Supplementary Information

Cycling between growth and production phases increases cyanobacteria bioproduction of lactate

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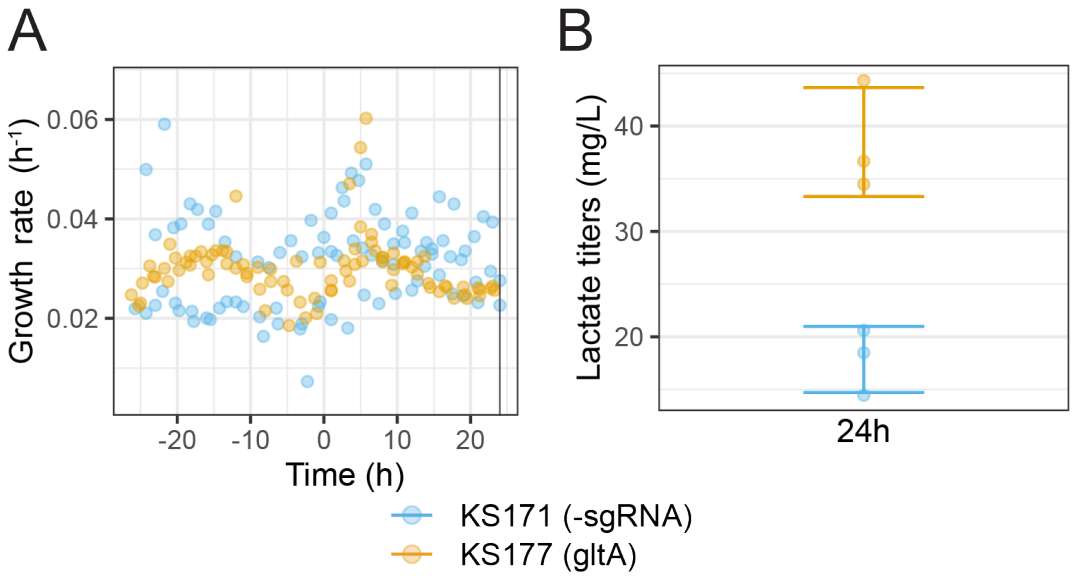

Figure S1. Growth arrest increases lactate titers compared to non-growth arrested strain. Growth rate and lactate titers for KS171 and KS177 grown in triplicates in turbidostat mode. (A) Growth rate. Addition of aTc to induce CRISPRi was at 0 h. Culture supernatant was sampled 24 h post induction (black line) for lacate titer. (B) Corresponding lactate titers after 24h. The cell density was OD = 0.4 with white light at 200 µE.

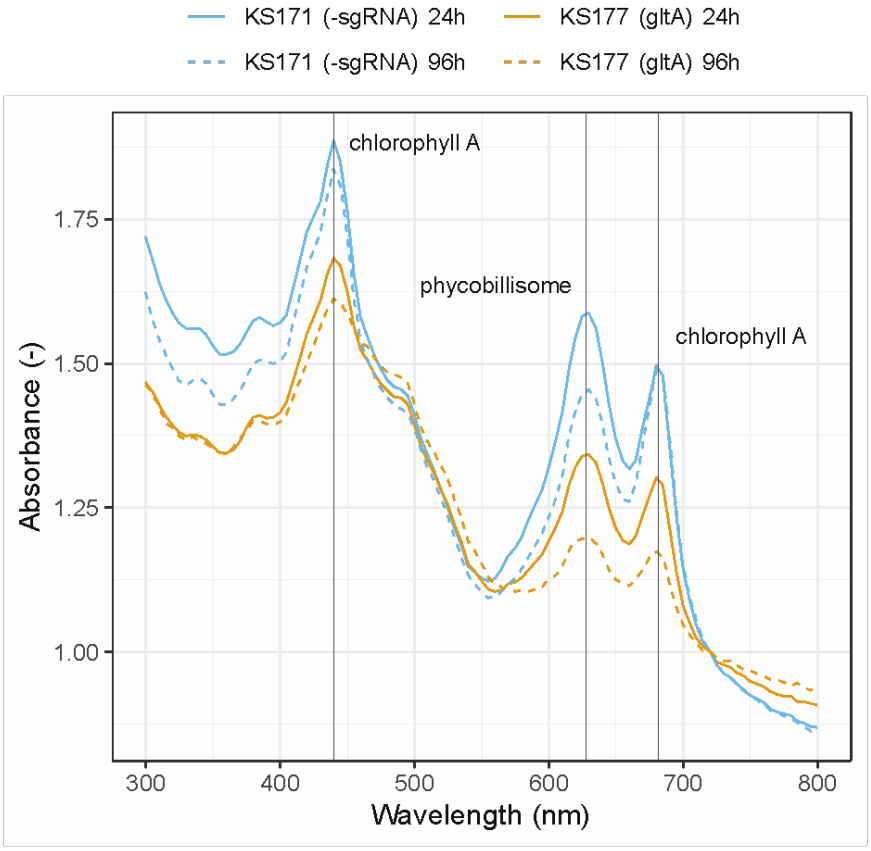

Figure S2. Absorbance spectrum for KS171 (no sgRNA) and KS177 (sgRNA targeting *gltA*) at 24 h and 96 h post-induction of CRISPRi. Absorbance peaks for chlorophyll A and the phycobilomsomes are indicated. Cells were grown in a multicultivator in batch mode with a light intensity of 300 µE. The induction of CRISPRi with aTc was at the start of the experiment when OD_{730} = 0.1.

