

# Metabolic Engineering of an ATP-Neutral Embden-Meyerhof-Parnas Pathway in *Corynebacterium glutamicum*: Growth Restoration by an Adaptive Point Mutation in NADH Dehydrogenase

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*Corynebacterium glutamicum* uses the Embden-Meyerhof-Parnas pathway of glycolysis and gains 2 mol of ATP per mol of glucose by substrate-level phosphorylation (SLP). To engineer glycolysis without net ATP formation by SLP, endogenous phosphorylating NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was replaced by nonphosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GapN) from *Clostridium acetobutylicum*, which irreversibly converts glyceraldehyde-3-phosphate (GAP) to 3-phosphoglycerate (3-PG) without generating ATP. As shown recently (S. Takeno, R. Murata, R. Kobayashi, S. Mitsuhashi, and M. Ikeda, Appl Environ Microbiol 76:7154–7160, 2010, <http://dx.doi.org/10.1128/AEM.01464-10>), this ATP-neutral, NADPH-generating glycolytic pathway did not allow for the growth of *Corynebacterium glutamicum* with glucose as the sole carbon source unless hitherto unknown suppressor mutations occurred; however, these mutations were not disclosed. In the present study, a suppressor mutation was identified, and it was shown that heterologous expression of *udhA* encoding soluble transhydrogenase from *Escherichia coli* partly restored growth, suggesting that growth was inhibited by NADPH accumulation. Moreover, genome sequence analysis of second-site suppressor mutants that were able to grow faster with glucose revealed a single point mutation in the gene of non-proton-pumping NADH:ubiquinone oxidoreductase (NDH-II) leading to the amino acid change D213G, which was shared by these suppressor mutants. Since related NDH-II enzymes accepting NADPH as the substrate possess asparagine or glutamine residues at this position, D213G, D213N, and D213Q variants of *C. glutamicum* NDH-II were constructed and were shown to oxidize NADPH in addition to NADH. Taking these findings together, ATP-neutral glycolysis by the replacement of endogenous NAD-dependent GAPDH with NADP-dependent GapN became possible via oxidation of NADPH formed in this pathway by mutant NADPH-accepting NDH-II<sup>D213G</sup> and thus by coupling to electron transport phosphorylation (ETP).

ATP generation occurs naturally by substrate-level phosphorylation (SLP), electron transport phosphorylation (ETP), photophosphorylation, and decarboxylation phosphorylation. The Embden-Meyerhof-Parnas (EMP) pathway of glycolysis supplies ATP by SLP in the absence of terminal electron acceptors or anaerobic conditions, as well as supplying direct precursors for biomass formation (1). Bacteria and archaea possess one or more of multiple biologically feasible routes for glucose catabolism, such as the EMP pathway, the Entner-Doudoroff (ED) pathway, and the phosphoketolase pathway, which yield zero to 3 ATP molecules per glucose molecule and exist in different variants. In natural habitats, a trade-off between the rate and the yield of ATP production is observed for heterotrophic organisms; faster but less efficient ATP production may be advantageous under conditions characterized by a low abundance of growth substrates (2, 3). For example, *Zymomonas mobilis* ferments sugars by the ED pathway with a net production of 1 mol of ATP per mol of glucose and requires 2- to 5-fold less energy to synthesize ED pathway enzymes than is required to accomplish the same glycolytic flux in the EMP pathway (3). The ED pathway flux in *Z. mobilis* and the specific ethanol productivity are higher than those in yeast. The glycolytic flux in *Saccharomyces cerevisiae* could not be increased by the introduction of the ED pathway, due to the lack of activity of the Fe-S cluster enzyme 6-phosphogluconate dehydratase (4). Overexpression of a plant gene encoding NADP-dependent nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (GapN) in *Escherichia coli*  $\Delta gapA$ , which lacks phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH), led to a strain

with functional glycolysis devoid of SLP (5). Recently, in the *E. coli*  $\Delta gapA$  strain, overexpression of NADP-dependent GapN from *Streptococcus mutans* led to increased levels of transhydrogenase (encoded by *udhA*), which generates NADH from NADPH, and decreased expression of the pentose phosphate pathway and of Krebs cycle genes to sustain energy levels. *E. coli* with  $\Delta gapA::gapN$  and plasmid-dependent expression of *gapN* (pTrc*gapN*) produced 22% higher levels of acetic acid to increase ATP levels by using the *ack-pta* system (6). The introduction of the phosphoketolase pathway into *Corynebacterium glutamicum* bypassed the pyruvate dehydrogenase reaction, which led to enhanced L-glutamate production and reduced CO<sub>2</sub> emission (7).

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*Corynebacterium glutamicum* is a workhorse of industrial biotechnology with a GRAS (generally recognized as safe) status and has been used for the production of L-glutamine and L-lysine in recent decades. The product spectrum of *C. glutamicum* has been widened for the overproduction of different amino acids (8–10), carotenoids (11, 12), alcohols (13), organic acids (14), glycolic acid (15) and diamines (16). *C. glutamicum* grows aerobically on a wide variety of carbon sources, including the sugars glucose, fructose, and sucrose, as well as organic acids, such as citrate, acetate, pyruvate, D-lactate, and L-lactate. Significant efforts have been focused on engineering *C. glutamicum* to utilize starch (17), glucans (18), crude glycerol (19), amino sugars (20, 21), and pentose sugars present in lignocellulosic hydrolysates (22), and the disaccharide cellobiose (23). However, glucose, fructose, and sucrose present in molasses or derived from starch hydrolysis are the main substrates used in industrial fermentations (24).

In *C. glutamicum*, glucose, fructose, and sucrose are imported and phosphorylated by the phosphoenolpyruvate-dependent carbohydrate:phosphotransferase system (PTS) and enter the EMP pathway as glucose-6-phosphate or fructose 1,6-bisphosphate (25, 26). As is typical, the EMP pathway yields 2 mol of ATP per mol of glucose in *C. glutamicum*. In addition, *C. glutamicum* can synthesize ATP by SLP in the tricarboxylic acid (TCA) cycle and by the conversion of acetyl coenzyme A (acetyl-CoA) to acetate by acetate kinase (27). Since oxygen (and nitrate) serves as a terminal electron acceptor in its respiratory energy metabolism, *C. glutamicum* generates ATP by ETP (28). By SLP and ETP with the cytochrome *bc<sub>1</sub>-aa<sub>3</sub>* branch, complete aerobic oxidation of glucose and acetate yields 26.7 and 7.3 mol of ATP, respectively (29, 30). ETP has been shown to be essential for growth with substrates that do not allow ATP generation by SLP (e.g., acetate); however, *C. glutamicum* mutants devoid of ETP grew with substrates allowing SLP, such as glucose (31). Glucose catabolism by *C. glutamicum*  $\Delta F_1F_0$  was slow and biphasic under oxygen-limiting conditions. In the first growth phase, ATP was generated via SLP in glycolysis, while the second phase was characterized by the formation of organic acids such as acetate, and ATP was apparently generated by SLP via an acetate kinase reaction (31).

