

ARTICLE

An “energy-auxotroph” *Escherichia coli* provides an in vivo platform for assessing NADH regeneration systems

Sebastian Wenk | Karin Schann | Hai He | Vittorio Rainaldi | Seohyoung Kim |
Steffen N. Lindner | Arren Bar-Even 

Systems and Synthetic Metabolism Lab, Max
Planck Institute of Molecular Plant Physiology,
Potsdam-Golm, Germany

Correspondence

Arren Bar-Even, Systems and Synthetic
Metabolism Lab, Max Planck Institute of
Molecular Plant Physiology, Am Mühlenberg 1,
14476 Potsdam-Golm, Germany.
Email: Bar-Even@mpimp-golm.mpg.de

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Abstract

An efficient in vivo regeneration of the primary cellular resources NADH and ATP is vital for optimizing the production of value-added chemicals and enabling the activity of synthetic pathways. Currently, such regeneration routes are tested and characterized mainly in vitro before being introduced into the cell. However, in vitro measurements could be misleading as they do not reflect enzyme activity under physiological conditions. Here, we construct an in vivo platform to test and compare NADH regeneration systems. By deleting dihydrolipoyl dehydrogenase in *Escherichia coli*, we abolish the activity of pyruvate dehydrogenase and 2-ketoglutarate dehydrogenase. When cultivated on acetate, the resulting strain is auxotrophic to NADH and ATP: acetate can be assimilated via the glyoxylate shunt but cannot be oxidized to provide the cell with reducing power and energy. This strain can, therefore, serve to select for and test different NADH regeneration routes. We exemplify this by comparing several NAD-dependent formate dehydrogenases and methanol dehydrogenases. We identify the most efficient enzyme variants under in vivo conditions and pinpoint optimal feedstock concentrations that maximize NADH biosynthesis while avoiding cellular toxicity. Our strain thus provides a useful platform for comparing and optimizing enzymatic systems for cofactor regeneration under physiological conditions.

KEYWORDS

auxotrophy, biosensor, energy metabolism, in vivo selection, methylotrophy

1 | INTRODUCTION

The growth of an organism requires three general components: reducing power, energy, and chemical elements—such as carbon and nitrogen. The former two are typically connected, as the transfer of reducing power from an electron donor to an acceptor (e.g., oxygen, nitrate, or an organic compound) is coupled to energy conservation in

the form of ATP biosynthesis. In heterotrophic metabolism, all component types are derived from a single growth substrate, where its catabolism not only retrieves reducing power and energy but also provides carbon building blocks for cell growth. However, in some cases, the carbon source is decoupled from that of reducing power and energy. The most notable example is autotrophic growth, where two different systems are responsible for carbon fixation and

Sebastian Wenk and Karin Schann contributed equally to this study.

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regeneration of NAD(P)H and ATP. Another example is the use of auxiliary substrates that are not converted to biomass but rather fully oxidized to provide the cell with more reducing power and energy, thus enabling a higher fraction of the primary substrate to provide carbon units for biosynthesis (Babel, 2009). This approach is also commonly used in cell-free systems, where the bioconversion process is supported by parallel systems for NAD(P)H and ATP regeneration (Claassens, Burgener, Vogeli, Erb, & Bar-Even, 2019).

To optimize the in vitro regeneration of NAD(P)H and ATP it usually suffices to choose an enzymatic system with a high $V_{\max}$, which can be measured and compared under the specific relevant conditions. However, optimization of in vivo regeneration of NAD(P)H and ATP is more delicate. First, the enzyme kinetic parameters can substantially change within the cellular environment, which is difficult to mimic in in vitro measurements (van Eunen & Bakker, 2014; van Eunen, Kiewiet, Westerhoff, & Bakker, 2012). Moreover, the physiological steady-state concentrations of the substrates and products affect the reaction thermodynamics and kinetics (Noor et al., 2016). Finally, overexpressed enzymes, native and heterologous alike, tend to misfold and degrade, thus lowering, sometimes dramatically, the amount of functional protein in the cell (Roodveldt, Aharoni, & Tawfik, 2005). Hence, there is a growing need to establish an in vivo platform to test and compare cofactor regeneration routes.

In this study, we construct an *Escherichia coli* strain which, when fed on acetate as a carbon source, cannot generate reducing power and energy for cell growth. Under these conditions, the strain depends on the introduction of an independent enzymatic system for NADH regeneration, which could further produce ATP via oxidative phosphorylation. We exemplify the use of this strain as a platform for testing and comparing the ability of different enzymes to produce NADH via the oxidation of either formate or methanol, two commonly used auxiliary substrates (Babel, 2009). We identify the most efficient enzymes under in vivo conditions and pinpoint optimal feedstock concentrations to maximize NADH regeneration rate while minimizing substrate toxicity. The results of this study pave the way for improved utilization of formate and methanol as energy sources for engineered autotrophic and methylotrophic growth of *E. coli* (Gleizer et al., 2019; Kim et al., 2020) and further provide a useful platform to compare other cofactor regeneration systems within the cellular environment.

2 | METHODS

2.1 | Reagents

Primers were synthesized by Eurofins (Ebersberg, Germany). Screening polymerase chain reaction (PCRs) were performed using DreamTaq polymerase (Thermo Fisher Scientific, Dreieich, Germany). PCR reactions for cloning were performed using PrimeSTAR MAX DNA Polymerase (Takara). Restrictions were carried out using FastDigest enzymes and ligations using T4 DNA ligase, all purchased from Thermo Fisher Scientific. Sodium acetate, sodium formate,

sodium formate- ^{13}C , methanol, methanol- ^{13}C , and sodium succinate were ordered from Sigma-Aldrich (Steinheim, Germany). D-Glucose, gluconate, xylose, and glycerol were ordered from Carl Roth (Karlsruhe, Germany).

2.2 | Bacterial strains

All strains used in this study are listed in Table 1. *E. coli* SIJ488 (Jensen, Lennen, Herrgard, & Nielsen, 2015), derived from *E. coli* K12 MG1655, was used as the parental strain for the Δlpd and $\Delta lpd \Delta frmRAB$ strains. The *lpd* gene and the *frmRAB* operon were first deleted by λ -Red recombineering (Jensen et al., 2015) in individual strains. Then, *lpd* was deleted in the $\Delta frmRAB$ strain thus generating the $\Delta lpd \Delta frmRAB$ genotype. *E. coli* K12 MG1655, $\Delta serA \Delta ltaE \Delta kbl \Delta aceA$ (Kim et al., 2020; Yishai et al., 2018) was used for engineering the formate assimilating strain used to test the activity of the methanol dehydrogenases. For all cloning procedures, the *E. coli* strain DH5 α was used.

2.3 | Genome engineering

Gene deletions were performed as described before (Wenk, Yishai, Lindner, & Bar-Even, 2018). For λ -Red recombineering in SIJ488, ~300 ng of a PCR amplified chloramphenicol resistance cassette (Datsenko & Wanner, 2000) with 50 bp homology arms on each side (targeting the up- and downstream region of the gene of interest) were electroporated into the SIJ488 target strain with arabinose-induced recombineering genes to replace the target gene with the resistance gene. After chloramphenicol selection, gene deletions were confirmed by PCR using verification (ver) primers that bind outside the target locus. The antibiotic marker was removed by inducing flippase expression in the SIJ488 strain. Marker removal was verified by taking colonies that grew only on plates without antibiotics and further confirmed by PCR using ver primers.

