Supporting Information

Title

Channeling anabolic side-products towards the production of non-essential metabolites: stable malate production in *Synechocystis* sp. PCC6803

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Additional Methods

Plasmids and generation of *Synechocystis* mutants. The strains and plasmids used in this work are listed in Table 1. In order to knock-out the malic enzyme (*me*) and malate dehydrogenase (*mdh*) genes from *Synechocystis*, a markerless deletion method has been used, as previously described in [1]. Briefly, two plasmids are needed for two consecutive transformation rounds. The first plasmid contains the up- and downstream homologous regions (~1 kb each) of the target gene. The second plasmid, in between the two homologous regions, carries a selection cassette composed of a kanamycin resistance and a nickel-induced *mazF* expression fragment [2]. MazF is an endoribonuclease that cleaves the mRNA at the ACA triplet sequence, acting as an inhibitor for protein synthesis [3]. In the first round of transformation, the native locus in the chromosome of *Synechocystis* is replaced by the selection cassette through homologous recombination, and the addition of kanamycin to the medium allows selecting the positive colonies. During the second round of transformation, the fully segregated selection cassette is removed from the chromosomes by using the plasmid containing just the homologous regions. In that case, the recombinant colonies are those able to grow in a nickel supplemented medium.

To build the plasmids, the corresponding homologous regions flanking the respective *me* and *mdh* genes were amplified from the genomic DNA of *Synechocystis*, and subsequently fused together using *Herculase II* Fusion DNA Polymerase (Agilent Technologies). In this step, a restriction site in between the two fragments has been inserted. The fused fragments were gel extracted and purified (Stratec Molecular) and then adenylated to their 3' ends using *Taq* DNA polymerase (Thermo Scientific). The extra adenosine ("A") allows the TA cloning of the fragments in the BioBrick "T" vector pFL-AN [4], resulting in pWD71 (for *mdh*) and pWD73 (for *me*) respectively. The selection cassette from pWD42 [5] contains an XbaI restriction site on both sides and can therefore be inserted into pWD71 and pWD73, resulting in pWD72 and pWD74, respectively. To overexpress the FumC enzyme in *Synechocystis, fumC* gene from *E. coli* was amplified by *Herculase II* Fusion DNA Polymerase (Agilent Technologies) with introduced NdeI to the 5' end and BamH1 to the 3' end. The fragment was purified (Stratec molecular) and cloned in the integration vector pHKH001 [6] at the *slr0168* genomic locus under the strong promoter PcpcBA, resulting in pBB1. All the fragments

amplified were confirmed by Sanger sequencing at Macrogen Europe (The Netherlands), and the primers used are listed in Table S1.

To transform *Synechocystis* with the plasmids, fresh cells were collected either from the plate or from liquid culture (OD_{730} ~1), and washed twice with fresh sterile BG-11 medium through centrifugation (5000 rpm, 5 min, Himac CT 15RE Centrifuge). Cells were then concentrated to a total volume of 200 µL, mixed with the specific plasmid to a final concentration of 10 µg mL^{-1} and illuminated for 5-6 h under white light of moderate intensity (~50 µmol photons m^{-2} s^{-1}). At the end of the incubation, the mixture was spread on a commercial membrane (Pall Corporation) resting on a BG-11 agar plate and illuminated for further 16-24 h. The membrane was then transferred to a new BG-11 plate containing the appropriate selection marker (kanamycin in the first round, nickel sulfate in the second round). Positive colonies, which grew in the presence of the selective pressure, were submitted to PCR confirmation. Segregation was monitored by using the primers pairing with the homologous regions of the target genes listed in Table S1 and several cycles of growth in liquid culture were performed when necessary.

Quantification of extracellular fumarate, malate and lactate. Extracellular concentrations of fumarate, malate or lactate were measured in samples collected from different strains. Briefly, 1 mL of culture sample was harvested and after centrifugation for 5 min at 14500 rpm (Himac CT 15RE Centrifuge) at 4 °C, the supernatant was filtered (Sartorius Stedin Biotech minisart, PES, 0,22 µm) and aliquoted for the measurement of these metabolites.