In the EMP pathway, SLP commences by a GAPDH catalyzing reaction that oxidizes glyceraldehyde-3-phosphate (GAP) to 1,3-bisphosphoglycerate (1,3-bPG) with simultaneous reduction of NAD to NADH. *C. glutamicum* possesses two GAPDH homologues encoded by *gapA* and *gapB*, respectively. 3-Phosphoglycerate kinase (3-PGK) uses a special “energy-rich” intermediate, 1,3-bisphosphoglycerate, to produce an additional 1 mol of ATP for a net balance of 2 mol of ATP per mol of glucose (32). Homodimeric 3-PGK in *C. glutamicum* is tightly regulated by the ADP concentration ( $K_p \approx 100 \mu\text{M}$ ) and plays a critical role in gluconeogenesis as well as being essential for glycolysis (33). To study the role of the absence of SLP in the glycolysis of *C. glutamicum*, SLP via 3-PGK was bypassed by replacing endogenous GAPDH with GapN. GapN catalyzes the irreversible oxidation of glyceraldehyde-3-phosphate to 3-phosphoglycerate (3-PG), bypassing SLP via 3-PGK in glycolysis (34). This ATP-neutral, NADPH-generating glycolytic pathway allowed for growth with glucose only if second-site suppressor mutations occurred (35). Unfortunately, the nature of the compensatory mutation(s) was not reported (35). The present study identifies a single point mutation in the gene of non-proton-pumping NADH:ubiquinone oxidoreductase (NDH-II) that enables *C. glutamicum* strains with

ATP-neutral glycolysis (no net ATP formation from glucose by SLP) to grow with glucose as the sole carbon source.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** The strains and plasmids used in this study are listed in Table 1. *C. glutamicum* strains were precultured in lysogeny broth (LB) medium (36), with antibiotics added when appropriate. *E. coli* strain DH5 $\alpha$  (37) was used as the host for cloning and heterologous expression. For growth experiments, exponentially growing cells of brain heart infusion (BHI) precultures (50 ml) were harvested by centrifugation (3,200  $\times$  g, 10 min) and were washed in CgXII medium (38) without a carbon source. Cultures in 50 ml of CgXII medium containing 4% (wt/vol) glucose, 100  $\mu\text{g/ml}$  spectinomycin or 25  $\mu\text{g/ml}$  kanamycin, and 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) were inoculated to a final optical density at 600 nm ( $\text{OD}_{600}$ ) of 1 and were incubated in 500-ml baffled shake flasks at 30°C. The  $\text{OD}_{600}$  was measured in dilutions resulting in an  $\text{OD}_{600}$  between 0.05 and 0.25 by using a Shimadzu (Duisburg, Germany) UV-1202 spectrophotometer. For the determination of enzymatic activity in cell extracts, cells were grown in LB medium to mid-exponential phase ( $\text{OD}_{600}$ , 3.5 to 4), harvested by centrifugation (for 10 min at 3,200  $\times$  g and 4°C), and washed in 100 mM triethanolamine hydrochloride (TEA-Cl) buffer (pH 7.4) or potassium phosphate buffer (KPB) (pH 7.5). The cells were stored at  $-20^\circ\text{C}$  until use. Cultivation was always performed in triplicate. Growth was monitored by measuring the OD at 600 nm using a V-1200 spectrophotometer (VWR, Radnor, PA, USA). For the screening of growth conditions, cells were grown in 48-well flower plates using the BioLector microfermentation system (m2p-labs GmbH, Aachen, Germany). One milliliter of medium was used per well with a shaking frequency of 1,100 rpm. Biomass formation was measured as backscattered light intensity sent at 620 nm with a signal gain factor of 20.

**DNA preparation, manipulation, and transformation.** Standard protocols were used for plasmid isolation, molecular cloning and transformation of *E. coli*, and electrophoresis (39). Chromosomal DNA and plasmids were isolated from *C. glutamicum* as described previously (40). Electroporation for the transformation of plasmids into *C. glutamicum* was performed as described previously (41). PCR amplifications were performed in a FlexCycler device (Analytik Jena) with *Taq* DNA polymerase (MBI Fermentas) or KOD DNA polymerase (Novagen) and the oligonucleotides listed in Table 2. All restriction enzymes and shrimp alkaline phosphatase were obtained from New England BioLabs, while T4 DNA ligase was obtained from Roche Diagnostics GmbH, and all were used according to the manufacturers’ instructions.

**Construction of *C. glutamicum* mutant strains.** For the deletion of *gapA* or *gapB*, a 1.6-kb insert with internal fragments of the genes was cloned into *Sma*I-digested pK19*mobsacB* (42), which is not replicable in *C. glutamicum*. The resulting vectors, pK19*mobsacBgapA* and pK19*mobsacBgapB*, were used to delete an internal fragment of chromosomal *gapA* and *gapB*, respectively. The deletion of the chromosomal *gapA* and *gapB* loci was verified by PCR amplification using the verification primers. The *gapA* and *gapB* mutants were designated *C. glutamicum*  $\Delta gapA$  (43), *C. glutamicum*  $\Delta gapB$ , and *C. glutamicum*  $\Delta gapAB$  (GSM0). The other strains used for this study are listed in Table 1.