2.4 | Gene expression

Expression units (individual genes or synthetic operons) were designed using Geneious 8 (Biomatters, New Zealand) and constructed as described before (Wenk et al., 2018). Genes were expressed with the ribosome binding site C under the control of a constitutive strong *pgi* promoter and a medium copy number origin of replication (p15A; Wenk et al., 2018). Genes not native to *E. coli* were codon optimized using JCat (PMID: 15980527) and de novo synthesized. The genes encoding the methanol dehydrogenases from *Bacillus stearothermophilus* (BsMDH, UniProt: P42327) and *Corynebacterium glutamicum* R (CgMDH, UniProt: A4QHJ5), were synthesized by Gen9 (Cambridge, MA). Methanol dehydrogenases from *B. methanolicus* (BmMDH; UniProt: I3DVX6) and its improved variant BmMDH* (carrying a Q5L and an A363L modification) (Roth et al., 2019) as well

TABLE 1 *Escherichia coli* strains and plasmids used and constructed in this study

Strain/plasmids	Genotype	Reference
Strain		
DH5 α	F ⁻ <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K⁻m_K⁺), λ⁻</i>	Hanahan (1983)
MG1655	F ⁻ λ ⁻ <i>ilvG⁻ rfb-50 rph-1</i>	Blattner et al. (1997)
SIJ488	MG1655 Tn7::para-exo-beta-gam; prha-FLP; xylSpm-lscl	Jensen et al. (2015)
Δ lpd	SIJ488 Δ lpd::cap	This study
Δ lpd Δ frmRAB	SIJ488 Δ frmRAB Δ lpd::cap	This study
gC ₁ M–gC ₂ M	MG1655, Δ serA Δ ltaE Δ kbl Δ aceA SS9:RBS _C :MeFtFL-MeFch-MeMtdA P _{STRONG} -RBS _C -gcvT-RBS _{NATIVE} -gcvH-RBS _{NATIVE} -gcvP	Kim et al. (2020)
Plasmids		
pZASS	Overexpression plasmid with p15A origin, streptomycin resistance, constitutive strong strength promoter (P _{STRONG})	Wenk et al. (2018)
ASS:C:adhP-mhpF	pZASS backbone for overexpression of RBS _C -adhP- RBS _C -mhpF from <i>E. coli</i>	This study
ASS:C:PsFDH	pZASS backbone for overexpression of RBS _C -PsFDH from <i>Pseudomonas</i> sp. 101(UniProt: P33160; Tishkov & Popov, 2006)	This study
ASS:C:CbFDH	pZASS backbone for overexpression of RBS _C -CbFDH from <i>Candida boidinii</i> (UniProt: O13437; Tishkov & Popov, 2006)	This study
ASS:C:BsmDH	pZASS backbone for overexpression of methanol dehydrogenase from <i>Bacillus stearothermophilus</i> (UnitProt, P42327) Whitaker et al. (2017)	This study
ASS:C:CgMDH	pZASS backbone for overexpression of methanol dehydrogenase from <i>Corynebacterium glutamicum</i> (UnitProt, A4QHJ5) Kotrbova-Kozak et al. (2007)	This study
ASS:C:BmMDH	pZASS backbone for overexpression of methanol dehydrogenase from <i>Bacillus methanolicus</i> (Unitprot, I3E949) Roth et al. (2019)	This study
ASS:C:BmMDH*	pZASS backbone for overexpression of engineered methanol dehydrogenase (Q5L A363L) from <i>Bacillus methanolicus</i> Roth et al. (2019)	This study
ASS:C:CnMDH	pZASS backbone for overexpression of engineered methanol dehydrogenase from <i>Cupriavidus necator</i> N-1 version CT4-1 (UnitProt, F8GNE5) Wu et al. (2016)	This study

as the engineered version CT4-1 of *Cupriavidus necator* N-1 MDH (CnMDH; UniProt: F8GNE5) (Wu et al., 2016) were synthesized by TWIST biosciences (South San Francisco, CA). The formate dehydrogenases from *Pseudomonas* sp.101 (PsFDH; UniProt: P33160) and *Candida boidinii* (CbFDH; UniProt: O13437) were obtained from collaborators at the Weizmann Institute of Science (Gleizer et al., 2019).

Native *E. coli* genes—ethanol dehydrogenase (*adhP*) and acet-aldehyde dehydrogenase (*mhpF*)—were PCR-amplified from the *E. coli* MG1655 genome. The individual genes were integrated into a high copy number cloning vector pNiv to construct a synthetic operon using the method described previously (Wenk et al., 2018). Then, operons or individual genes were transferred into a pZ based expression vector. All gene sequences are listed in the Supporting Information.

2.5 | Media and growth conditions

LB medium (1% NaCl, 0.5% yeast extract, 1% tryptone) was used for SIJ488 strain engineering (genomic modifications and transformations with expression plasmids) and cloning of recombinant genes in DH5 α . Antibiotics were used at the following concentrations:

streptomycin: 100 μ g/ml; chloramphenicol: 30 μ g/ml, and ampicillin: 100 μ g/ml. Growth experiments were performed in M9 minimal medium (47.8 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.6 mM NaCl, 18.7 mM NH₄Cl, 2 mM MgSO₄, and 100 μ M CaCl₂), supplemented with trace elements (134 μ M ethylene diamine tetrachloroacetic acid, 31 μ M FeCl₃·6H₂O, 6.2 μ M ZnCl₂, 0.76 μ M CuCl₂·2H₂O, 0.42 μ M CoCl₂·2H₂O, 1.62 μ M H₃BO₃, and 0.081 μ M MnCl₂·4H₂O). Carbon sources were added according to the experimental conditions as detailed in the text.

For plate reader growth experiments, strains were precultured in 4 ml M9 medium with carbon sources that allowed growth of all tested strains (glycerol 20 mM, acetate 20 mM, and succinate 20 mM). The precultures were harvested and washed three times in M9 medium before used to inoculate the test medium (M9 medium supplemented with the indicated carbon sources) to a starting OD₆₀₀ of 0.01. In 96-well microplates (Nunclon Delta Surface, Thermo Fisher Scientific), 150 μ l of culture were added to each well and covered with 50 μ l mineral oil (Sigma-Aldrich) to avoid evaporation. (We note that mineral oil enables gas exchange and thus keeps the medium aerobic). The culture was incubated at 37°C in a microplate reader (EPOCH 2, BioTek). The shaking program cycle (controlled by Gen5 3.04) has four shaking phases, lasting 60 s each: linear shaking 567 cpm (3 mm), orbital shaking 282 cpm (3 mm), linear shaking

731 cpm (2 mm), orbital shaking 365 cpm (2 mm). Absorbance OD₆₀₀ in each well was measured and recorded after every three shaking cycles (~12 min). Raw data from the plate reader were calibrated to normal cuvette measured OD₆₀₀ values according to $OD_{cuvette} = OD_{plate}/0.23$. Growth parameters were calculated using Matlab based on three technical replicates; the average values were used to generate the growth curves. In all cases variability between triplicate measurements were less than 5%. Growth curves were calculated using a sliding window methodology. First, we converted the linear values of the OD₆₀₀ into logarithmic values. Then, using linear fitting (polyfit function) we calculated the growth rate for given windows of 5–20 hr: starting from each time point (increments of 12.5 min), we defined a period of 5–20 hr, and fitted the logarithmic OD₆₀₀ values in this time window into linear function, the slope of which corresponds to the growth rate within this window. We choose the maximal slope calculated across all time windows to represent the growth rate of the strain.