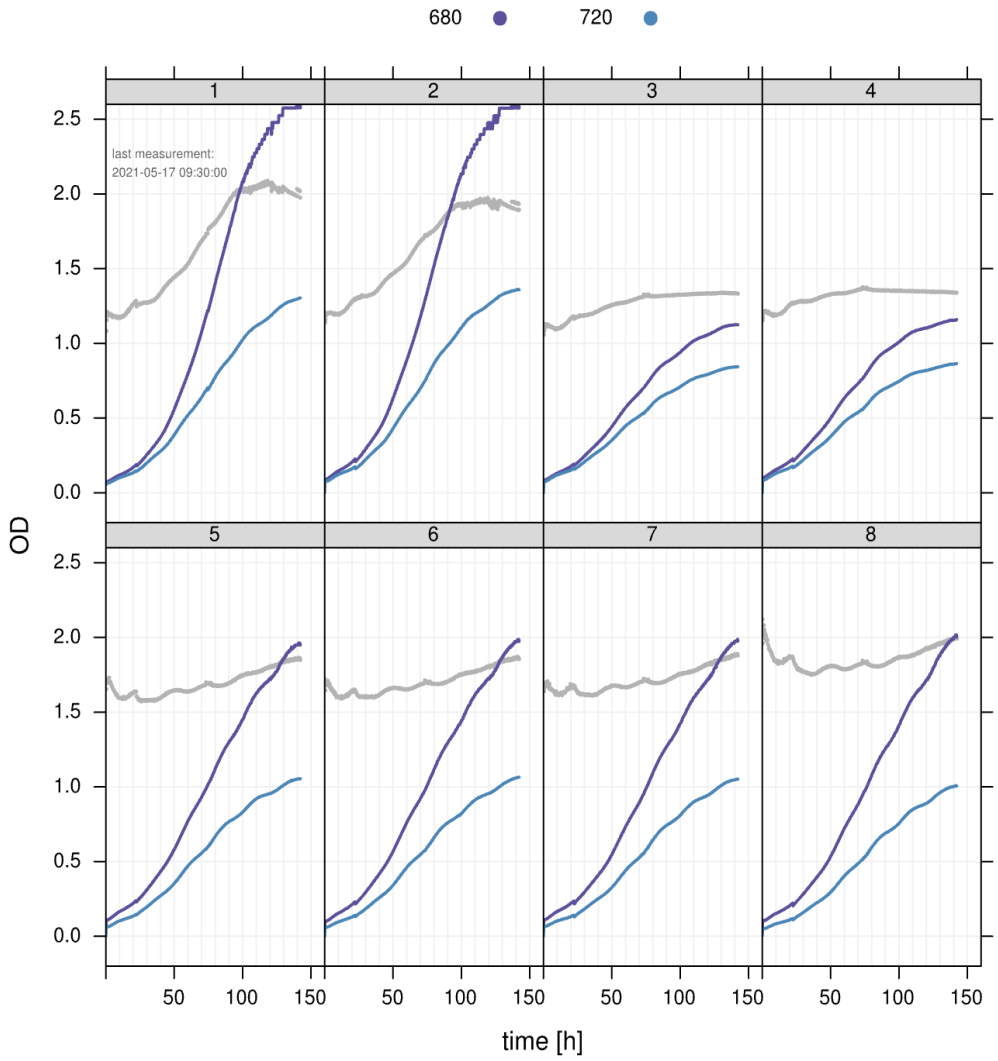

Figure S3. Growth curves for strain KS177 during growth arrest, with added external glutamate and aspartate to some cultures. Cultures 1-2 are KS177 without aTc (non-arrest control). Channels 3-4 are KS177 with aTc (growth arrested). Channels 5-6 are KS177 with added aTc and added 5 mM L-glutamate (pH-adjusted). Channels 7-8 are KS177 with added aTc and 5 mM glutamate and 5 mM aspartate (pH-adjusted). Addition of glutamate with or without aspartate could mitigate the growth arrest but not fully. All cultivations were done in batch mode in an MC-1000 multicultivator. Starting OD_{730}= 0.05, temperature was 30°C, 1% CO2 was bubbled into the cultures, and white light was constant at 150 µmol photons/m^2/s. Purple lines: recorded OD_{680}. Blue lines: recorded OD_{720}. Gray lines: OD_{680}/OD_{720}, a measure of pigment content per cell.

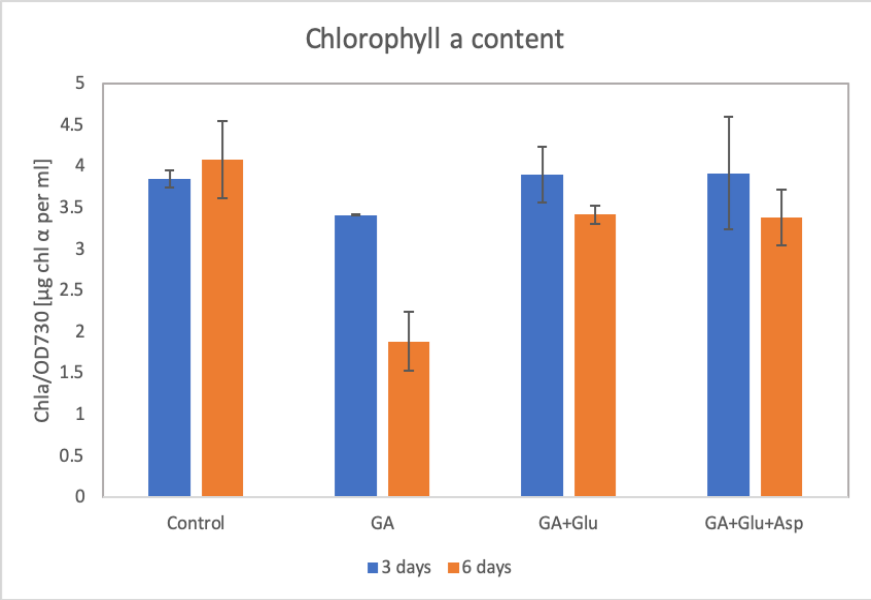

Figure S4. Chlorophyll A content for strain KS177 during normal growth, during growth-arrest with CRISPRi (GA), and during growth arrest with added L-glutamate and L-aspartate (5 mM each, pH-adjusted). Addition of L-glutamate to growth-arrested cells increases chlorophyll A levels. Starting OD_{720} was 0.05 and cells were grown in a Multicultivator at 30°C with 1% CO_2 atmosphere and light at 150 µmol photons/m^2/s.