**Construction of expression vectors.** For IPTG-inducible overexpression, the pEKEx3 and pVWEx1 vectors were used. The *gapN* and *udhA* (b3962) genes were amplified via PCR from genomic DNA of wild-type (WT) *Clostridium acetobutylicum* or *E. coli* MG1655 by using the oligonucleotide primers listed in Table 2. For the overexpression of *gapA*, *gapB*, and *ndh*, the genes were amplified via PCR from genomic DNA of WT *C. glutamicum* using the oligonucleotide primers listed in Table 2. The PCR products of *gapA* and *gapB* were cloned into the *Sma*I-restricted blunt-end vector pEKEx3, resulting in pEKEx3-*gapA* and pEKEx3-*gapB*, respectively. *ndh* and its mutants were cloned into the *Xba*I-restricted vector pVWEx1, and the resulting plasmids are listed in Table 1. The integrity of the constructs was confirmed by sequencing.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Function and relevant characteristic(s)	Source or reference
<b>Strains</b>		
<i>E. coli</i> DH5 $\alpha$	General cloning host [ $F^-$ <i>thi-1 endA1 hsdR17</i> ( $r_K^-$ $m_K^-$ ) <i>supE44 <math>\Delta</math>lacU169 <math>\lambda^-</math> <math>\phi</math>80d<math>\Delta</math>lacZ<math>\Delta</math>M15 <i>recA1 gyrA96 relA1</i>]</i>	35
<i>C. glutamicum</i>		
ATCC 13032	WT strain, auxotrophic for biotin	This work
$\Delta$ <i>gapA</i> strain	In-frame deletion of the <i>gapA</i> gene of the WT strain	This work
$\Delta$ <i>gapAB</i> strain	In-frame deletion of the <i>gapA</i> and <i>gapB</i> genes of the WT strain	This work
$\Delta$ <i>ndh</i> strain	In-frame deletion of the <i>ndh</i> gene of the WT strain	This work
GSM0	In-frame deletion of the <i>gapA</i> and <i>gapB</i> genes of the WT strain with pEKEx3- <i>gapN</i> <sup>Cac</sup> overexpression	This work
GSM1/2/3/4	GSM0 strain with evolved <i>ndh</i> (D213G)	This work
<b>Plasmids</b>		
pEKEx3	Spec <sup>r</sup> ; <i>C. glutamicum</i> - <i>E. coli</i> shuttle vector ( <i>P</i> <sub>lac</sub> <sup>q</sup> ; <i>pBL1 oriV</i> <sub>Cg</sub> <i>oriV</i> <sub>Ec</sub> )	48
pEKEx3- <i>gapA</i> <sup>Cg</sup>	Derived from pEKEx3, for regulated expression of <i>C. glutamicum gapA</i>	43
pEKEx3- <i>gapB</i> <sup>Cg</sup>	Derived from pEKEx3, for regulated expression of <i>C. glutamicum gapB</i>	This work
pEKEx3- <i>gapN</i> <sup>Cac</sup>	Derived from pEKEx3, for regulated expression of <i>Clostridium acetobutylicum gapN</i>	This work
pVWEx1	Kan <sup>r</sup> <i>P</i> <sub>lac</sub> <i>lacI</i> <sup>q</sup>	This work
pVWEx1- <i>udhA</i> <sup>Ec</sup>	Derived from pVWEx1, for regulated expression of <i>E. coli udhA</i>	This work
pVWEx1- <i>ndh</i> <sup>Cg</sup>	Derived from pVWEx1, for regulated expression of <i>C. glutamicum ndh</i>	This work
pVWEx1- <i>ndh</i> <sup>D213G</sup>	Derived from pVWEx1, for regulated expression of <i>C. glutamicum ndh</i>	This work
pVWEx1- <i>ndh</i> <sup>D213N</sup>	Derived from pVWEx1, for regulated expression of <i>C. glutamicum ndh</i>	This work
pVWEx1- <i>ndh</i> <sup>D213Q</sup>	Derived from pVWEx1, for regulated expression of <i>C. glutamicum ndh</i>	This work
pK19 <i>mobsacB</i>	Km <sup>r</sup> ; <i>E. coli</i> - <i>C. glutamicum</i> shuttle vector for construction of insertion and deletion mutants in <i>C. glutamicum</i> ( <i>pK18 oriV</i> <sub>Ec</sub> <i>sacB lacZ<math>\alpha</math></i> )	This work
pK19 <i>mobsacB</i> $\Delta$ <i>gapA</i>	pK19 <i>mobsacB</i> with a <i>gapA</i> deletion construct	This work
pK19 <i>mobsacB</i> $\Delta$ <i>gapB</i>	pK19 <i>mobsacB</i> with a <i>gapB</i> deletion construct	This work

**Genome sequence analysis.** Genomic DNA was isolated, and libraries were prepared, as described previously (44). Libraries were sequenced on a Genome Analyzer IIX platform (Illumina, San Diego, CA, USA) by using a Single-Read Cluster Generation kit (version 4) according to the manufacturer's instructions. The SARUMAN program (45) was used for the mapping of 32-bp sequence reads to the genome sequence of *C. glutami-*

*cum* (46). The coverage was obtained by multiplying the respective read start by the read length. A Perl programming language script was implemented for parsing of the read start information and calculation of read start numbers and coverage.

**Amino acid, glucose, and organic acid determination.** Extracellular amino acids, carbohydrates, and organic acids were quantified by means

TABLE 2 Sequences of oligonucleotide primers

Name	Sequence (5'-3')	Function and relevant characteristic(s) <sup>a</sup>
<i>gapA</i> _Del_A	GGCTGATCCTCAAATGACCAAG	Del of <i>gapA</i>
<i>gapA</i> _Del_B	CCCATCCACTAAACTTAAACAACCAACACGAATGGTCATGTTG	Del of <i>gapA</i>
<i>gapA</i> _Del_C	TGTTTAAAGTTTGTGGATGGGCTGCGTCTGACCGAGCTCGTAG	Del of <i>gapA</i>
<i>gapA</i> _Del_D	CACCGAAGCCGTCAGAAACGAATG	Del of <i>gapA</i>
<i>gapA</i> _Del_Seq	GTTCGTTCCCTGCAAAAATATTAG	Del of <i>gapA</i>
<i>gapA</i> _Del_Ver_fw	CCAACCTCGACGATGCCAATC	Verification of <i>gapB</i> deletion
<i>gapA</i> _Del_Ver_rv	CTCTGGTGATTCTGCGATCTTTTC	Verification of <i>gapB</i> deletion
<i>gapB</i> _Del_A	GACGGTGACCAATCCGGAG	Del of <i>gapB</i>
<i>gapB</i> _Del_B	CCCATCCACTAAACTTAAACACTTGTGGTTGTGCGTCATAAAAAGT	Del of <i>gapB</i>
<i>gapB</i> _Del_C	TGTTTAAAGTTTGTGGATGGGTTGTACCCGAGCGCAGGCAG	Del of <i>gapB</i>
<i>gapB</i> _Del_D	GCCACAATATTGGCTTTGAGGTTG	Del of <i>gapB</i>
<i>gapB</i> _Del_Seq	CTCACTTAACCGGAGATCTTGGAC	Del of <i>gapB</i>
<i>gapB</i> _Del_Ver_fw	GATTGAGCAATGGGTGGGAG	Verification of <i>gapB</i> deletion
<i>gapB</i> _Del_Ver_rv	GATGACAGTGCACGATCATCATG	Verification of <i>gapB</i> deletion
<i>gap</i> _Cacet_fw	GATCTAGAGAAAGGAGGCCCTTCAGATGTTTAAAAATATATCATCAAATGGAGTT TATAAAAATC	OE of <i>C. acetobutylicum gapN</i> ; start RBS
<i>gap</i> _Cacet_rv	GATCTAGATTATAGGTTTAAACTATTGATTTATGCCTTGTG	OE of <i>C. acetobutylicum gapN</i>
<i>ndh</i> -fw	CTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCAGATGTGCAAGTAAACCAACCCG	OE of <i>ndh</i>
<i>ndh</i> -rev	CGGTACCCGGGGATCTTACTTTCCGCTGAAACGCTG	OE of <i>ndh</i>
<i>udhA</i> _fw	GCTCTAGAGAAAGGAGGCCCTTCAGATGCCACATTCCTACGATTACG	OE of <i>udhA</i>
<i>udhA</i> _rev	GCTCTAGATTAATAACAGCGGTTTAAACCG	OE of <i>udhA</i>

<sup>a</sup> Abbreviations: OE, overexpression; Del, deletion; RBS, ribosomal binding site.