2.6 | Estimation of substrate utilization rates and biomass yields

Flux balance analysis (FBA) was conducted in Python with COBRApy (Ebrahim, Lerman, Palsson, & Hyduke, 2013). We used the *E. coli* core metabolic model (Orth et al., 2010) with several curations: (a) transhydrogenase (THD2) translocates one proton instead of two (Bizouarn, van Boxel, Bhakta, & Jackson, 2005); (b) pyruvate formate lyase (PFL) was deactivated as it operates only under anaerobic condition; (c) the phosphogluconate dehydrogenase reaction was removed to block an unrealistic oxidation cycle in which a phosphosugar substrate is completely oxidized to CO₂, thus replacing the tricarboxylic acid (TCA) cycle (Aslan, Noor, Benito Vaquerizo, Lindner, & Bar-Even, 2020); (d) secretion of C_{≥2} compounds was blocked as we did not observe it experimentally. Based on the WT strain growing solely on acetate, the maximum acetate uptake rate was estimated to be 13 mmol·gCDW⁻¹·hr⁻¹, which is within the range previously reported (Edwards, Ibarra, & Palsson, 2001). When glucose was used as a carbon source, its maximum uptake rate was set to be 10.5 mmol·gCDW⁻¹·hr⁻¹ (Varma & Palsson, 1994); this value also matches the growth rate of the Δlpd strain cultivated on glucose and acetate. For the gC1M–gC2M strain, we added to the model the reactions producing serine, glycine and C₁-tetrahydrofolate and amended the biomass function such that serine, glycine, and C₁-tetrahydrofolate are consumed instead of 3-phosphoglycerate (He et al., 2018). Biomass yields were calculated as the ratio between the growth rate and the uptake/utilization rate of the limiting substrate; the resulting number was converted to units of OD₆₀₀/mM by dividing with 0.39 gCDW/L/OD₆₀₀ (Milo, Jorgensen, Moran, Weber, & Springer, 2010). The optimal stoichiometric ratio of substrate coutilization was calculated as the ratio between their uptake/utilization rates. The full code, including changes to the model, reactions specific to each energy module, and the calculations can be found at <https://github.com/he-hai/PubSuppl> within the “2020_Energy_Auxotroph” directory.

3 | RESULTS

3.1 | Construction and validation of an *E. coli* strain auxotrophic to reducing power and energy

Dihydrolipoyl dehydrogenase, encoded by *lpd*, catalyzes the oxidative regeneration of lipoic acid, as part of three enzyme complexes: pyruvate dehydrogenase, 2-ketoglutarate dehydrogenase, and the glycine cleavage system (Guest & Creaghan, 1972; Pettit & Reed, 1967; Steiert, Stauffer, & Stauffer, 1990). Deletion of *lpd* is expected to render these enzyme complexes inactive, effectively blocking the known routes for the complete oxidation of acetyl-CoA (Figure 1): the canonical TCA cycle that relies on the activity of 2-ketoglutarate dehydrogenase, and the alternative PEP-glyoxylate cycle (Fischer & Sauer, 2003) that relies on the activity of pyruvate dehydrogenase. On the other hand, the glyoxylate shunt, which serves to assimilate acetyl-CoA into biomass, is not blocked by this deletion. Hence, the Δlpd strain is expected to be auxotrophic to reducing power and energy when cultivated on acetate: acetate can be activated to acetyl-CoA and assimilated via the glyoxylate shunt but cannot be oxidized to provide the cell with reducing power and energy. Conversely, when provided with carbon sources that enter upper metabolism, the Δlpd strain is expected to behave as an acetyl-CoA auxotroph: reducing power and energy can be obtained by the oxidation of these carbon sources, but the biosynthesis of acetyl-CoA is blocked due to the disruption of pyruvate dehydrogenase.

To test these hypotheses, we constructed the Δlpd strain and characterized its growth on various carbon sources (Figure 2). As expected, growth on glucose, glycerol, and gluconate—all entering upper metabolism—was made possible only upon addition of acetate. (We note that this finding contrasts a previous study, using a different *E. coli* strain—BW25113 instead of K-12 MG1655—in which the deletion of *lpd* was compensated with the activation of pyruvate oxidase, thus providing an alternative route for acetyl-CoA biosynthesis (Li, Ho, Yao, & Shimizu, 2006). On the other hand, growth on carbon sources that enter lower metabolism—pyruvate and succinate—was not observed even upon addition of acetate, as the cells cannot extract reducing power from these feedstocks (Figure 2). Furthermore, as originally anticipated, growth on acetate was not possible.

To validate that the inability to grow on acetate stems from lack of reducing power, we aimed to cultivate the Δlpd strain on ethanol. Similarly to acetate, ethanol is assimilated via its conversion to acetyl-CoA. However, in contrast to acetate, metabolism of ethanol to acetyl-CoA produces two NADH molecules that could provide the cell with sufficient reducing power. Yet, *E. coli* cannot grow on ethanol, due to the low expression levels of *adhE*, which encodes a bifunctional ethanol dehydrogenase/acetylating aldehyde dehydrogenase, as well as the oxygen sensitivity of the enzyme (Membrillo-Hernandez et al., 2000). We, therefore, chose to overexpress the oxygen-tolerant enzymes ethanol dehydrogenase AdhP (Thomas et al., 2013) and acetylating aldehyde dehydrogenase MhpF (Fischer et al., 2013), which together are expected to convert ethanol to acetyl-CoA. The Δlpd strain expressing the genes encoding for these two enzymes was able to grow on ethanol as a sole carbon source. (We note that we