Fumarate concentration was measured on a HPLC system (LC-20AT, Prominence, Shimadzu) equipped with a Photo Diode Array UV/VIS detector (SPD-M30A, NexeraX2, Shimadzu) and a Refractive Index Detector (RID 20A, Shimadzu). A volume of 10 µL of sample was injected through an autosampler (SIL-20AC, Prominence, Shimadzu) onto a Rezex ROA-Organic Acid H+ (8%) column (250x 4.6 mm; Phenomenex). The compounds were eluted at 45 ºC with an isocratic flow rate of 0.15 mL min^{-1} of 5 mM H_2SO_4. The detection UV wavelength was set at 210 nm. Peaks identity and quantification were assigned using external standards. In these conditions, the detection limit for fumarate was 20 µM.

Malate and lactate were quantified by using commercial enzymatic kits (Megazyme) according to the manufacturer's instructions. The assays, consisting of two consecutive enzymatic reactions, were performed in the 96-well plate (SpectraPlate 96 MB, PerkinElmer) at 30 °C. In the case of the L-malic acid kit, the first reaction is catalyzed by the glutamate-oxaloacetate transaminase, which in the presence of L-glutamate consumes the oxaloacetate in the sample by converting it into 2-oxoglutarate and L-aspartate. During the second reaction, the L-malate dehydrogenase oxidizes the L-malic acid to oxaloacetate by using the reduction of nicotinamide-adenine dinucleotide (NAD^+) to NADH. The formation of NADH is stoichiometrically coupled to the conversion of L-malic acid into oxaloacetate. In the case of the L-lactic acid kit, the first reaction consists in the conversion of pyruvate to D-alanine and 2-oxoglutarate, with the enzyme D-glutamate-pyruvate transaminase in the presence of a large excess of D-glutamate. The second reaction is catalyzed by L-lactate dehydrogenase, which oxidizes L-lactic acid to pyruvate by using the reduction of NAD^+ to NADH. The formation of NADH is stoichiometrically coupled to the conversion of L-lactic acid into pyruvate. The amount of NADH produced during the reactions, quantifiable as an increase in absorbance at 340 nm, was measured using a plate reader (SPECTROstar Nano, BMG LABTECH). For quantification, a standard curve was performed for calibration of the enzymatic assay. According to the kit, the detection limit was 1.9 μM for malate and 2.3 μM for lactate.

Sequencing of the *ldh* and *fumC* cassettes. Sequencing analysis of the *ldh* and *fumC* cassettes was performed on single colonies isolated from previously restreaked samples of the populations in BG-11 agar plates. Colony PCR with *Herculase II* Fusion DNA Polymerase (Agilent Technologies) was used to amplify the sequence encoding for either the *ldh* gene and the upstream P*trc* promoter or the *fumC* gene and the upstream P*cpcBA* promoter. After PCR product purification (Stratec molecular), fragments were sent for sequencing at Macrogen Europe (The Netherlands) using the primers listed in Table S1.

Tables

Table S1. Primers used in this study

Primer	Sequence (5'-3' direction)
mdh-up-Fwd	AGTTCCCACGGTGGATTTGA
mdh-up-Rev	CAGCAAAATGTCGCCAACGACTAGTGAGTGGAAGATATTCTCGAAGTGC
mdh-down-Fwd	GCACTTCGAGAATATCTTCCACTCACTAGTCGTTGGCGACATTTTGCTG
mdh-down-Rev	CAGGCTTTGTAACTGGTGGA
me-up-Fwd	TGAGTCTTATATCCCGTCCGT
me-up-Rev	ACTGCACTGGCTACGGTAGTTCTAGACTGACGCTATAACTCGGATTGG
me-down-Fwd	CCAATCCGAGTTATAGCGTCAGTCTAGAACTACCGTAGCCAGTGCAGT
me-down-Rev	CAACTCAGCATGGATATTAAGCACG
me-seq	ATGGTAGGACACCTTCTCCA
mdh-seq	TCGCACAGTTCGTAGGCAA
FumC_ecoli_Fwd	GCCATATGAATACAGTACGCAGCGA
FumC_ecoli_Rev	ACGGATCCTTAACGCCCGGCTTTCATA
FumC_ecoli_Seq	GCTGGGTAGCGATGCTC
phkh_H1seq_F	CCTCTGGTTAGCCACCA
phkh_Kanseq_R	TTGAGACACAACGTGGC
Pcpcb_rev	ATATGGCCGCTGCTGTG
H1_Fwd	TGTCGCCGCTAAGTTAGA
LDH_Rev_3	GTAGGTCAAGATGTCCACGG
LDH_Rev_2	CATCTTGGAACACGGACACG
Kan_Rev	TCCCGTTGAATATGGCTC