TABLE 3 Specific activities of GAPDH and GapN in various strains

<i>C. glutamicum</i> strain	Sp act (nmol/min/mg protein) of <sup>a</sup> :	
	GAPDH	GapN
WT	90 ± 1	<5
GSM0	<5	31 ± 1
GSM1	<5	31 ± 1

<sup>a</sup> Cell extracts were obtained from the indicated *C. glutamicum* strains cultivated in BHI medium with 1 mM IPTG and 100 µg ml<sup>-1</sup> spectinomycin. Averages and standard deviations for triplicate cultivations are shown.

of high-pressure liquid chromatography (1200 series; Agilent Technologies Deutschland GmbH, Böblingen, Germany). Cell culture extracts were centrifuged (13,000 × g, 10 min), and the supernatant was used for analysis. For the detection of amino acids, samples were derivatized with *ortho*-phthalaldehyde (OPA), separated on a system consisting of a pre-column (LiChrospher 100 RP-18 EC [particle size, 5 µm; length, 40 mm; inside diameter, 4 mm]; CS-Chromatographie Service GmbH, Langerwehe, Germany) and a main column (LiChrospher 100 RP-18 EC [particle size, 5 µm; length, 125 mm; inside diameter, 4 mm]; CS-Chromatographie), and detected with a G1321A fluorescence detector (FLD) (1200 series; Agilent Technologies). L-Asparagine was used as an internal standard. For the detection of carbohydrates and organic acids, the analyte was separated with a column for organic acids (length, 300 mm; inside diameter, 8 mm; particle size, 10 µm; pore diameter, 25 Å; CS-Chromatographie), and a G1362A refractive index detector (RID) (1200 series; Agilent Technologies) was used. Derivatization and quantification were carried out as described previously (47).

**Measurement of enzyme activities.** For determination of the specific activity of glyceraldehyde-3-phosphate dehydrogenase, cells were harvested by centrifugation (3,220 × g, 4°C, 10 min), washed in the appropriate buffer, and stored at -20°C until use. Cells were resuspended in 1 ml of the buffer, and cell extracts were prepared by sonication as described previously (48). All enzyme activity measurements were carried out at 30°C. Protein concentrations were determined with bovine serum albumin as the standard using Bradford (49) reagents (Sigma, Taufkirchen, Germany). Glyceraldehyde 3-phosphate dehydrogenase (GapA) activity was measured according to the method of Omumasaba et al. (50), modified as follows. The assay mixture contained 1 mM NAD, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.2 mM EDTA, and 2.5 mM glyceraldehyde-3-phosphate in 50 mM TEA-Cl buffer (pH 8.5). One unit of enzyme activity corresponds to 1 µmol NADH formed per min. GapN activity was measured by adding the enzyme to the assay mixture containing 50 mM Tricine buffer (pH 8.5), 1 mM NADP, and 1 mM glyceraldehyde-3-phosphate (GAP) at 30°C. The variation in absorbance at 340 nm was followed (51). Transhydrogenase activity (UdhA) was measured at 375 nm and 30°C in a mixture containing 50 mM Tris-HCl (pH 7.6), 2 mM MgCl<sub>2</sub>, 500 µM NADPH, 1 mM 3-acetyl pyridine adenine dinucleotide, and 10 to 100 µl crude cell extract. The specific activity was then determined by dividing the measured slope by the protein concentration (52).

The NAD(P)H oxidase activity of NADH:ubiquinone oxidoreductase

was measured at 30°C in a reaction mixture consisting of appropriate amounts of the enzyme (10 µl), 50 mM KPB, and 50 µM Q<sub>1</sub> dissolved in dimethyl sulfoxide, as well as 0.2 mM NADH/NADPH. Activity was measured spectrophotometrically at 340 nm by following the decrease in the NADH/NADPH concentration at 30°C. The reaction was started by the addition of 50 µM coenzyme Q<sub>1</sub> solution. The amount of enzyme oxidizing 1 mmol of NADH or NAD(P)H per min was defined as 1 unit, where a millimolar extinction coefficient of 6.2 was used for the calculation (53). Enzymatic kinetics was analyzed by fitting the data to the Michaelis-Menten equation using the nonlinear regression equation  $V_i = (V_{max} \cdot [S]) / (K_m + [S])$ , where  $V_i$  is the initial velocity and  $[S]$  is the concentration of the substrate.

## RESULTS

**Design of a *C. glutamicum* strain with ATP-neutral glycolysis.** Gene deletion mutants devoid of GAPDH activity (GapA and/or GapB) were constructed to avoid SLP in the subsequent reaction catalyzed by 3-PGK. The deletion was confirmed by enzyme activity measurements; WT GapA has a specific activity of 0.09 ± 0.01 U/mg, and strain GSM0 showed no detectable activity (Table 3). The growth of the *C. glutamicum* WT,  $\Delta gapA$ ,  $\Delta gapB$ , and  $\Delta gapAB$  strains was compared in minimal medium with different carbon sources. In the absence of GapA, no growth in glucose minimal medium and impaired growth in pyruvate minimal medium were observed, while the  $\Delta gapB$  strain grew similarly to the WT strain. The double deletion mutant showed no growth with either glucose or pyruvate as the sole carbon source. Ectopic expression of *gapA* complemented the mutants lacking GapA (Table 4).