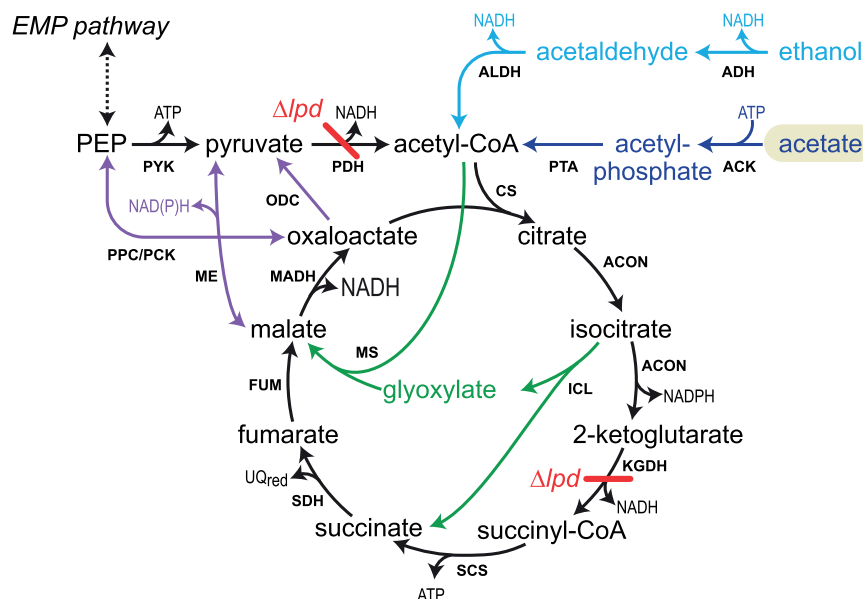


FIGURE 1 Central carbon metabolism of the Δlpd strain. The deletion of the *lpd* gene renders the pyruvate dehydrogenase complex and the 2-ketoglutarate dehydrogenase complex inactive, interrupting lower (EMP) glycolysis, and the TCA cycle (indicated with red bars). When acetate is provided as a feedstock (dark blue), the strain can assimilate acetyl-CoA into biomass via the glyoxylate shunt (indicated in green) but is expected to be auxotrophic to reducing power and energy since acetyl-CoA oxidation is blocked. On the other hand, the assimilation of ethanol (light blue) produces two molecules of NADH, which should be sufficient to provide the cell with reducing power and energy. ACK, acetate kinase; ACON, aconitase; ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; CS, citrate synthase; EMP, Embden–Meyerhof–Parnas; FUM, fumerase; ICL, isocitrate lyase; KGDH, 2-ketoglutarate dehydrogenase complex; MADH, malate dehydrogenase; ME, malic enzyme; MS, malate synthase; ODC, oxaloacetate decarboxylase; PDH, pyruvate dehydrogenase complex; PPC, PEP carboxylase; PPCK, PEP carboxykinase; PTA, phosphate acetyltransferase; PYK, pyruvate kinase; SCS, succinyl-CoA synthetase; TCA, tricarboxylic acid; SDH, succinate dehydrogenase [Color figure can be viewed at wileyonlinelibrary.com]

used 50 mM of ethanol to overcome the thermodynamic barrier of ethanol oxidation to acetaldehyde [Shafqt et al., 1999]: $\Delta_r G^m = +18$ kJ/mol, where $\Delta_r G^m$ is the reaction change in the Gibbs energy under reactant concentration of 1 mM, pH 7.5, and ionic strength of 0.25 M [Flamholz, Noor, Bar-Even, & Milo, 2012]). After a few cultivation cycles in test-tubes, the growth rate and final OD_{600} improved considerably. We sequenced the genome and plasmid of the adapted strain and found a single mutation in the *mhpF* gene on the plasmid: Asp209Asn. Notably, this residue was previously found to be involved in the activation of the active site cysteine in an ortholog enzyme (Manjasetty, Powlowski, & Vrielink, 2003) and hence probably also participates in the catalytic cycle of MhpF. When the mutated plasmid was transferred to a native (nonevolved) Δlpd strain, efficient growth on ethanol was directly observed (Figure 3).

These results confirm that, when cultivated on acetate, the Δlpd strain is auxotrophic to reducing power and energy and could thus serve as a platform for the testing and comparison of different NADH regeneration systems under physiological conditions.

3.2 | Testing formate as a reducing power source

Formate can be efficiently produced via the abiotic reduction of CO_2 , for example, electrochemical reduction of CO_2 (Jouny, Luc, &

Jiao, 2018) or hydrogenation of CO_2 (Alvarez et al., 2017). Hence, it represents a promising renewable electron donor for metabolic transformations (Claassens, Sanchez-Andrea, Sousa, & Bar-Even, 2018; Yishai, Lindner, Gonzalez de la Cruz, Tenenboim, & Bar-Even, 2016). The utilization of formate as a source of reducing power and energy was demonstrated before in cellular and cell-free systems (Mattozzi, Ziesack, Voges, Silver, & Way, 2013; Schwander, Schada von Borzyskowski, Burgener, Cortina, & Erb, 2016). However, *E. coli* does not harbor an NAD-dependent formate dehydrogenase (FDH) and hence cannot use this compound as a source of reducing power.

To test whether formate can serve as sole source of reducing power and energy for growth of the Δlpd strain on acetate, we cloned and overexpressed two NAD-dependent FDHs that are widely used in enzyme and metabolic engineering studies: *Pseudomonas* sp.101 FDH (PsFDH) and *Candida boidinii* FDH (CbFDH; Balzer, Thakker, Bennett, & San, 2013; Berrios-Rivera, Bennett, & San, 2002; Mattozzi et al., 2013; Tishkov & Popov, 2006; Uppada, Bhaduri, & Noronha, 2014). Overexpression of either FDH variant enabled the growth of the Δlpd strain on acetate only when formate was added (green and purple lines in Figure 4a). Addition of formate without FDH expression did not enable growth (gray line in Figure 4a). PsFDH supported a higher growth rate (doubling time of ~5 hr) than CbFDH (doubling time of ~15 hr). As mentioned above, rather than reflecting only the in vitro measured kinetics of the enzymes, this finding corresponds to

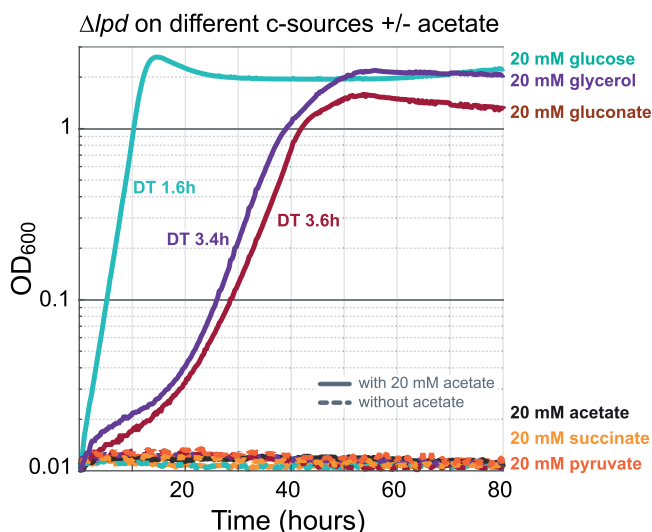


FIGURE 2 Growth of the Δlpd strain on different carbon sources. The Δlpd strain behaves like an acetyl-CoA auxotroph when provided with carbon sources from upper metabolism: it can grow on 20 mM glucose, 20 mM glycerol, or 20 mM gluconate only when 20 mM acetate is added to the medium. The Δlpd strain cannot grow on carbon sources that enter lower metabolism (20 mM pyruvate or 20 mM succinate), even when 20 mM acetate are added, as it cannot obtain reducing power and energy. For the same reason, the Δlpd strain could not grow on acetate. Experiments were conducted within 96-well plates and were performed in triplicates, which displayed identical growth curves ($\pm 5\%$), and hence were averaged. Doubling times (DT) are shown in the figure. All experiments were repeated three times, which showed highly similar growth behavior. OD, optical density [Color figure can be viewed at wileyonlinelibrary.com]

their expression, stability, and activity within the cellular context. It therefore seems that PsFDH is more suitable for NADH regeneration within the *E. coli* cellular environment.