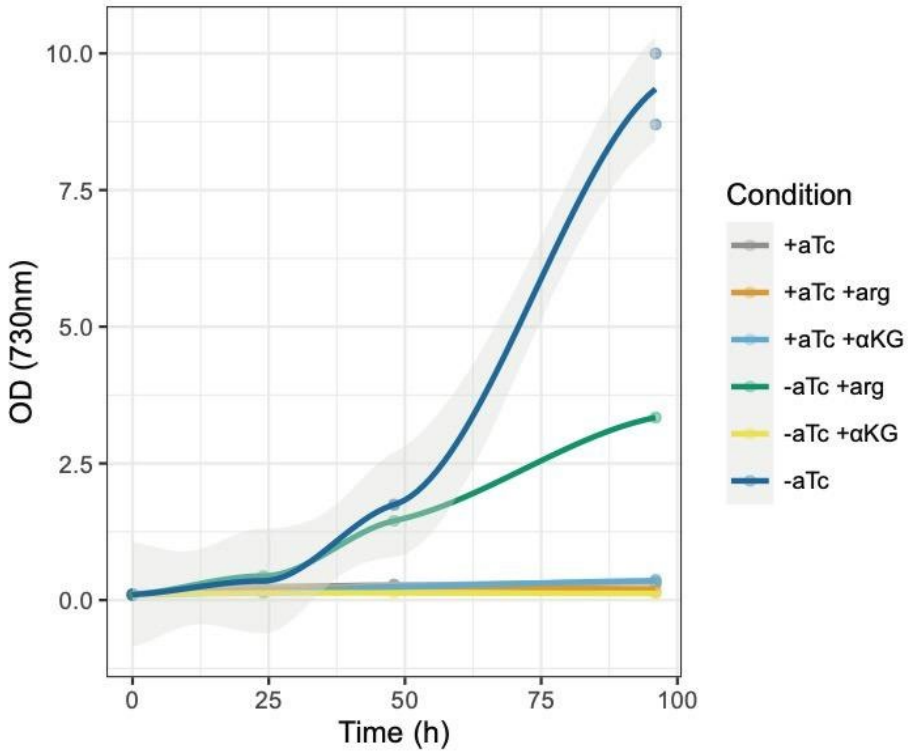

Figure S5. Growth curves for strain KS201 (containing the *ktgP* transporter from *E. coli*) during normal growth or during growth arrest with added external metabolites 2-oxoglutarate (alph-ketoglutarate; aKG) at 1 mM or L-arginine at 1 mM (added at t=0 h). Addition of aKG to 1 mM was lethal to cells, and addition of L-arginine to 1 mM was detrimental to growth even without induction of CRISPRi (-aTc samples). Strains where CRISPRi was induced had aTc (+aTc) had aTc added to 1 µg/mL 1 day prior to t=0 h. Starting OD_{730} was 0.1 and cells were grown in flasks in an incubator at 30°C with 1% CO_2 atmosphere and constant white light with estimated intensity of 40-80 µmol photons/m^2/s.

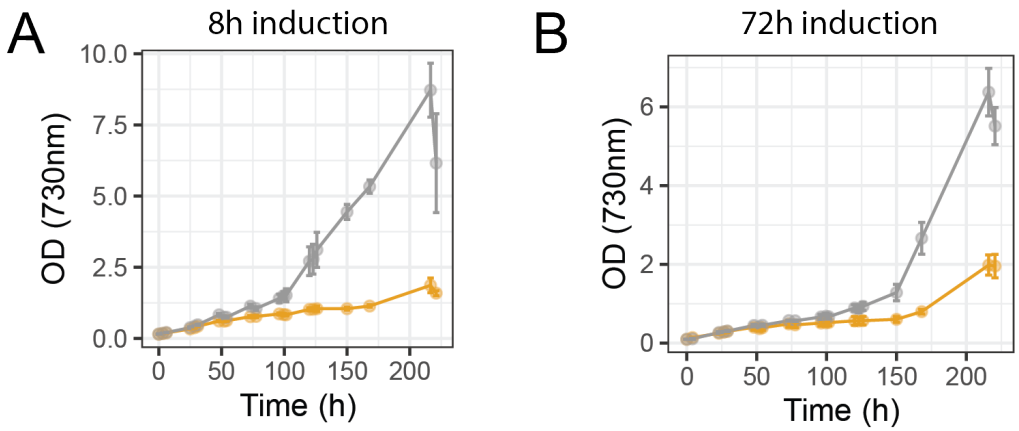

Figure S6. Effect of inducer removal on growth-arrested KS177 strain. A seed culture of induced KS177 was growth arrested by addition of aTc in a flask (light intensity was 40-80 µmol photons/m^2/s). At selected time points after induction (8 h, 72 h) aliquots were taken out, washed in BG-11, and used to seed new cultures at OD_{720} = 0.1, without aTc (gray lines) or with aTc present (orange lines). Cell growth (OD_{720}) was recorded for the new cultures.