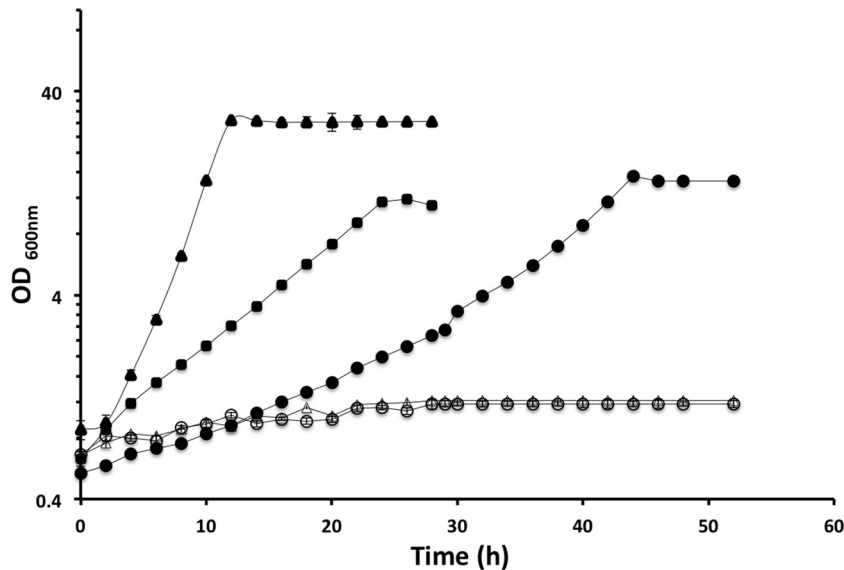
The *Clostridium acetobutylicum gapN* gene was expressed from the IPTG-inducible plasmid pEKEx3 in *C. glutamicum*  $\Delta gapAB$ , and the resulting strain was designated GSM0. In GSM0, the oxidation of glucose to pyruvate in the ATP-neutral variant of glycolysis should yield 2 mol of NADPH per mol of glucose, but neither NADH nor ATP, while WT *C. glutamicum* gains 2 mol of NADH and 2 mol of ATP per mol of glucose in glycolysis. GapN activity (30 ± 0.15 mU/mg) was observed in crude extracts of GSM0 but was absent from the control carrying an empty vector (Table 3). Crude extracts of both the  $\Delta gapAB$ (pEKEx3) and GSM0 strains lacked detectable GAPDH activity (<0.05 mU/mg) (Table 3). *C. glutamicum* GSM0 grew like the WT in BHI medium but did not grow in glucose minimal medium (Fig. 1).

**Influence of heterologous transhydrogenase on the growth of GSM0 in glucose minimal medium.** Since ATP-neutral glycolysis in strain GSM0 yields NADPH instead of NADH, we tested whether equilibrating NADPH and NADH by heterologous transhydrogenase enabled growth with glucose as the sole carbon source. For this purpose, the *udhA* gene encoding soluble transhydrogenase from *E. coli* (52) was expressed from expression vector

TABLE 4 Growth of various *C. glutamicum* strains on CgXII minimal medium agar plates with either glucose or pyruvate as the carbon source

Strain genotype	Growth <sup>a</sup> of the strain carrying the indicated vector with the following carbon source:							
	Glucose				Pyruvate			
	pEKEx3	pEKEx3- <i>gapA</i> <sup>Cg</sup>	pEKEx3- <i>gapB</i> <sup>Cg</sup>	pEKEx3- <i>gapN</i> <sup>Cac</sup>	pEKEx3	pEKEx3- <i>gapA</i> <sup>Cg</sup>	pEKEx3- <i>gapB</i> <sup>Cg</sup>	pEKEx3- <i>gapN</i> <sup>Cac</sup>
WT	+	+	+	+	+	+	+	+
$\Delta gapA$	-	+	-	-	+	+	+	+
$\Delta gapB$	+	+	+	+	+	+	+	+
$\Delta gapA \Delta gapB$	-	+	-	-	-	+	+	-

<sup>a</sup> +, growth; -, no growth.



**FIG 1** Growth of the *C. glutamicum* WT (filled triangles), GSM1 (filled squares),  $\Delta gapAB(pEKEEx3)(pVWEx1)$  (open circles), GSM0 [ $\Delta gapAB(pEKEEx3-gapN)$ ] (open triangles), and  $\Delta gapAB(pEKEEx3-gapN)(pVWEx1-udhA)$  (filled circles) strains in glucose minimal medium. All cultivations were carried out in 50 ml of CgXII with 100 mM glucose at 30°C in 500-ml Erlenmeyer flasks with shaking at 120 rpm. Averages and standard deviations for triplicate cultivations are shown.

pVWEx1. Crude extracts of *C. glutamicum* GSM0(pVWEx1-*udhA*) contained transhydrogenase with a specific activity of  $17 \pm 1 \text{ nmol} \cdot \text{min}^{-1} \text{ mg}^{-1}$ , while the control carrying an empty vector showed no detectable transhydrogenase activity ( $<5 \text{ nmol} \cdot \text{min}^{-1} \text{ mg}^{-1}$ ). *C. glutamicum* GSM0(pVWEx1-*udhA*) could grow in glucose minimal medium (Fig. 1). However, the growth of GSM0(pVWEx1-*udhA*) was much slower than that of the WT strain ( $0.06 \pm 0.00 \text{ h}^{-1}$  compared to  $0.35 \pm 0.01 \text{ h}^{-1}$ ), and a 2-fold-lower final OD was reached (Fig. 1). No organic acids were detected in supernatants, suggesting that the metabolic perturbations did not lead to carbon overflow. Taken together, these results indicate that NADPH formation in the engineered ATP-neutral glycolytic pathway prevented growth with glucose as the sole carbon source. Partial restoration of growth was possible when NADPH and NADH were equilibrated by heterologous transhydrogenase.

**Adaptive evolution of *C. glutamicum* strain GSM0.** To test if adaptive evolution can overcome the impairment of GSM0 growth in glucose minimal medium, incubation was prolonged for 5 to 6 days, and finally, overgrowth of the culture was observed. After plating onto a CgXII agar plate with 2% glucose, four colonies were selected for further experiments. When incubated in glucose minimal medium, all four colonies grew ( $\mu$ ,  $0.14 \pm 0.02 \text{ h}^{-1}$ ) (Fig. 2) and thus were designated suppressor mutants GSM1 to GSM4. Sequencing of *gapN* in the suppressor mutants revealed that *gapN* did not carry any mutation, a finding supported by enzyme activity measurements (Table 4). Accordingly, retransformation of plasmid pEKEEx3-*gapN* from GSM1 into *C. glutamicum*  $\Delta gapAB$  did not result in growth with glucose. Thus, one or more mutations present in the suppressor mutants may restore growth in glucose minimal medium.