Next, we wanted to determine the optimal concentration of formate supporting NADH regeneration. We focused on PsFDH due to its apparent superiority. We cultivated the Δlpd strain overexpressing PsFDH on 20 mM acetate and varying concentrations of formate: from 1.7 mM, increasing stepwise by a factor of 1.5, up to 100 mM. We found that the final OD₆₀₀ of the culture increased monotonically with increasing formate concentration (Figure 4b). This indicates that even at the highest formate concentration tested, the availability of reducing power limits bacterial growth, rather than the supply of carbon from acetate. The doubling time was rather constant (5.2–5.8 hr) at the formate concentration range 10–70 mM (at lower concentrations the doubling time was difficult to define as the culture did not spend sufficient time in exponential phase but rather reached saturation quite fast). A higher formate concentration, 100 mM, lowered the growth rate, probably due to the toxicity of formate (Nicholls, 1975; Warnecke & Gill, 2005). Our results thus indicate an optimal range within which the concentration of formate should be kept to enable *E. coli* to use this C₁ compound efficiently as a source of reducing power without adverse effects.

We performed FBA using a model of *E. coli* core metabolism (Orth, Fleming, & Palsson, 2010) to estimate the utilization rates of acetate and formate as well as the expected biomass yields (i.e., final OD₆₀₀ per substrate consumed) of the Δlpd strain expressing PsFDH (Section 2). For the observed doubling times (5.2–5.8 hr; Figure 4b), we calculated that the oxidation (i.e., utilization) rate of formate needs to be approximately fivefold higher than the utilization rate of acetate (assuming optimal cointegration of the substrates). Hence, at 20 mM acetate, the tested formate concentrations, 1.7–100 mM, should limit biomass production, as confirmed by the growth experiments (Figure 4b).

As compared to a WT strain growing on acetate, the Δlpd strain growing with acetate and formate displayed a lower growth rate (Figure 4), suggesting that the formate oxidation rate is limiting. Indeed, the estimated utilization rate of acetate in a WT strain was $\approx 13 \text{ mmol} \cdot \text{gCDW}^{-1} \cdot \text{hr}^{-1}$, whereas in the Δlpd strain, a growth-limiting formate oxidation rate of $\approx 16 \text{ mmol} \cdot \text{gCDW}^{-1} \cdot \text{hr}^{-1}$ reduced the requirement for acetate to only $\approx 3 \text{ mmol} \cdot \text{gCDW}^{-1} \cdot \text{hr}^{-1}$. According to the formate oxidation rate, we calculated the expected biomass yield of the Δlpd strain on formate to be $\approx 0.02 \text{ OD}_{600}/\text{mM}$. However, the observed yield was 2.5–4-fold lower, that is 0.005–0.008 OD₆₀₀/mM (in contrast to a WT strain growing on acetate, where the estimated yield, 0.047 OD₆₀₀/mM, was similar to the observed one, 0.04 OD₆₀₀/mM). This suggests that the cellular use of formate-derived NADH might not be optimal, that is imbalanced with acetate assimilation such that too much NADH is directed to respiration thus wasting reducing power required for biomass formation.

3.3 | Testing methanol as a reducing power source

Methanol can be either derived cheaply from fossil methane (Pfeifenschneider, Brautaset, & Wendisch, 2017) or renewably produced via the hydrogenation of CO₂ (Szyma & Cormos, 2018). As such, methanol has gained a lot of attention as a microbial feedstock (Antoniewicz, 2019; Schrader et al., 2009) and is actively researched as a source of reducing power and energy (Guo et al., 2019). However, *E. coli* lacks a methanol dehydrogenase (MDH) and cannot oxidize methanol.

We cloned and overexpressed five MDH variants—previously explored in studies aimed to achieve synthetic methylotrophy—and tested their ability to restore growth of the Δlpd strain on acetate upon addition of methanol: *Bacillus stearothermophilus* MDH (BsMDH; Whitaker et al., 2017), *Bacillus methanolicus* MDH (BmMDH; Roth, Woolston, Stephanopoulos, & Liu, 2019) and its improved variant (BmMDH*, carrying Q5L A363L modifications) (Roth et al., 2019), *Corynebacterium glutamicum* MDH (CgMDH; Kotrbava-Kozak, Kotrba, Inui, Sajdok, & Yukawa, 2007), and *Cupriavidus necator* N-1 MDH (CnMDH; Wu et al., 2016). We found that only cells expressing BsMDH could grow on acetate and methanol (Figure 5a). Hence, it seems that the activity of this enzyme is sufficient to enable the cells

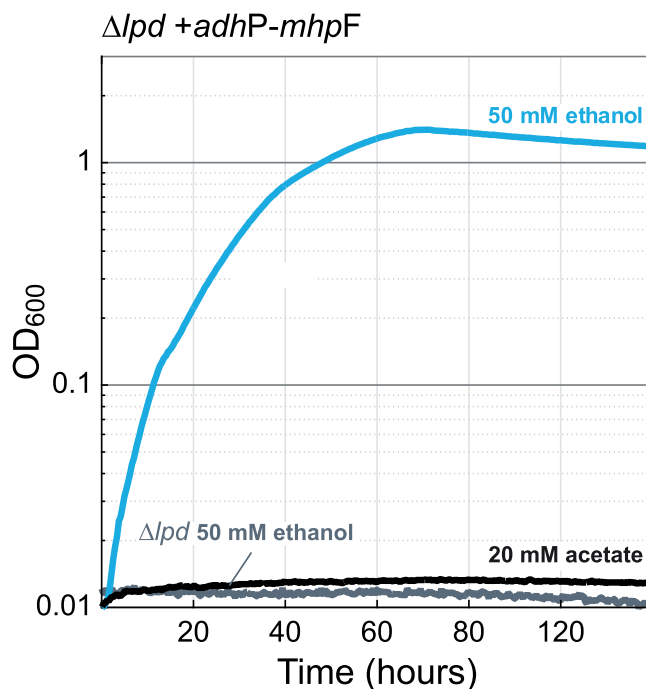


FIGURE 3 Ethanol metabolism provides both acetyl-CoA and reducing power to the Δlpd strain. The Δlpd strain could grow on 50 mM ethanol, but not on acetate, upon expression of the oxygen-tolerant enzymes ethanol dehydrogenase AdhP and the acetylating aldehyde dehydrogenase MhpF. Shown is the growth of a strain adapted to grow on ethanol (see main text). Experiments were conducted within 96-well plates and were performed in triplicates, which displayed identical growth curves ($\pm 5\%$), and hence were averaged. All experiments were repeated three times, which showed highly similar growth behavior. OD, optical density [Color figure can be viewed at wileyonlinelibrary.com]

to use methanol as sole source of reducing power and energy, while the activity of the other MDH variants is too low.

