inoc	BG11	Inoc	BG11	Inoc	BG11	Inoc	BG11	Inoc	BG11	Inoc
A	0	100	100	100	0	100	100	100	0	100	100	100	0	100	100	100	0	100	100	100
B	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10
C	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10
D	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10
E	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10
F	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10
G	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10
H	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10	90	10

10 µL



Transformation efficiency can be calculated by dividing the amount of colonies on the BG-11 plate by the amount of colonies on the BG-11 + Kan plate. The ratio of fully segregated colonies can be calculated by dividing the amount of colonies on the BG-11 + Kan plate by the amount of colonies on the BG-11 + Kan + Nickel plate. If all clones are fully segregated, this will result in a ratio of 1. Segregation efficiency can be calculated by dividing the amount of colonies on the BG-11 plate by the amount of colonies on the BG-11 + Kan + Nickel plate.

Large scale transformation of the glyoxylate shunt promoter library DNA into Synechocystis

From the segregation dynamics, the transformation and segregation efficiency was calculated to estimate the amount of transformations needed to represent the entire genome. A pre-culture (BG-11) of 20 mL per transformation was used (OD_{730} between 1 and 2). The culture was spun down (3900 rpm; 10 min), washed twice with fresh BG-11 to remove antibiotics, and concentrated 100 times to a volume of 200 µL per transformation. Next, the library DNA was added (30 µg DNA/mL culture) and incubated for 5 hours (30 °C ; ~30 µmol/m2/s of constant white light). After five hours, the 200 µL transformed culture was diluted in fresh BG-11(20 mL) without antibiotics and incubated for ~22 hours (day 0). Subsequently, Kanamycin was added (50 µg/mL) to push segregation (day 1) and cultivated for an additional three days. At day 4, the culture was spun down (3900 rpm; 10 min) and concentrated (OD_{730} ~40), after which 100 µL per plate was spread on BG-11 plates (1% agar) containing Kanamycin (50 µg/mL) and Nickel (20 µM). The plates were incubated for 8 days (30 °C; ~30 µmol/m2/s of constant white light) until colonies appeared, resulting in the first fully segregated glyoxylate shunt promoter library in *Synechocystis*.

Figure 2.16 *Pipetting scheme in order to make the droplet dilution series and the plating design.*

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▶ Supplementary Material