Development of a high-throughput, *in vivo* selection platform for NADPH-dependent reactions based on redox balance principles

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Supplementary information:

1. Plasmids and Strains

The strains and plasmids used in this study are summarized in Table S1.

Table S1 Strains and Plasmids

Strains	Description	Reference
XL-1 blue	Cloning strain	Stratagene
BL21 (DE3)	Protein expression strain	Invitrogen
W3CG	*E. coli* W3110 gapA::Tn10, TetR	[1]
JCL166	BW25113/F′ [traD36 proAB +lacIqZ M15 (Tetr)] *adhE ldhA frdBC*	[2]
Plasmids	**Description**	**Reference**
pLZ2	pRSF P_{BAD}::*Sm gapN,* Spec^r	This study
pLZ3	pRSF P_{BAD}::*Ca gapN,* Spec^r	This study
pLZ4	pRSF P_{BAD}::*Ca gapC,* Spec^r	This study
pLZ5	pRSF P_{BAD}::*Bs gapB,* Spec^r	This study
pLZ6	pUC19 P_{Lac}::*Kp budC*, Amp^r	This study
pLZ7	pUC19 P_{Lac}::C*b adh*, Amp^r	This study
pLZ75	pUC19 P_{Lac}::*Ld ldhA* WT, Amp^r	This study
pLZ77	pUC19 P_{Lac}::*Ld ldhA* D176S-I177R-F178T, Amp^r	This study
pLZ82	pUC19 P_{Lac}::*Ld ldhA* D176-I177-F178 NNK library, Amp^r	This study
pQElac	Amp^r ; ColE1 ori; P_{LlacO1}. Expression vector	[3]
pEK28	pQElac 6×His *Ec gapA*	This study
pLZ108	pQElac 6×His *Ld ldhA* WT, Amp^r	This study
pLZ109	pQElac 6×His *Ld ldhA* D176S-I177R-F178T, Amp^r	This study
pLZ110	pQElac 6×His *Ld ldhA* D176S-I177R-F178S, Amp^r	This study
pLZ111	pQElac 6×His *Ld ldhA* D176S-I177S, Amp^r	This study
pLZ112	pQElac 6×His *Ld ldhA* D176A-I177F-F178S, Amp^r	This study
pLZ113	pQElac 6×His *Ld ldhA* D176G-I177L-F178W, Amp^r	This study
pLZ121	pQElac 6×His *Ld ldhA* D176V-I177W-F178G, Amp^r	This study
pLZ122	pQElac 6×His *Ld ldhA* D176S-I177L-F178G, Amp^r	This study

Abbreviations indicate source of the genes: Ec, *Escherichia coli*; Ld, *Lactobacillus delbrueckii*; Kp, *Klebsiella pneumoniae*; Cb, *Clostridium beijerinckii*; Sm, *Streptococcus mutans*; Ca, *Clostridium acetobutylicum*; Bs, *Bacillus subtilis*.

2. Plasmid and library construction

The *Streptococcus mutans gapN* gene was amplified from a synthesized DNA template. The *Clostridium acetobutylicum gapN* and *gapC* and *Bacillus subtilis gapB* genes were amplified from genomic DNA. After PCR and gel extraction, all gene fragments were inserted into the vector backbone (pRSF ori, Spec^r) using Gibson isothermal DNA assembly method [4], resulting in plasmids pLZ 2,3,4 and 5.

Both *Klebsiella pneumoniae budC* and *Clostridium beijerinckii adh* were amplified from synthesized DNA templates. Both sequences were reported in previous studies [5]. After PCR and gel extraction, both gene fragments were inserted into the vector backbone (pUC19, Amp^R) using Gibson isothermal DNA assembly method, resulting in plasmids pLZ 6 and 7.