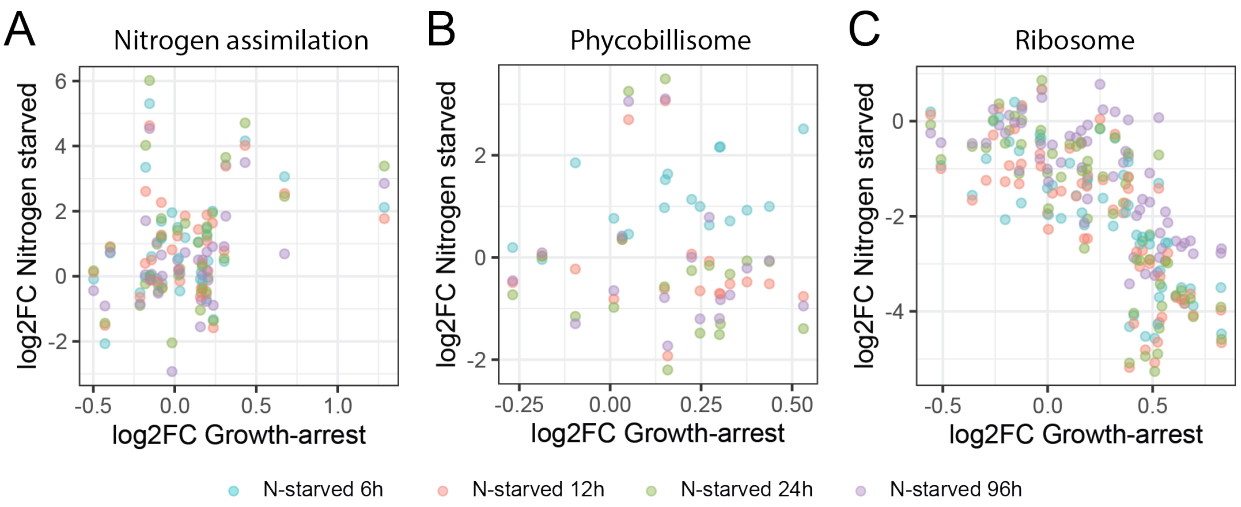

Figure S7. Correlation analysis between the *Synechocystis* growth-arrest transcriptome response (this work, 24h after induction, OD_{720} = 0.4, light = 200 µmol photons/m^2/s) and a published transcriptome dataset for nitrogen starved *Synechocystis* (Krasikov *et al.*, 2012). Spearman correlations were performed for the following subsets: (A) Nitrogen assimilation genes, (B) phycobilisome genes and (C) Ribosome genes. Each dot represents one gene. Spearman correlations were 0.27, -0.19 and -0.82, respectively. Gene classifications into nitrogen assimilation, phycobilisome and ribosome subcategories were obtained from von Wobeser *et al.*

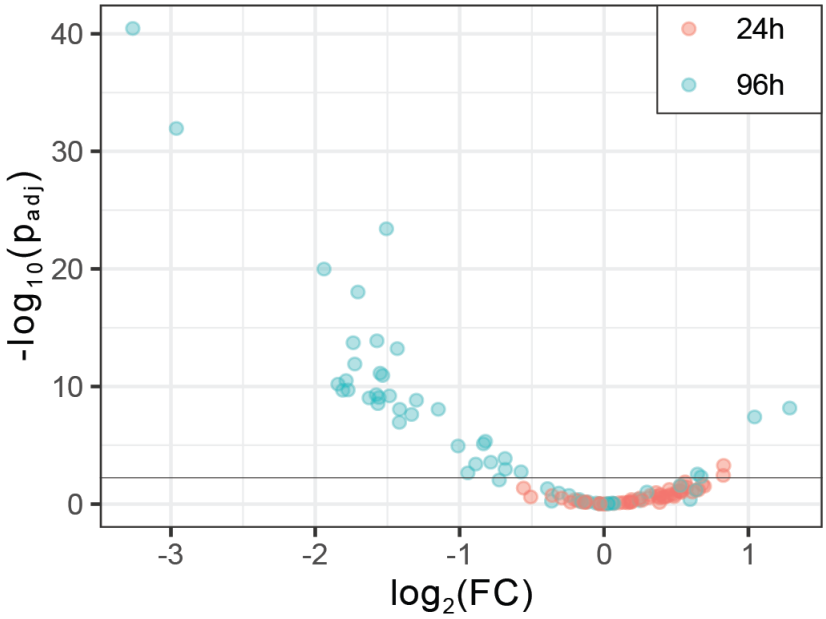

Figure S8. Volcano plot of the transcriptome response of ribosomal genes for KS177 at 24 h and 96 h post-induction of growth arrest. KS177 was grown in triplicate in turbidostat mode (OD_{720} = 0.4, light = 200 µmol photons/m^2/s). The horizontal line represents the significance line (P_{adj} = 0.005).