Growth and biomass formation by suppressor mutant GSM1 with various carbon sources were compared to those of the WT. With glucose, the growth rate and final biomass concentrations observed for GSM1 were almost two times lower than those for the

WT (Fig. 1 and 2). The specific glucose consumption rates for the WT and GSM strains are  $0.78$  and  $1.1 \text{ mmol} \cdot \text{g (dry weight)}^{-1} \cdot \text{h}^{-1}$ , respectively. Similarly, the growth of GSM1 in minimal medium with fructose or sucrose was slower than that of the WT (Fig. 2 and 3). GSM1, GSM2, GSM3, and GSM4 showed no growth on acetate, which was expected, since the GapN reaction operates irreversibly in the glycolytic direction but not in the gluconeogenic direction (51).

**Genome sequencing of suppressor mutants.** Since the growth of the suppressor mutants in glucose minimal medium was not due to mutation of heterologous *gapN*, the genomes of the suppressor mutants GSM1 to GSM4 were sequenced and compared to the genome of the WT strain (see Table S1 in the supplemental material). Single point mutations (single nucleotide polymorphisms [SNPs]) and insertions or deletions (indels) relative to the WT genome were found (see Table S2 in the supplemental material). The relatively large numbers of SNPs and small deletions and insertions (about 200) are not unprecedented and have been found, e.g., in a recently constructed prophage-free variant of WT *C. glutamicum* (54). To identify the mutation(s) responsible for restoring growth in glucose minimal medium, all SNPs and indels in the suppressor mutants were compared with the genome of a strain derived from the same wild-type stock and used as a control. Only the following mutations affected genes and were common to all suppressor mutants isolated: a SNP in the *ndh* gene and the  $\Delta gapA$  and  $\Delta gapB$  deletions (see Table S2). The SNP in *ndh* (cg1656) encoding the non-proton pumping NADH:ubiquinone oxidoreductase NDH-II led to replacement of the aspartic acid residue at position 213 by glycine (*ndh*<sup>D213G</sup>). PCR amplification of this region from the WT strain and all four suppressor mutants, and subsequent sequence analysis, confirmed the observed point mutation in the suppressor mutants and its absence from the WT. It was also observed that GSM1 and GSM4 carried 9 base deletions in the gene for 30S ribosomal protein S1 (cg1531; *rpsA*) near the C terminus.

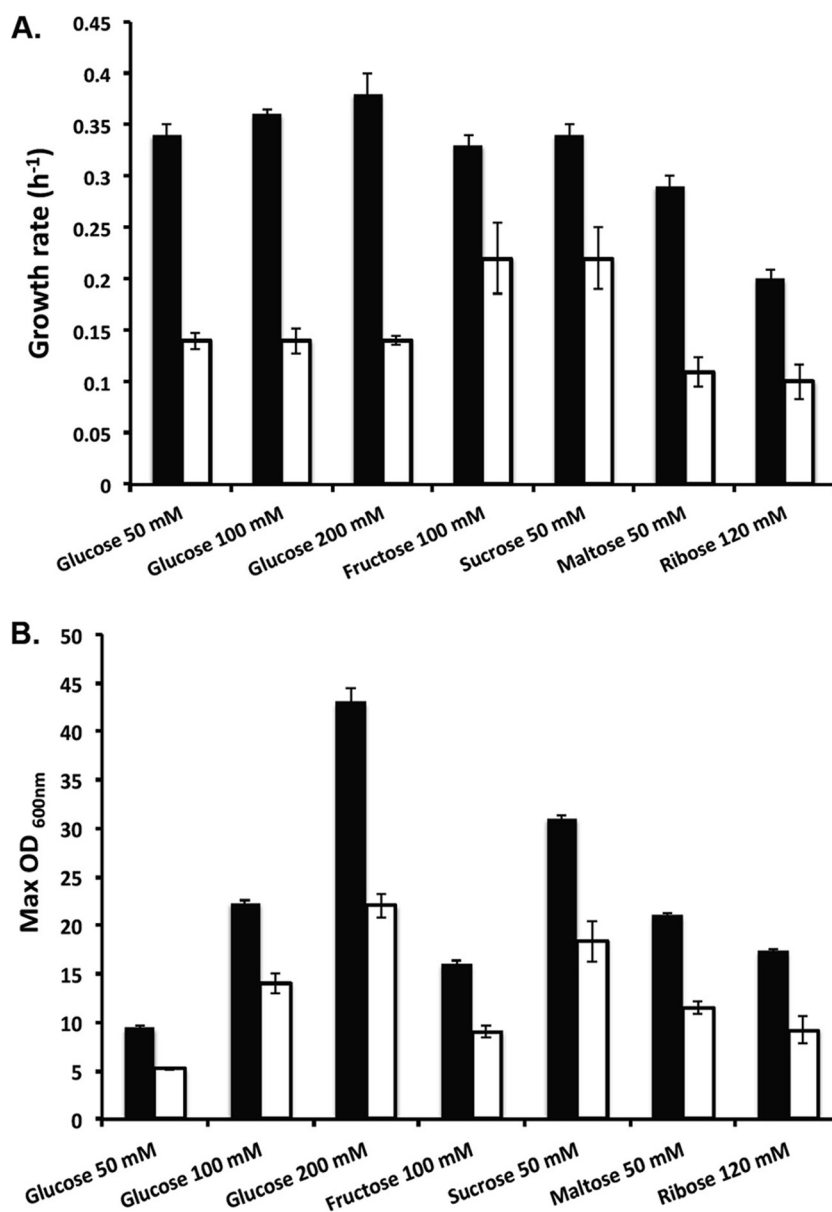


FIG 2 Analysis of the growth of the *C. glutamicum* WT (filled bars) and GSM1 (open bars) strains with different carbon sources. (A) Growth rates; (B) maximal OD<sub>600</sub>. Strains were cultivated in 50 ml of CgXII medium with different carbon sources. Average values and standard deviations for triplicate cultivations are shown.

**Consequences of the amino acid exchange D213G present in NDH-II of the suppressor mutants.** Two types of NDHs are known in nature, but *C. glutamicum* possesses only NADH:ubiquinone oxidoreductase (NDH-II) and lacks a proton-pumping NADH:ubiquinone oxidoreductase (NDH-I). NDH-II is a 55-kDa flavoprotein that couples the transfer of 2 electrons from NADH to ubiquinone without translocation of protons (29). NDH-II has two conserved flavin adenine dinucleotide (FAD) binding domains with a glycine-rich consensus sequence (GXGXXG) along with an NADH binding domain containing a negatively charged residue (D or E) at the end of the second  $\beta$ -sheet, which determines the nucleotide specificity (55). Previous studies revealed that NDH-II was solely responsible for NADH oxidation (NADH:ubiquinone oxidoreductase), with maxi-

mal activity at pH 6.5, whereas NADPH:ubiquinone oxidoreductase activity was detectable *in vitro* at pH 4.5 but negligible at pH 6.5 (53).