The *Lactobacillus delbrueckii ldhA* gene was amplified from genomic DNA and assembled with vector backbone (pUC19, Amp^R) to yield plasmid pLZ75. The D-Ldh library pLZ82 was constructed as follows: A forward primer containing three consecutive NNK codons at D176, I177 and F178 positions was used together with a reverse primer which primes at the 3'-end of the *ldhA* gene in PCR, yielding the insert in Gibson assembly. The remainder of the plasmid pLZ75, which contains the remaining half of the *ldhA* gene and the vector, was also amplified as the backbone. The insert and backbone were assembled together, purified, and transformed into ElectroMAX DH10B cells (Invitrogen, U.S.A.) using electroporation. Subsequently, the cells were rescued with SOC medium at 37 °C for 1 hour and then added to 20 mL LB medium with 200 μg/mL ampicillin. 2 μL of culture was immediately taken from the culture and plated on an LB agar plate with 200 μg/mL ampicillin. The liquid culture was incubated at 37 °C with agitation for 12 hours, before the library DNA was extracted. The plate was incubated at 37 °C overnight, and the number of colonies formed was counted to estimate the library size. 10 single colonies were also cultured individually to extract plasmids, which were sequenced as representatives of the population. The results showed that all 10 plasmids sequenced contained unique mutation patterns, and no other mutations outside the intended mutagenesis sites.

After selection, the selected D-Ldh variants, as well as the wild type, were amplified from the corresponding pUC19 plasmids, and moved into pQE (ColE1 ori, Amp^R) which has a N-terminal 6×His tag on the backbone for protein purifications. The resulting plasmids were named pLZ 108, 109, 110, 111, 112, 113, 121, and 122.

3. Enzyme assays of *Streptococcus mutans* GapN

The plasmid pLZ2 carrying *S. mutans gapN* was transformed into W3CG strain. The cells were cultured in LB medium with 15 g/L glycerol, 4 g/L malate, and 50 μg/mL spectinomycin in shake flasks at 37°C in a rotary shaker (250 rpm) to an OD_{600nm} of 0.5. The cultures were then induced with 0.2% arabinose, and incubated at 30°C with 250 rpm agitation for 24 h. The cells were harvest with centrifugation, and lysed with bead-beating in lysis buffer (20mM Tris pH 8.5, 3mM 2-mercaptoethanol). The cell lysate was centrifuged at 15,000rcf for 15min at 4°C, and the supernatant was used in the enzyme assays. The reaction mixture contained 50mM Tris-Cl pH 8.5, 3mM 2-mercaptoethanol,

1.5mM DL-glyceraldehyde 3-phosphate, and 5mM $NADP^+$ or NAD^+. The reaction was initiated by adding cell lysate and the initial reaction rate as measured by the increase of absorbance at 340 nm, which was monitored by a spectrophotometer at room temperature. The background levels of glyceraldehyde 3-phosphate dehydrogenase activity in the host were measured using the same method as with the W3CG strain with no plasmid transformed. The final activities represent the difference between with plasmid and without plasmid (Figure S1).

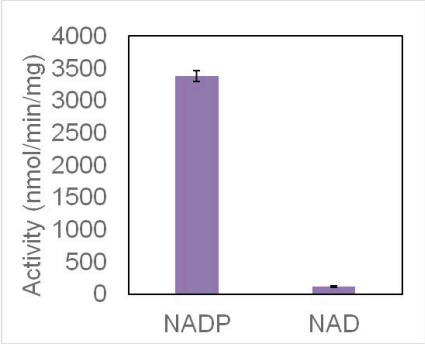

Fig S1 Activities of *S. mutans gapN* for NAD^+ and $NADP^+$. Error bars represent standard deviations of 3 replicates. n=3.