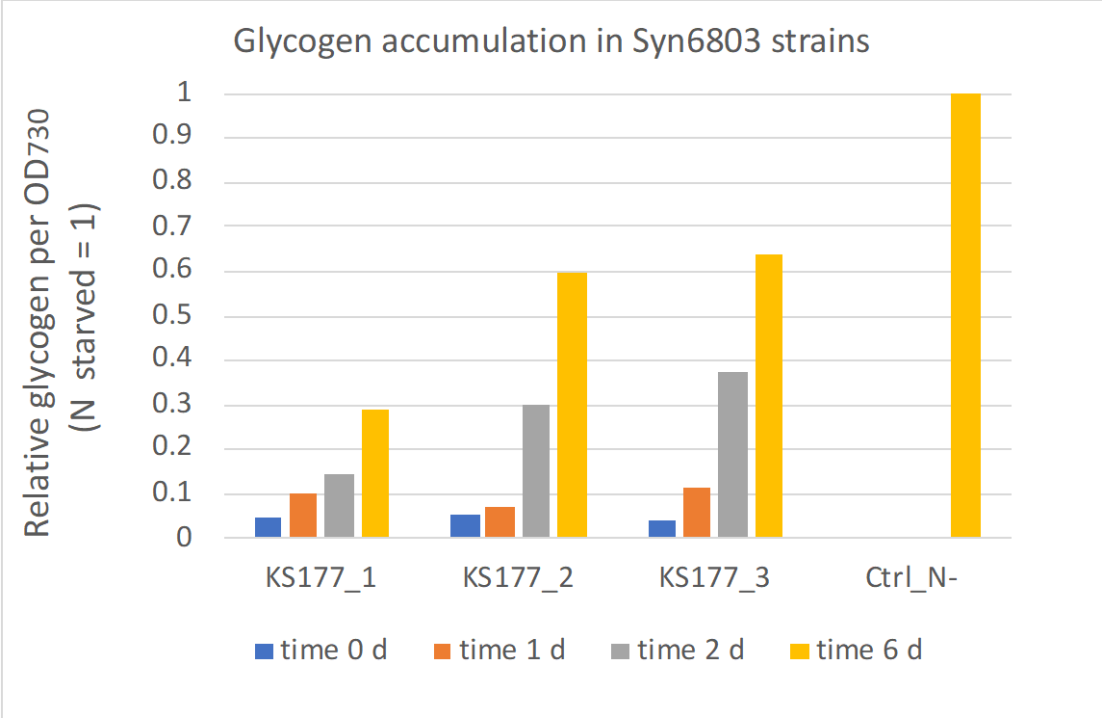

Figure S9. Glycogen accumulation in KS177 as it enters growth arrest. Three separate cultures of KS177 were started in turibodstat (OD720 =0.2, light = 100 uE). After cultures were stabilized, aTc was added to induce growth arrest (t 0). Samples (15 mL) were taken at 1 d, 2 d, and 6 d post induction for glycogen assay following Koch et al., 2020. By 6 d all KS177 cultures had severely reduced growth rate (50-80% reduction). As a control, a separate KS177 strain (no aTc induction) was washed and resuspended in BG110 media, which has no nitrate. Nitrogen starvation is known to cause glycogen accumulation in *Synechocystis* (Koch et al., 2020). After 6 days, this sample was assayed for glycogen. The relative glycogen content in KS177 cells are reported as normalized ot the glycogen content in nitrogen starved cells.