Another explanation for the inability of the Δlpd strain to grow on acetate and methanol with the other MDHs might be that these variants are completely inactive. To test if this is the case, we applied a different selection scheme that requires a lower methanol oxidation flux to enable growth. Specifically, we used a strain deleted in the endogenous glycine and serine biosynthesis routes ($\Delta serA \Delta ltaE \Delta kbl \Delta aceA$) which overexpresses *Methylobacterium extorquens* enzymes that convert formate to methylene-THF (Ftfl, Fch, and MtdA) as well as the endogenous enzymes of the glycine cleavage system (Figure 6a; Kim et al., 2020). We previously demonstrated that this strain, termed gC₁M–gC₂M (Kim et al., 2020), is capable of producing glycine and serine via the assimilation of formate and CO₂. We also showed that expression of MDH enabled the replacement of formate with methanol in the medium (Kim et al., 2020): methanol is oxidized to formaldehyde via MDH activity, while the endogenous glutathione system metabolizes this highly reactive intermediate to formate (Gutheil, Kasimoglu, & Nicholson, 1997; Figure 6a). Using FBA, we confirmed that the gC₁M–gC₂M strain is indeed expected to be considerably more sensitive to methanol than the Δlpd strain, that is requiring substantially lower oxidation rates for growth: while a methanol oxidation rate as low as 1 mmol·gCDW^{−1}·hr^{−1} is expected to support a doubling time of approximately 2 hr of the former strain, an oxidation rate of as high as 5 mmol·gCDW^{−1}·hr^{−1} supports a doubling time of more than 30 hr of the Δlpd strain.

When we overexpressed the different MDHs in the gC₁M–gC₂M strain, we were able to detect growth on glucose (as main carbon source) and methanol (for glycine and serine biosynthesis) with all MDH variants except *Bm*MDH (Figure 6b). While *Bs*MDH, *Cn*MDH, and *Bm*MDH* supported similar growth (doubling time 2.7–3 hr, from which we derived a methanol oxidation rate of ≈ 0.7 mmol·gCDW^{−1}·hr^{−1}), the strain expressing *Cg*MDH showed a much poorer growth (doubling time 10 hr, from which we derived a methanol oxidation rate of ≈ 0.2 mmol·gCDW^{−1}·hr^{−1}). Upon further addition of formate, all strains showed comparable growth (thin dashed lines in Figure 6b, doubling time of ≈ 2 hr), indicating that the inability of *Bm*MDH to support growth and the poor growth supported by *Cg*MDH are not related to the burden of enzyme overexpression or to the deleterious accumulation of formaldehyde.

Overall, it is clear that most MDH variants are indeed active in vivo. Still, only *Bs*MDH can support a sufficiently high NADH regeneration rate to enable the cell to gain all of its reducing power and energy from methanol (Figure 5a, where the required methanol oxidation rate is more than sixfold higher than in the gC₁M–gC₂M strain). This demonstrates that predicting in vivo activity from in vitro kinetic measurements is inaccurate: while *Bs*MDH does not have the best kinetic parameters among the MDH variants tested (Whitaker et al., 2017), it is the only enzyme that could support sufficient NADH regeneration within the cellular context.

Next, we tested the dependency of growth on methanol concentration. We cultivated the Δlpd strain expressing *Bs*MDH on

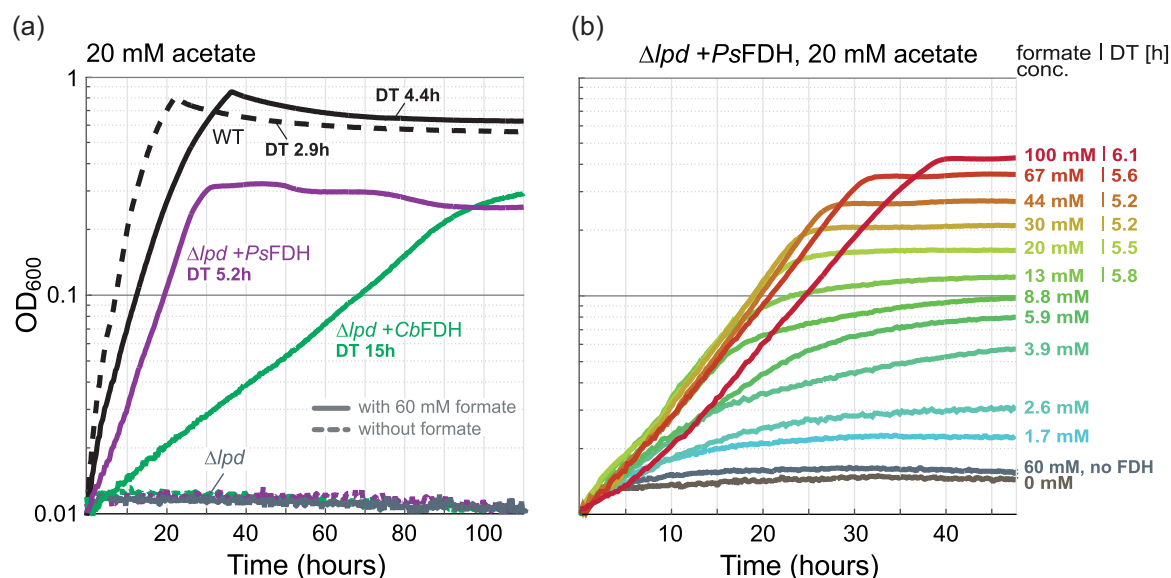


FIGURE 4 Expression of an NAD-dependent FDH enables the Δlpd strain to grow on acetate and formate. NAD-dependent formate dehydrogenases from different organisms—*Ps* corresponds to *Pseudomonas* sp.101 and *Cb* corresponds to *Candida boidinii*—were expressed in the Δlpd strain and growth of the strains on acetate with or without formate was tested. (a) Both FDH variants enabled growth of the Δlpd strain on 20 mM acetate only when 60 mM formate were added to the medium. *PsFDH* supported a higher growth rate than *CbFDH*. (b) The Δlpd strain expressing *PsFDH* was cultivated on 20 mM acetate and formate at concentrations ranging from 1.7 to 100 mM (increasing stepwise by a factor of 1.5). The final OD₆₀₀ of the culture increased with increasing formate concentration. Experiments were conducted within 96-well plates and were performed in triplicates, which displayed identical growth curves ($\pm 5\%$), and hence were averaged. Doubling times (DT) are shown in the figure; only doubling times associated with formate concentrations higher than 10 mM were calculated as for lower concentrations the culture did not spend sufficient time in exponential phase to enable reliable calculation. All experiments (in triplicates) were repeated three times, which showed highly similar growth behavior. OD, optical density [Color figure can be viewed at wileyonlinelibrary.com]

20 mM acetate and varying concentrations of methanol: from 26 mM, increasing stepwise by a factor of 1.5, up to 1,500 mM. We found that the final OD₆₀₀ was monotonically increasing with methanol concentrations up to 667 mM. Addition of 1,000 mM methanol resulted in a lower OD₆₀₀ and 1,500 mM methanol completely abolished growth (Figure 5b), presumably due to cell dehydration and protein precipitation (Hobro & Smith, 2017) as well as increased levels of formaldehyde, the concentration of which is (thermodynamically) coupled to that of methanol (Kim, Kim, & Oh, 2003). The growth rates associated with the different methanol concentrations were difficult to define as they were not constant but rather seem to decrease during the growth phase. This can be attributed to evaporation of methanol from the culture and to the unfavorable thermodynamics of methanol oxidation ($\Delta_r G'^m = +31$ kJ/mol [Flamholz et al., 2012]) such that the oxidation rate decreases with the consumption of the substrate. Still, it seems that the highest growth rates were attained at methanol concentrations of 444 and 667 mM, indicating an optimal concentration range in which methanol should be added as a source of reducing power and energy.