4. Anaerobic growth mediated by *Clostridium acetobutylicum gapC*

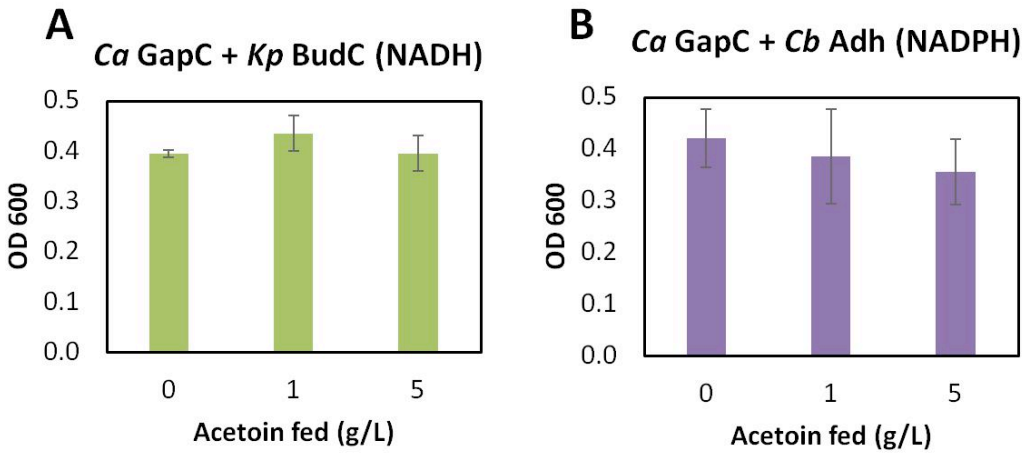

Fig S2 Anaerobic growth behavior of *Clostridium acetobutylicum gapC* mediated glycolysis. In W3CG strain transformed with plasmid pLZ4, both *K. pneumoniae* BudC (A) and *C. beijerinckii* Adh (B) -mediated fermentation pathways enabled growth. Both strains also grew without the substrate acetoin, which was presumably enabled by the redox balance-restoring function of the native mixed-acid fermentation pathways.

5. Determination of the transformation efficiency of W3CG/pLZ2

The electro-competent cells of W3CG/pLZ2 were made as follows: The cells were cultured in 200mL LB medium with 4 g/L glucose, 0.2% arabinose and 50 μg/mL spectinomycin at 30 °C with shaking until OD_{600nm} reached 0.4-0.6. The culture was chilled on ice for 15 min and the cells were pelleted at 4 °C, 4000×g. The cells were finally resuspended with 5mL 10% glycerol in water (sterile, ice cold), and aliquoted 50uL in each microcentrifuge tube before stored at -80 °C.

The transformation was performed as follows: Thaw W3CG/pLZ2 electro-competent cells on ice, add 1 μg pUC19 plasmid DNA within 100ul competent cells, and mix by gentle pipetting. Transfer the cell-DNA mixture into an ice chilled 1 mm gap electroporation cuvette. Tap the cuvette to ensure cells span the entire base of the cuvette. Remove water droplets exterior and immediately electroporate. Electroporate the cells at 2kV, 129Ω, 50uF, resistance 2.5kV. Immediately add 1 mL SOC-2% arabinose to the cuvette, mix by pipetting and transfer into a microcentrifuge tube. Rescue at 37 °C with shaking for ~1 hour. Serial dilution of the cells was performed and then plated on LB agar plate with 4 g/L glucose, 0.2% arabinose, 50 μg/mL spectinomycin, and 200 μg/mL ampicillin. After incubation at 37 °C overnight aerobically, colonies formed were counted.

6. Protein expression and purification of D-Ldh variants

E. coli BL21 (DE3) transformed with plasmids pLZ108, 109, 110, 111, 112, 113, 121, 122 were cultured in LB medium with appropriate antibiotics at 37°C overnight. The overnight cultures were then used to inoculate 20 mL LB medium in the presence of antibiotics at 37°C in a rotary shaker (250 rpm) to an OD_{600nm} of 0.5. The cultures were then induced with 0.5 mM IPTG, and incubated at 30°C with 250 rpm agitation for 24 h. The recombinant proteins were purified using His-Spin Protein Miniprep kit (Zymo research Corporation, CA), according to the manufacturer's instructions. The concentrations of the purified proteins were quantified by Bradford assay. In addition, all purified proteins appeared >90% pure as examined by SDS-PAGE.

7. Protein expression levels of selected D-Ldh variants

The protein purification yield of selected D-Ldh variants is summarized in Table S2. The protein expression condition is described above.

Table S2 Protein purification yields of selected D-Ldh variants

Protein variants	Mutations	Protein yield (μg protein /ml culture)
1	D176S-I177R-F178S	4.3
2	D176A-I177F-F178S	17.6
3	D176S-I177R-F178T	11.5
8	D176G-I177L-F178W	21.3
11	D176V-I177W-F178G	27.7
13	D176S-I177L-F178G	98.1
18	D176S-I177S-F178F	19.9
WT	WT	63.3

8. Mutations in the 20 D-Ldh variants obtained from anaerobic selection

Table S3 Mutations in selected D-Ldh variants.