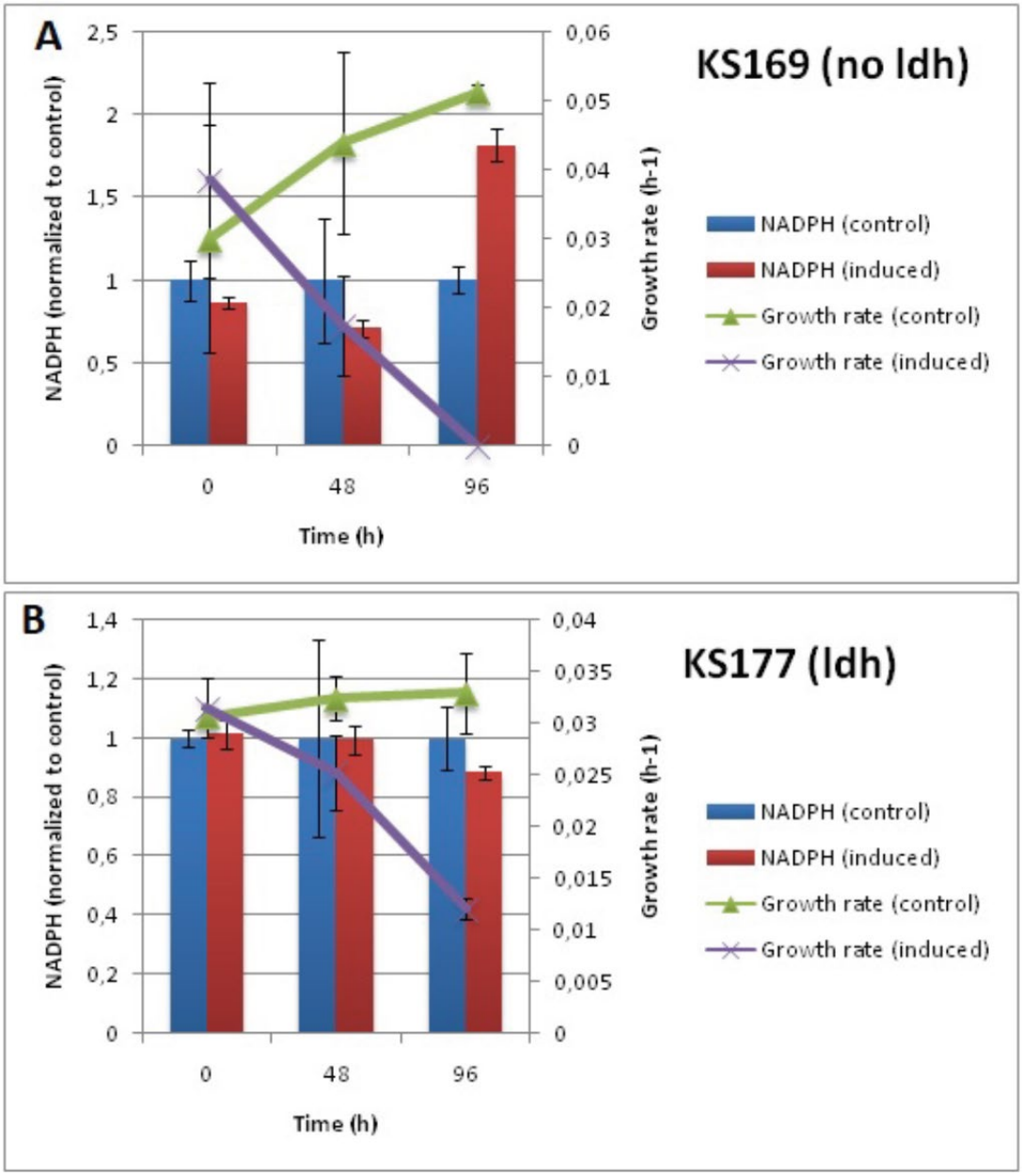

Figure S10. A comparison of the effect of the lactate sink (Ldh-catalyzed lactate formation) on growth-arrest and resulting NADPH accumulation. KS169 is a strain containing CRISPRi targeting gltA, but no Ldh gene (cannot synthesize lactate). KS177 is a *Synechocystis* strain containing CRISPRi and the Ldh gene (can synthesize lactate). Strains were grown in turbidostat mode (OD_{730} = 0.4, 200 µmol photons/m^2/s) in a Multicultivator. At time t=0, aTc was added to induce growth-arrest. Growth rate was recorded and intracellular NADPH was assayed using a fluorescence-based assay (Sigma). NADPH levels for each strain are normalized to the uninduced (control) culture at each timepoint. The KS169 strain accumulates NADPH during growth-arrest, while the lactate-producing KS177 does not. The KS169 strain was completely growth arrested after 96 h, while KS177 retained some growth at that time point.

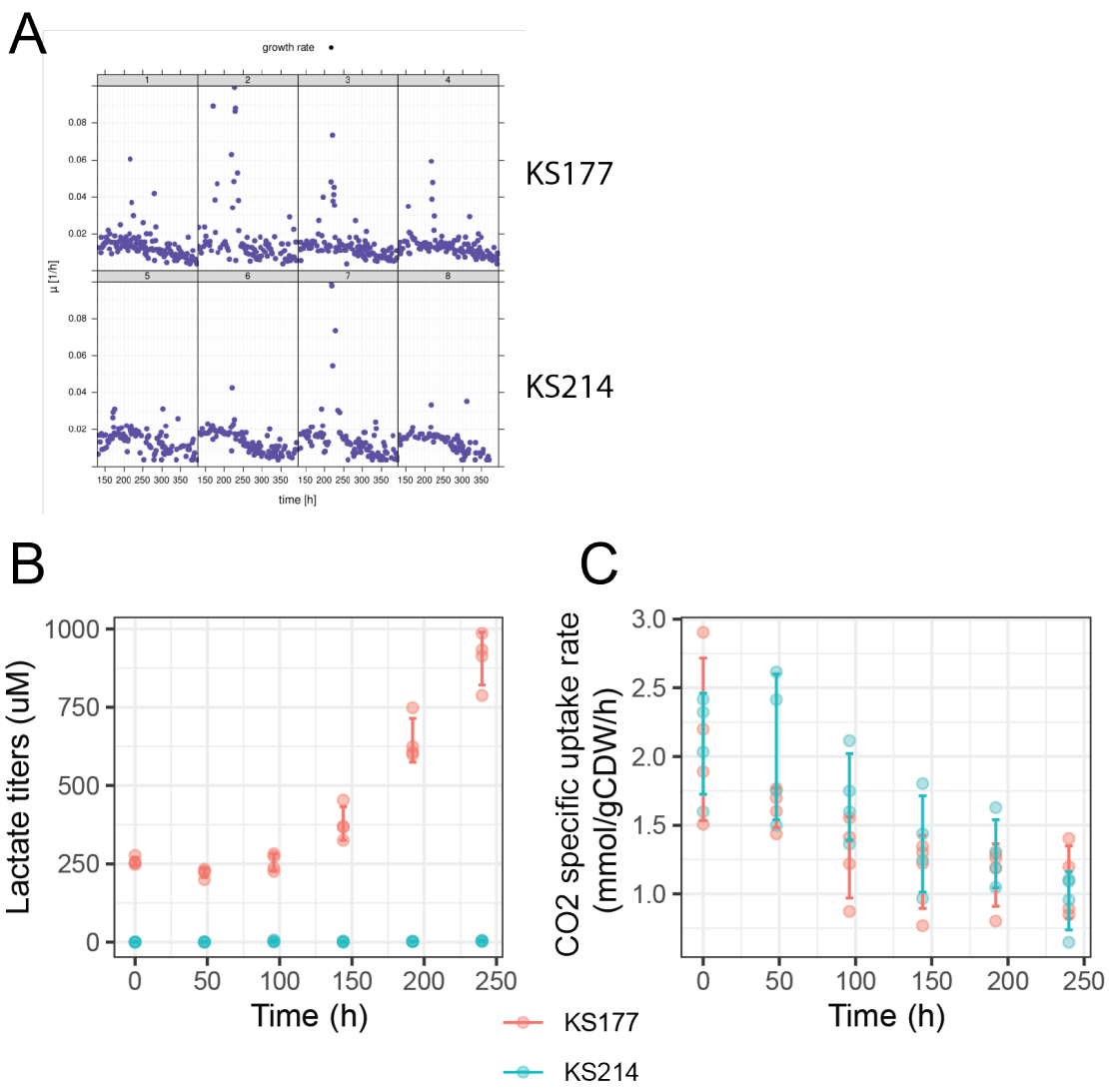

Figure S11. Effect of expression of cytochrome P450 CYP1A1 on growth-arrest in lactate-producing *Synechocystis*. (A) Growth rates for KS177 (top panel, no CYP1A1) and KS214 (bottom panel, with CYP1A1 from *Mus musculus* expressed). (B) Lactate amount in the turbidostat for both strains. (C) Specific CO_2 uptake rates for both strains. Cells were cultivated in a turbidostat mode with an OD_{720} = 0.4 and a white light intensity of 60 µmol photons/m^2/s.. KS214 had CYP1A1 under PpsbA2 promoter inserted at the *slr0397* locus. Active CYP1A1 enzyme in KS214 was confirmed using the ethoxyresorufin O-deethylation (EROD) assay as described in Berepiki *et al.*, 2016 (data not shown).