Notably, the observed increase in OD₆₀₀ with increasing methanol concentration up to 667 mM contradicts the results of a FBA which indicated that the concentration of methanol needs to be only ninefold higher than that of acetate (assuming a representative doubling time of 30–40 hr). This can be explained by noting, as

mentioned above, that methanol oxidation is thermodynamically unfavorable, such that very high concentrations, well beyond the stoichiometric requirements, are needed to push the reaction forward. Therefore, growth with methanol is expected to slow down and stop well before it is being depleted. Yet, it could still be the case, just as with formate, that the allocation of methanol-derived NADH is not optimal in the Δlpd strain (i.e., overrespiration at the expense of acetate assimilation), such that methanol remains the limiting substrate even at concentrations well above ninefold that of acetate (>200 mM).

Due to the unfavorable thermodynamics, methanol oxidation depends on the presence of a strong metabolic sink for formaldehyde. As mentioned above, in *E. coli*, the glutathione system serves to metabolize formaldehyde via the activity of S-(hydroxymethyl)glutathione dehydrogenase and S-formylglutathione hydrolase, encoded by *frmA* and *frmB*, respectively (Gutheil et al., 1997). We speculated that deletion of these two genes would abolish growth with methanol due to the accumulation of formaldehyde, which would halt methanol oxidation and, by reacting and modifying macromolecules, would reduce cell viability (Metz et al., 2004). Indeed, a $\Delta lpd \Delta frmRAB$ strain overexpressing *BsMDH* was not able to grow on acetate and methanol (Figure 5a), indicating that the metabolic sink for formaldehyde is required for methanol to serve as an electron source.

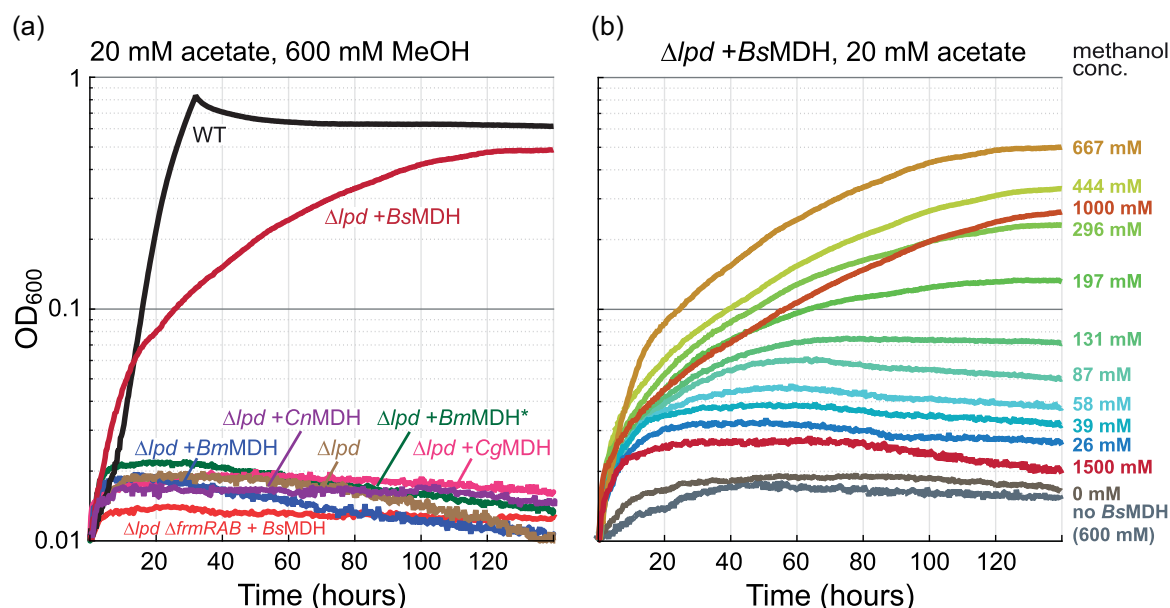


FIGURE 5 Expression of an NAD-dependent MDH enables the *Δlpd* strain to grow on acetate and methanol. (a) NAD-dependent methanol dehydrogenases from different organisms were expressed in the *Δlpd* strain from a plasmid. The different strains were tested for growth on 20 mM acetate and 600 mM methanol in 96-well plates. Only BsMDH enabled growth. Upon deletion of the glutathione-dependent formaldehyde-oxidation system (*frmRAB*), growth on acetate and methanol was abolished. (b) The *Δlpd* strain expressing BsMDH was cultivated on 20 mM acetate and methanol at a concentration ranging from 26 to 1,500 mM (increasing stepwise by a factor of 1.5). The final OD₆₀₀ of the culture increased with increasing methanol concentration up to a methanol concentration of 667 mM. Higher methanol concentrations impaired growth. Experiments were conducted within 96-well plates and were performed in triplicates, which displayed identical growth curves ($\pm 5\%$), and hence were averaged. All experiments (in triplicates) were repeated three times, which showed highly similar growth behavior. BsMDH, *Bacillus stearothermophilus* MDH; BmMDH, *Bacillus methanolicus* MDH; BmMDH*, engineered *Bacillus methanolicus* MDH; CgMDH, *Corynebacterium glutamicum* MDH; CnMDH, *Cupriavidus necator* N-1 MDH [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

In this study, we demonstrated the applicability of an *E. coli* strain auxotrophic to reducing power and energy to serve as a platform to test and compare different enzymatic systems for NADH regeneration. This platform uses a simple readout—cell growth—to replace laborious enzymatic assays and measurements of protein expression, folding and stability, each of which provides only partial information on the *in vivo* suitability of the regeneration system. While in this study we focused on comparing FDH and MDH variants, this strain can be easily used to test various other NADH regeneration systems, based on both organic and inorganic electron donors. For example, higher chain alcohols, such as isopropanol, which cannot be assimilated into biomass (at least in *E. coli*), can be tested and used as reducing power source. Similarly, phosphite dehydrogenase can be tested for its ability to use the inorganic compound phosphite as an *in vivo* electron donor (Vrtis, White, Metcalf, & van der Donk, 2001).

The *Δlpd* strain should be considered as auxotrophic to reducing power rather than to NADH alone since the strain can be regarded also as auxotrophic to NADPH, the regeneration of which ultimately relies on the external electron donor. Biosynthesis of NADPH in the *Δlpd* strain using formate or methanol as electron donors probably takes place mainly via the membrane-bound transhydrogenase (PntAB), which transfers electrons from NADH to NADP⁺ at the

expense of a proton imported from the periplasm to the cytosol (Sauer, Canonaco, Heri, Perrenoud, & Fischer, 2004). A reverse electron transfer could also take place, that is, electrons derived from the external electron donor to regenerate NADPH could be transferred to produce NADH via the soluble transhydrogenase (SthA; Sauer et al., 2004). Hence, our “biosensor” strain cannot effectively differentiate between enzymes that regenerate NADH and those producing NADPH, as both could relieve its auxotrophy to reducing power. If such distinction is required, further deletion of *sthA* would be necessary, such that enzymes that transfer electrons primarily to NADP⁺ could not support the growth of the *Δlpd* strain.