	Residues		
Variants	176	177	178
1	**S**	**R**	**S**
2	**A**	**F**	**S**
3	**S**	**R**	**T**
4	Y	L	G
5	A	G	Q
6	S	Y	Y
7	W	G	R
8	**G**	**L**	**W**
9	A	G	W
10	Q	S	L
11	**V**	**W**	**G**
12	G	C	F
13	**S**	**L**	**G**
14	I	Y	G
15	S	P	S
16	R	F	R
17	G	V	L
18	**S**	**S**	**F**
19	W	S	T
20	L	V	V
WT	D	I	F

Variants in bold were further characterized using purified proteins.

9. NADH-dependent activities of selected D-Ldh variants

Similar method as described in the section 2.5 was used to characterize the NADH-dependent activities of D-Ldh variants 1, 2, 3, 8, 11, 13, 18, as well as the wild type (Figure S3). While Figure 4C shows that the NADPH-dependent activities of the variants were consistently higher than wild type, the results in Figure S3 suggest that variants could have either higher or lower NADH-dependent activities. These results indicate that no selection pressure was imposed on NADH-dependent activities.

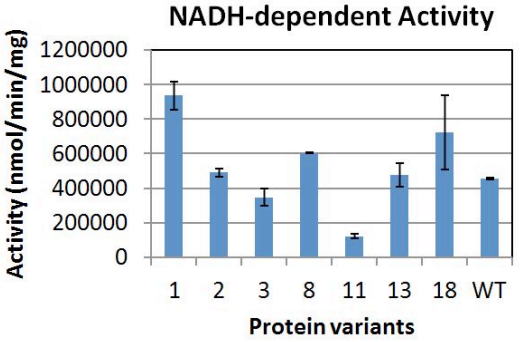

Fig S3 NADH-dependent activities of selected D-Ldh variants

10. Aerobic and anaerobic growth rescue conditions

The aerobic growth rescue condition used in Figure 2 was as follows: Briefly, the strains tested were first cultured in LB with 4 g/L glucose under aerobic conditions at 37°C overnight with appropriate antibiotics and inducers. Next, the overnight cultures were inoculated with the ratio of 0.25% (v/v) to 0.3mL M9 minimal medium with 10 g/L glucose in a 96-well culture plate, with appropriate antibiotics and inducers. The 96-well culture plate was sealed with a breathable membrane and incubated at 37°C in a rotary shaker, and OD_{600nm} was measured using a 96-well plate reader. The anaerobic growth rescue condition used in Figure 3 is adapted from previous reports [6]. Briefly, the strains tested were first cultured in LB with 4 g/L glucose under aerobic conditions at 37°C overnight with appropriate antibiotics and inducers. Next, the overnight cultures were inoculated with the ratio of 0.25% (v/v) to 0.3mL fresh LB medium in a 96-well culture plate, supplemented with 10 g/L glucose and set amounts of the substrates acetoin, with appropriate antibiotics and inducers. The 96-well culture plate was sealed with a breathable membrane and placed in a BD GasPak EZ anaerobic bag (Maryland, U.S.A). After culturing at 37°C in a rotary shaker for 24 h, OD_{600nm} was measured using a 96-well plate reader. Similar conditions were used for plate-based tests.

11. Rosetta modeling of D-Ldh variants

To model the binding of NADPH in LDH-SRT and LDH-GLW, we introduced the mutations onto the crystal structure for WT LDH (PDB: 1J49) and performed docking of NADPH. NADPH coordinates were extracted from crystal structures of NADPH-bound LDH homologues (PDB: 2DBQ, 3BAZ, 4E5M, 4XYB, 5AOV, 5V6Q) and a conformer library was produced through OpenBabel. The starting position for NADPH docking was chosen by superposing the NADPH onto the co-crystallized NADH position in the 1J49 crystal structure. A total of 500 decoys were produced per variant. The docking protocol involved Monte Carlo evaluation of side chain rotamer sampling, backbone torsion perturbation, and loop remodeling combined with NADPH rotation/translation moves.

Output models with more favorable total Rosetta energies (an aggregate of residue energy terms describing Van der Waals interactions, electrostatics, rotamer conformation probabilities, etc. that serves as an indicator of complex stability) than the relaxed starting structure were ranked by interface energies (the difference in energy with the ligand separated from the complex that reflects the predicted favorability of ligand binding) [7]. Visual inspection of the top 10 models for each variant selected by interface energies was performed to ensure realistic geometries. No structural artifacts were observed in the selected decoys, and representative models for NADPH bound LDH-SRT and LDH-GLW were chosen as the respective decoys with the lowest interface energies.

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