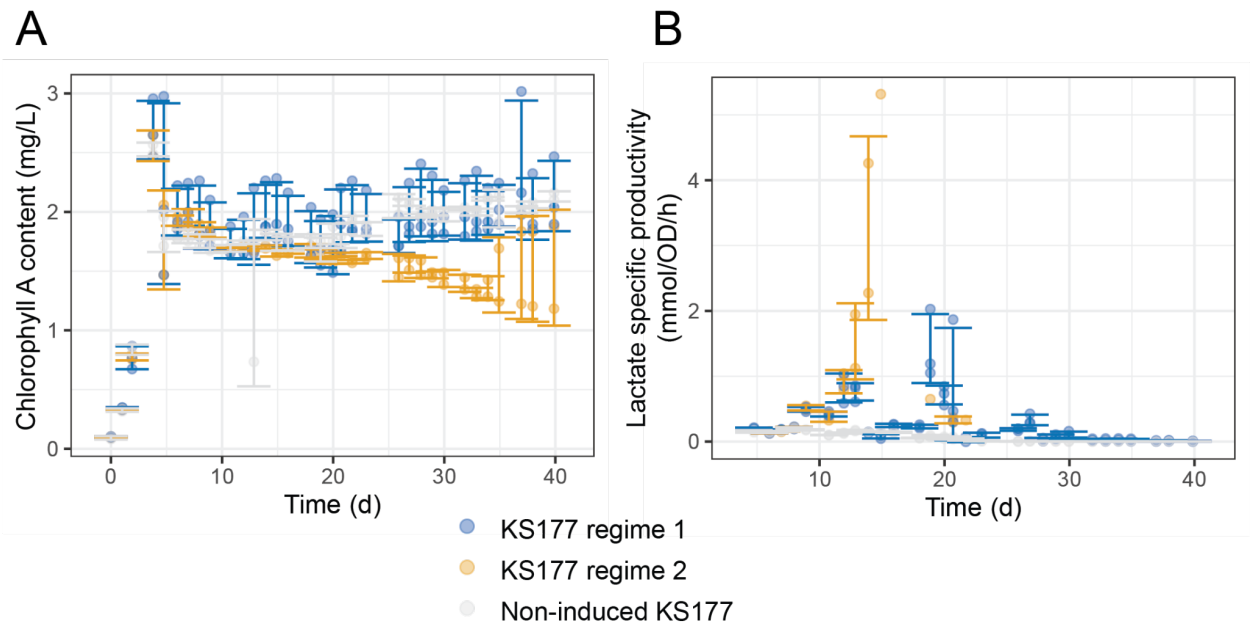

Figure S12. Chlorophyll A and lactate specific productivity for the intermittent growth-arrest experiments. Chlorophyll A contents were estimated from A_{680} and A_{720} absorbance values (Supplementary Note 2). Cells were cultivated in a turbidostat mode with an OD_{720} = 1 and a light regime of 110 µmol photons/m^2/s. Regime 1 was performed in triplicate cultivations. Regime 2 and the control regime were performed in duplicate cultivations. Specific lactate productivity of each culture was calculated using two sequential lactate concentration measurements and the dilution rate of the turbidostat.

Table S1. GO term enrichment for genes significantly upregulated (P_{adj} <0.005) at the transcriptome level in KS177 at 24 h after CRISPRi induction.

GO biological process complete	fold enrichment	raw pvalue	false-discovery rate
response to heat (GO:0009408)	28.59	4.74E-05	4.45E-02
response to temperature stimulus (GO:0009266)	28.59	4.74E-05	2.97E-02
protein folding (GO:0006457)	13.54	1.46E-05	2.74E-02

Table S2. List of *Synechocystis* PCC 6803 strains used in this study.

Strain ID	Genotype	Phenotype
KS169	ΔpsbA1::PL22 dCas9 SpR Δslr2030−2031::PL22 sgRNA-gltA_33 gltA_155 NatR	Can be growth-arrested by addition of aTc. Not lactate producing
KS171	ΔpsbA1::PL22 dCas9 SpR Δslr0168::Ptrc ldh CmR	Cannot be growth-arrested by addition of aTc .Lactate producing.
KS177	ΔpsbA1::PL22 dCas9 SpR Δslr0168::Ptrc ldh CmR Δslr2030−2031::PL22 sgRNA-gltA_33 gltA_155 NatR	Can be growth-arrested by addition of aTc. Lactate producing
KS201	ΔpsbA1::PL22 dCas9 SpR Δslr0168::Ptrc ldh CmR Δslr2030−2031::PL22 sgRNA-gltA_33 gltA_155 NatR Δslr0397::PpsbA2 kgtP GmR	Can be growth-arrested by addition of aTc. Lactate producing. Constitutive expression of KgtP transporter from *E. coli*.
KS214	ΔpsbA1::PL22 dCas9 SpR Δslr0168::Ptrc ldh CmR Δslr2030−2031::PL22 sgRNA-gltA_33 gltA_155 NatR Δslr0397::PpsbA2 CYP1A1 GmR	Can be growth-arrested by addition of aTc. Lactate producing. Constitutive expression of cytochrome P450 enzyme CYP1A1 from *M. musculus.*

Sp: spectinomycin; Km: kanamycin, Gm: gentamicin, Cm: chloramphenicol; Nat: nourseothricin

Supplementary Note 1: Protocol for proteome analysis.