Table S2. Mutational analysis of the *ldh* expression cassette from DNA sequence of the isolated single colonies of the SAA023 strain.

Mutation type	Positon (start from ATG)	Translation analysis
Deletion of 12 nucleotides	190-201	a.a. 65 to 68 missing
Point mutation (A→C)	180	K61Q
Point mutation (T→A)	293	N98K
Single nucleotide insertion (T)	132	46 aa
Single nucleotide insertion (T)	305	60 aa
Deletion of 6 nucleotides	711-717	a.a.238 and 239 missing

Figures

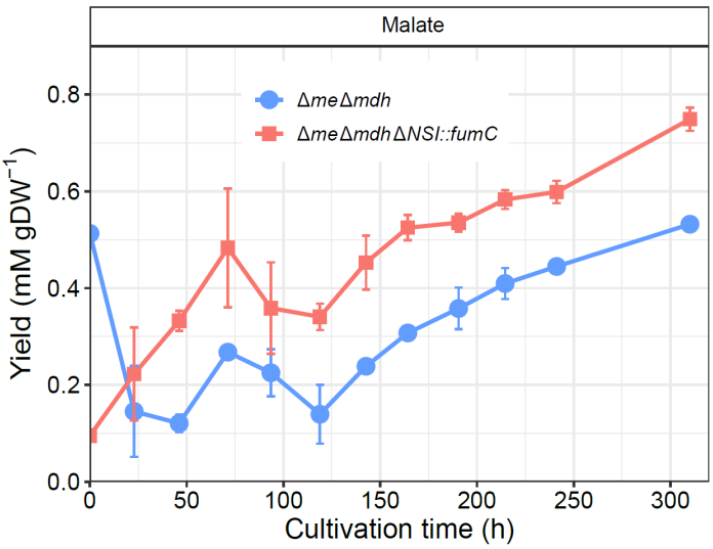

Figure S1. Malate yield of the *Synechocystis* sp. PCC6803 mutants with a conversion of OD_{730} to gram dry weight (gDW) of 148 mg L^{-1} OD_{730}^{-1}.[7]

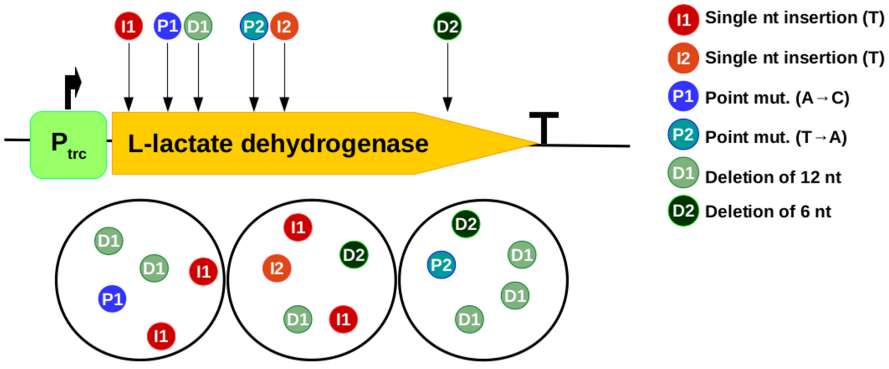

Figure S2. Mutations (and their positioning) found after sequencing of the *ldh* cassette of colonies of SAA023. The distribution of the mutation types amongst the three experimental populations is reported in the circles.

Sequences

1. **Plasmid construct to delete *mdh*** with font colors indicating the upstream (green) and downstream (blue) homologous regions, and the grey color highlighting the open reading frame.