Since the D213G mutation may affect the nucleotide specificity of NDH-II enzymes, the protein sequences of biochemically characterized NADH-dependent and NADPH-dependent NDH-II enzymes were aligned (Fig. 3). NADPH-dependent NDH-II enzymes possess a neutral amino acid residue at position 213 (numbering according to *C. glutamicum* NDH-II); the *Solanum tuberosum* and *Neurospora crassa* enzymes, for example, have glutamine at this position (Fig. 3). NDH-II enzymes specific for NADH as a cofactor possess a charged aspartic acid (as in the wild-type NDH-II of *C. glutamicum*) or glutamic acid (as in the NDH-II enzymes of *S. cerevisiae* and *E. coli*) residue in position 213 (Fig. 3). Since the NDH-II enzymes of the evolved strains GSM1 to GSM4

<i>Corynebacterium</i>	AERERLLTFVVV <b>GAGPTG</b> VELAGQLAEMAHRTLAGEYKNFNTNSAKI ILL <b>D</b> GAPQV	NADH
<i>Mycobacterium</i>	-RRAKLLTFTVV <b>GAGPTG</b> VEMAGQIAELAEHTLKGAFRHIDSTKARVILL <b>D</b> AAPA-	NADH
<i>Synechocystis</i>	-RQQAWLTFVIV <b>GAGPTG</b> VELAGAI AEIAHSSLLKDNFHRIDTRQAKILLI <b>E</b> GVDR-	NADH
<i>Bacillus</i>	---EADATILIG <b>GGGLTG</b> VELVGEADIM-PNLAKKY-GVDHKEIKLKL <b>V</b> EAGPK-	NADH
<i>Escherichia</i>	---NGKVNIAIV <b>GGGATG</b> VELSAELHNAVVKQLHSYGYKGLTNEALNVT <b>L</b> VEAGERI	NADH
<i>Saccharomyces</i>	---ARLLSFVVV <b>GGGPTG</b> VEFAAELRDYVDQDLRKWMP <b>E</b> L-SKEIKVTL <b>V</b> EALPNI	NADH
<i>Neurospora</i>	---KRLLSFVVC <b>GGGPTG</b> VEFAAELFDLLNEDLTLHF <b>P</b> RLLRNEISVHLI <b>Q</b> SRDHI	NADPH
<i>Solanum</i>	---RTNLHFVIV <b>GGGPTG</b> VEFAAELHDYVYEDLVKIYPSV-KDFVKIT <b>V</b> I <b>Q</b> SGDHI	NADPH
GSM	AERERLLTFVVV <b>GAGPTG</b> VELAGQLAEMAHRTLAGEYKNFNTNSAKI ILL <b>G</b> GAPQV	??

**FIG 3** Sequence alignment of various NAD(P)H:quinone oxidoreductases. The conserved glycine-rich sequence is in boldface; the most conserved negatively charged residues are underlined. The boldface underlined residue is an adaptive neutral residue. The GenBank accession numbers of the sequences are as follows: for *Corynebacterium glutamicum*, YP\_225750.1; for *Mycobacterium tuberculosis*, YP\_001283183.1; for *Synechocystis* sp. strain PCC 6803, BAA17787.1; for *Bacillus subtilis*, YP\_007533169.1; for *Escherichia coli*, YP\_489377.1; for *Saccharomyces cerevisiae*, NP\_010198.1; for *Neurospora crassa*, CAB41986; and for *Solanum tuberosum*, CAB52797.

possessed the neutral amino acid residue glycine at position 213, the encoded NDH-II variant may accept NADPH rather than NADH.

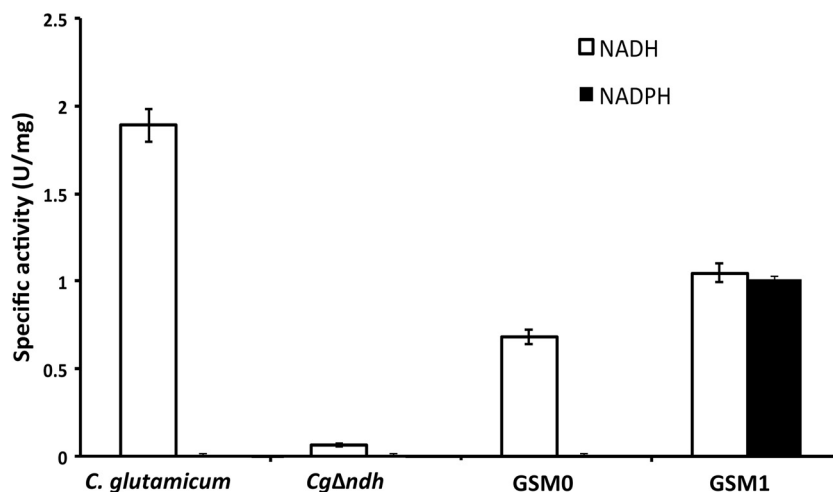
In order to test the specific activities of NDH-II with NADH and NADPH as cofactors, crude extracts of the *C. glutamicum* WT,  $\Delta ndh$ , and GSM1 strains were prepared, and ubiquinone oxidoreductase activities with either NADH or NADPH were determined at pH 7.5 (Fig. 4). Previous studies with purified NDH-II have shown different activities at different pHs (56). At a lower pH (pH 5.5), NDH-II activities with NADPH as a cofactor were higher in crude extracts of GSM1 ( $0.06 \pm 0.01$  U/mg) than in crude extracts of the WT strain ( $0.01 \pm 0.00$  U/mg). Similar results were obtained with crude extracts of strains GSM2, GSM3, and GSM4 (data not shown). In addition, D213N and D213Q variants of *C. glutamicum* NDH-II were constructed, and crude extracts of *C. glutamicum*  $\Delta ndh$  (carrying pVWEx1-*ndh*<sup>D213N</sup> or pVWEx1-*ndh*<sup>D213Q</sup>) were prepared and analyzed. As reported previously (53), the crude extracts of the wild-type strain had no detectable ubiquinone oxidoreductase activity with NADPH but did show activity with NADH ( $k_{cat}$ ,  $88 \text{ s}^{-1}$ ;  $K_m$ ,  $50 \mu\text{M}$ ) (Table 5). In contrast, crude extracts of GSM1 with NDH-II<sup>D213G</sup> showed high ubiquinone oxidoreductase activity with NADPH (with a  $k_{cat}$  of  $34 \text{ s}^{-1}$  and a  $K_m$  of  $100 \mu\text{M}$ , corresponding to a catalytic effi-

ciency of  $0.33 \text{ s}^{-1} \mu\text{M}^{-1}$ ) and NADH ( $k_{cat}$ ,  $32 \text{ s}^{-1}$ ;  $K_m$ ,  $78 \mu\text{M}$ ) (Table 5). For crude extracts with NDH-II<sup>D213N</sup> or NDH-II<sup>D213Q</sup>, NADH:ubiquinone oxidoreductase activities ( $k_{cat}$  values,  $80 \text{ s}^{-1}$  and  $63 \text{ s}^{-1}$ , respectively) and NADPH:ubiquinone oxidoreductase activities ( $k_{cat}$  values,  $32 \text{ s}^{-1}$  and  $56 \text{ s}^{-1}$ , respectively) were estimated (Table 5). Taking the findings together, neutral amino acid residues (G, N, or Q) at position 213 of *C. glutamicum* NDH-II resulted in activities with NADPH that were comparable to, or higher than, those than with NADH.