Lysis and protein quantification

Frozen samples were brought out of -80 °C storage and thawed on ice. They were then lysed mechanically through rigorous shaking by a FastPrep-24 5G lysis machine over six cycles of 45 seconds, 6.5 m/s with 30 seconds on ice between cycles. The lysate was centrifuged for 5 min at 21,000g and 4 °C. The resulting supernatant was run through a Zeba Spin Desalting column for 2 min at 1,500g and 4 °C. The protein concentration was then evaluated through a Bradford assay.

Digestion

The samples were then incubated at 96 °C for 3 min. Afterwards, sodium deoxycholate and DTT were added to all samples to final concentrations of 5 % and 10 mM respectively after which the samples were again incubated at 96 °C for 10 min. Iodoacetamide was added to each sample to a final concentration of 10 mM for alkylation, followed by 30 min of room temperature incubation in the dark. Finally, the samples were diluted 10X in ammonium bicarbonate 100 mM and digested by 0.2 µg/µL LysC at 37 °C, 400 RPM, for 3h followed by digestion by 0.2 µg/µL Trypsin at 37 °C, 400 RPM, for 16h overnight. Digestion was halted by addition of formic acid to reduce pH below 2 which caused sodium deoxycholate to precipitate. Samples were then centrifuged at 14,000g for 10 min after which the supernatant was removed and stored at -20 °C.

Peptide purification and LC – MS/MS analysis

Frozen peptide mixes were thawed to room temperature and loaded 325 µL onto activated C18 columns. The columns were then washed with 0.1 % formic acid after which the samples were eluted with 80 % acetonitrile – 0.1 % formic acid. The resulting peptide mixes were dried in a vacuum centrifuge and stored in -20 °C for several weeks until the mass spectrometry machine was available. At that point, the peptides were resuspended in 10 µL 0.1 % formic acid and analyzed by LC – MS/MS utilizing a Q-exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled with an UltiMate 3000 RSLCnano System with an EASY-Spray ion source. 2 µL of each sample was loaded onto a C18 Acclaim PepMap 100 trap column (75 µm x 2 cm, 3 µm, 100 Å) with a flow rate of 7 µL per min, using 3 % acetonitrile, 0.1 % formic acid and 96.9 % water as 6 solvent. The samples were then separated on ES802 EASY-Spray PepMap RSLC C18 Column (75 µm x 25 cm, 2 µm, 100Å) with a flow rate of 3.6 µL per minute for 40 minutes using a linear gradient from 1 to 32 % with 95 % acetonitrile, 0.1 % formic acid and 4.9 % water as secondary solvent. After separation MS analysis was performed using one full scan (resolution 30,000 at 200 m/z, mass range 300 – 1200 m/z) followed by 30 MS2 DIA scans (resolution 30,000 at 200 m/z, mass range 350 – 1000 m/z) with an isolation window of 10 m/z. Precursor ion fragmentation was performed with high-energy collision-induced dissociation at an NCE of 26. The maximum injection times for the MS1 and MS2 were 105 ms and 55 ms respectively, and the automatic gain control was set to 3e6 and 1e6 respectively. The EncyclopeDIA and Prosit workflows were used to generate a predicted library from a fasta file of *Synechocystis* proteome against which an EncyclopeDIA search was performed to generate a list of detected peptides.

Data analysis

The output of the LC – MS/MS analysis was in the form of a list of peptides, their protein of origin and detected intensity. Each timepoint was considered a separate experiment in which the intensity of every detected peptide was compared to the intensity of that same peptide in the first timepoint sample. Peptides not present in all timepoints were excluded from the result. Statistical analysis was performed group-wise with the MSstats package in R. A significant peptide was defined as one that has an absolute log2FC of at least 1 and a q-value of less than 0.01, meaning the False Discovery Rate (FDR) was set to 1 %. The data was searched using EncyclopeDIA (Searle *et al.*, 2018) against a predicted library generated with Prosit (Gessulat *et al*., 2019).

Supplementary Note 2. Calculation of turbidostat production parameters.

The carbon partitioning and cumulative lactate titer in the turbidostat experiment were estimated as follows. First the dilution percentage for each turbidostat cycle was calculated based on the recorded Multi-Cultivator OD_{720} before and after pumps were activated. Using the dilution percentage, the theoretical lactate concentration was calculated assuming no lactate was produced after each lactate measurement. The difference between this value and the measured lactate concentration in the consecutive sampling point was taken as the amount of lactate produced. The same approach was followed to estimate the amount of biomass produced between sampling points, based on the external OD_{730} measurements.

The cumulative lactate titer was then calculated as the sum of all the differences between theoretical and measured lactate concentrations. The carbon partitioning was calculated as the amount of carbon diverted into lactate divided by the sum of carbon in lactate and biomass. To obtain the amount of carbon in biomass, a conversion factor of 0.148 g / L * OD_{730} was applied and the biomass molecular composition was assumed to be $C_4H_7O_2N$ (Du et *al*., 2016).

In cases where Chl a content was estimated from cell absorbance spectra, we used an empirical formula, $10.186*(OD_{680}-OD_{730})-0.08$ = mg Chl a L^{-1} (Inskeep and Bloom, 1985).

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