Identifying and utilizing efficient enzymatic systems for the *in vivo* regeneration of NAD(P)H can improve microbial growth and bioproduction. This is in line with the auxiliary substrate concept (Babel, 2009). For example, addition of formate was found to improve the growth of *Hansenula polymorpha* on glucose (Babel, Müller, & Markuske, 1983), *Vibrio natriegens* on glucose (Linton, Griffiths, & Gregory, 1981), and *Paracoccus Denitrificans* on mannitol (Van Verseveld & Stouthamer, 1980). Furthermore, addition of formate to *Penicillium chrysogenum* growing on glucose was shown to increase the volumetric productivity of penicillin (Harris, van der Krogt, van Gulik, van Dijken, & Pronk, 2007). While these studies were based on native FDHs to extract reducing power from formate, our study shows that by choosing an appropriate FDH variant and formate

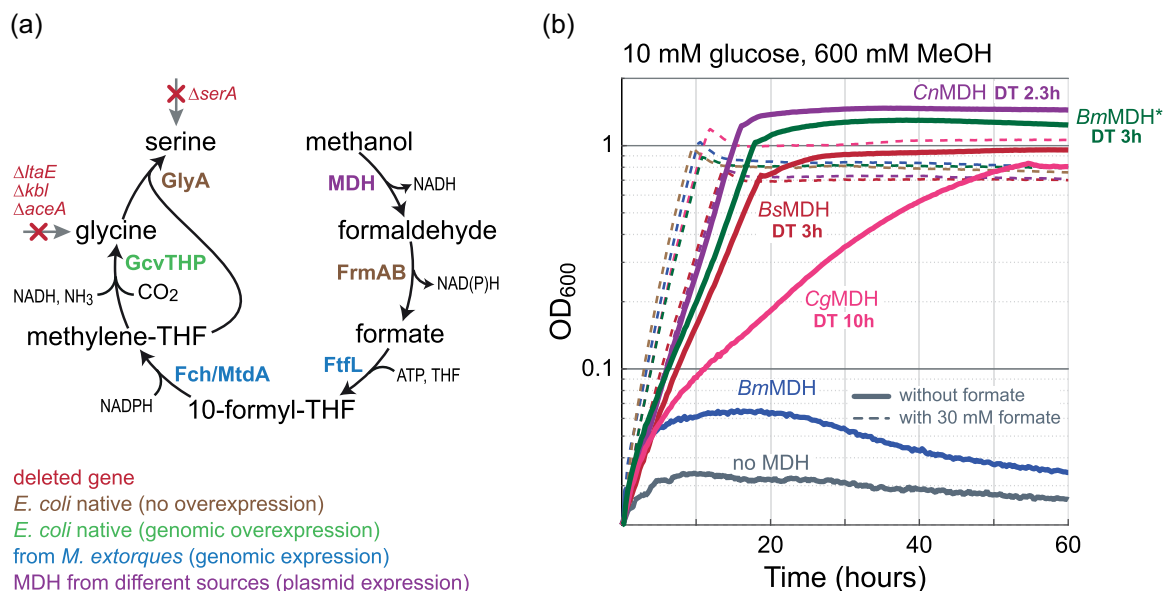


FIGURE 6 Most MDH variants are active in vivo. All methanol dehydrogenase variants were expressed and tested in a strain that requires a lower methanol oxidation flux to enable its growth. (a) The gC₁M–gC₂M strain (Kim et al., 2020), deleted in the endogenous glycine and serine biosynthesis routes ($\Delta serA$ ΔtaE Δkbl $\Delta aceA$), overexpresses *Methylobacterium extorques* enzymes that convert formate to methylene-THF (FtfL, Fch, and MtdA) as well as the endogenous enzymes of the glycine cleavage system. Upon methanol oxidation by MDH and conversion of formaldehyde to formate via the glutathione system (FrmAB), the strain could assimilate formate and CO₂ to produce glycine and serine (Kim et al., 2020). Therefore, an active MDH is expected to enable the growth of the strain with methanol. (b) The strain expressing the different MDH variants was tested for growth on 10 mM glucose and 600 mM methanol in a 96-well plate at 10% CO₂. CnMDH, BsMDH, BmMDH*, and CgMDH restored growth of the strain, although the later variant supported a low growth rate. Upon further addition of 30 mM formate, all strains showed comparable growth, indicating that the failure of BmMDH to support growth is not related to protein burden or accumulation of formaldehyde. Experiments were conducted within 96-well plates and were performed in triplicates, which displayed identical growth curves ($\pm 5\%$), and hence were averaged. Doubling times (DT) are shown in the figure. All experiments (in triplicates) were repeated five times, which showed highly similar growth behavior. BsMDH, *Bacillus stearothermophilus* MDH; BmMDH, *Bacillus methanolicus* MDH; BmMDH*, engineered *Bacillus methanolicus* MDH; CgMDH, *Corynebacterium glutamicum* MDH; CnMDH, *Cupriavidus necator* N-1 MDH [Color figure can be viewed at wileyonlinelibrary.com]

concentrations, it should be possible to implement a similar approach in *E. coli*, where addition of formate, or another electron donor, enhances growth and bioproduction.

Establishing efficient in vivo regeneration of NAD(P)H is central to the ongoing efforts to achieve autotrophic growth of *e. coli* (Antonovsky et al., 2016; Gleizer et al., 2019; Mattozzi et al., 2013). The identification of the most active FDH and MDH variants in this bacterium, as well as the determination of optimal concentrations of formate and methanol, could assist in supporting carbon fixation, whether via the Calvin Cycle (Antonovsky et al., 2016), the 3-hydroxypropionate bicycle (Mattozzi et al., 2013), or any other route. Similarly, recent efforts to establish *E. coli* growth on formate or methanol (Chou, Clomburg, Qian, & Gonzalez, 2019; He, Edlich-Muth, Lindner, & Bar-Even, 2018; Kim et al., 2020; Lu et al., 2019; Meyer et al., 2018; Siegel et al., 2015; Wang et al., 2017; Yang et al., 2019; Yishai, Bouzon, Doring, & Bar-Even, 2018) would greatly benefit from the information gathered in this study, showing how these feedstocks—besides assimilated by the synthetic pathways—can efficiently provide reducing power and energy for cell growth.

The results obtained here are specific to the cellular environment of *E. coli*. In other microorganisms, different FDH and MDH variants might show higher activity, and different concentration ranges of formate and methanol might prove optimal. Therefore, the analysis we demonstrated here should be repeated in each organism of interest. This probably holds true for the optimization of other metabolic systems as well. Rather than solely relying on in vitro measurements, the efficiency of enzymatic transformations should always be tested, compared, and optimized within dedicated cellular systems with a simple readout.

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ORCID

Arren Bar-Even  <http://orcid.org/0000-0002-1039-4328>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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