TAGTCGTGAATTTTCGTAACCCAGTTCCCACGGTGGATTTGATCATCGAAATGATTGATCAACCCCGCCGACCTATTTTATT
AATTGAGCGGCAAAATCCGCCCCACGGCTGGGCCCTGCCCGGAGGCTTTGTGGACTACGGCGAAACCCTAGAAGCGGCA
GCCCTGCGGGAAGCCAAGGAAGAAATTTCCCTGGACGTCCAGCTTATCGAAATGTTTTATGCCTACTCAGATCCCCGCCG
GGATCCCCGGCAACACACCCTCAGTGTGGTATTCCTGGCGGCGGCCACCGGCAAACCCCAAGCGGCGGACGATGCTAAA
ACCGTTGGCTTTTTTGATTTGTGGGAATTTCCCGACCCAGTTTGCTTTGATCATGGACAAATTTTGCGGGATTATCGCCGCT
ACCGCTATTATCGTCAACGCCCCGATCCCCACCGGAATTATTTGGGGGACTAACGGGCAAAGCATTAGTTCCTTGGCTCCT
TCTAGACCAATAGTGCAAATCTAACAAATTCAATGGGGGATGGCTTCCGAACAAATATTGTTGATGAATTTCACCCCATCT
TCCTTGGGTATCAGTTCTTGTAAATAAAAATCCACCGTTTCATTACTTTCGTTGGTCACTAGCACCTTAGCTTGGTGCCCCTT
TTGTAATACCTCACGGCGATCGCACAGTTCGTAGGCAAAAATAATCAGGCGATCGCCTGGTTGACAAAGCAAGGCGGCTC
CACCGTTGGGGCAAATTTCCTTGGAATGGGCTTGACCGGGCAGGACATAGGTGGAAAAGCGATTACCGTTGCTGAGATT
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CGGGCCTGCGGGGTAGACCAGCGGTAATTACCACCACATCGGAGCCGGCGGTGGCCTCGTATTCATTGGTGCCAATGATT
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CTGCCAGGACTGACAGGCGATCGGAGCATACTCCAAAATATTCATGGCACCCTCCGGGGTGTTGATGGTGGAGAAAAGC
AACCTAAGCCAGGGCTTCGATTTGGTCTAAGCGCAGCCAGACGTTGGGGGTAGGGACATAAAATCGCACTAGGGCATAT
TCTTCGTTTAAATCCAACACTTCTCCTTTGCTATGGAAAAGGTAGTCGGGTAAACGGCGATCGCTGGCTTGGGCTTCAAGG
CTGTTTTCCAATTGTGCGCGGATAACCCGCACTAAACTGCCCTTTTTCATTTTAGCGGCCATGGTTTTGCCCTCGTTGGGAG
ACTGGGAAATTAACTCTAAATTGTTACATAACCCCAAAATTTTGTAATTCTTATTTTTGGCTAGTTTCTCCCCAGGCCATTCC
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CAAAGCAGCTCCGCCCTTTGACAGAGAGAAGAACCATGGATACCTCCGTTAGTGTTTTTGCTGAAATTCCCGAAGACCTGC
ACCAGTCCTTGACCACCTATCTGGATAAACATCCCCATTGGGACCTAGACCGGGTGTTTTCCGCTGCCCTGTCCCTGTTCCT
GCTGCAAAATGAGGGAGGCTACGGCACCAAACCCATGTCCACCAGTTACAAAGCCTGTGCCCGGGTCTATCTGGA

2. **Plasmid construct to delete *me*** with font colors indicating the upstream (green) and downstream (blue) homologous regions, and the grey color highlighting the open reading frame.

CCTGCTTGGGGGGCTAGGGTTCCCATTTTGAGTCTTATATCCCGTCCGTCGAAGGATATGGACTTGTCAAACTCTTGCATTT
AACGTGTTTTCCTTATGTGCTATGACGTTTCTTCTTCTCAGCAGATCCTAACACCATTGACGAACCCTGGGCCGACCAAAGG
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3. **Plasmid construct for the *fumC* overexpression** cassette

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GCGTTTATATACTAGAGAGAGAATATAAAAAGCCAGATTATTAATCCGGCTTTTTTATTATTT

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