## DISCUSSION

GAPDH is important in glycolysis for the generation of the reduction equivalent NADH and for ATP generation by SLP in the subsequent reaction of 3-PGK; thus, GAPDH is important for growth and amino acid production by *C. glutamicum*. NAD-dependent GAPDH could be replaced by NADP-dependent GapN only when secondary mutations occurred. This was reported previously; however, the nature of the(se) mutation(s) remained elusive (35). Here we report on the identification of such a suppressor mutation, namely, an amino acid exchange in non-proton-pumping NDH-II that enabled it to accept NADPH as the substrate, suggesting that excess NADPH inhibited growth.

Recently, a different modification of glycolysis in *C. glutami-*



**FIG 4** Specific activities of NDH-II in crude extracts from various strains. Activity was measured at pH 7.5 for NADH and NADPH, using  $Q_1$  ( $50 \mu\text{M}$ ) as an acceptor, in the reaction medium. Enzyme activity was measured by monitoring of NADH or NADPH consumption, expressed in  $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ . The cell extracts were from the indicated *C. glutamicum* strains cultivated in BHI medium with  $1 \text{ mM IPTG}$  and  $100 \mu\text{g ml}^{-1}$  spectinomycin. Average values and standard deviations for triplicate cultivations are shown.

TABLE 5 Kinetic parameters of NADH and NADPH oxidation by wild-type and mutated NDH-II proteins<sup>a</sup>

Enzyme	$K_m$ ( $\mu\text{M}$ )		$V_{\text{max}}$ (U/mg)		$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	
	NADH	NADPH	NADH	NADPH	NADH	NADPH
WT NDH-II	50 $\pm$ 4	<5	10 $\pm$ 1	<5	88 $\pm$ 2	<5
NDH-II <sup>D213G</sup>	78 $\pm$ 4	103 $\pm$ 1	4 $\pm$ 1	4 $\pm$ 1	32 $\pm$ 2	34 $\pm$ 2
NDH-II <sup>D213N</sup>	150 $\pm$ 9	180 $\pm$ 1	9 $\pm$ 1	4 $\pm$ 1	80 $\pm$ 3	32 $\pm$ 3
NDH-II <sup>D213Q</sup>	110 $\pm$ 11	172 $\pm$ 16	7 $\pm$ 1	7 $\pm$ 1	63 $\pm$ 3	56 $\pm$ 3

<sup>a</sup> Kinetic parameters were measured at pH 7.5 for NADH and NADPH, using  $Q_0$  (100  $\mu\text{M}$ ) as an acceptor, in the reaction medium.

*cum* was followed when endogenous NAD-dependent GAPDH was engineered to accept NADP as the substrate in addition to NAD (57). Homology modeling studies predicted a highly conserved Asp35 in the NAD binding site as part of a network of hydrogen bonds interacting with the 2'- and 3'-hydroxyl groups of the adenosine ribose ring of NAD. Changing Asp35 to glycine, together with changes of Leu36 and Thr37, which determine the flexibility of the loop between the second  $\beta$ -strand and the subsequent  $\alpha$ -helix, and of Pro192, to improve NAD/NADP binding, resulted in a variant (D35G L36T T37K P192S) with high catalytic efficiency for both NAD (1,640  $\pm$  108  $\text{mM}^{-1} \text{min}^{-1}$ ) and NADP (5,868  $\pm$  352  $\text{mM}^{-1} \text{min}^{-1}$ ) as cofactors (57). Interestingly, the engineered GapA variant had increased L-lysine productivity. However, this came at the cost of perturbed growth and was suggested to be due to excess NADPH, levels of which may be even higher in strains that do not produce L-lysine, an NADPH sink (57). In line with the strict NADP dependence of GapN, growth perturbation was more pronounced, as shown here (Fig. 1) and previously (35), than the growth perturbation observed with the engineered NAD- and NADP-accepting GapA variant (57). The concept that excess NADPH inhibited growth was tested directly by heterologous expression of *udhA* from *E. coli*, encoding soluble transhydrogenase (52), which catalyzes the reversible transfer of reducing equivalents between NAD and NADP pools. Indeed, heterologous expression of *udhA* improved growth with GapN dramatically, although wild-type growth rates were not reached (Fig. 1). Similarly, *udhA* overexpression in *C. glutamicum* and *E. coli*  $\Delta\text{pgi}$  strains increased growth by oxidizing NADPH with NAD under conditions of NADPH excess due to the redirection of flux through the pentose phosphate pathway (58–60). In contrast, the membrane-bound transhydrogenase PntAB from *E. coli*, which uses the proton motive force to drive the reduction of NADP to NADPH by the oxidation of NADH to NAD (61), was employed to increase the provision of NADPH in various *C. glutamicum* strains thought to exhibit NADPH limitation, e.g., to increase the production of L-lysine, L-valine, L-ornithine, and isobutanol by *C. glutamicum* (13, 62–64).

The finding that the suppressor mutation D213G in non-proton-pumping NADH:ubiquinone oxidoreductase enabled it to accept NADPH as the substrate and relieved the growth inhibition due to NADP-dependent GapN (Fig. 4) supports the notion that excess NADPH inhibits growth. In *E. coli*, NADPH stress due to restructuring of the metabolic network (MG1655  $\Delta\text{udhA}$   $\Delta\text{pgi}$   $\Delta\text{qor}$   $\Delta\text{edd}$ ) was overcome by a suppressor mutation in proton-pumping NADH:ubiquinone oxidoreductase (NDH-I) subunit *nuoF* (E183A), which encodes a fragment that can oxidize NADPH for catabolism (65). *C. glutamicum* lacks NDH-I and

relies on non-proton-pumping NDH-II. In *C. glutamicum*, proton motive force is generated largely via cytochrome *bc*<sub>1</sub>-*aa*<sub>3</sub> supercomplex and/or less efficiently by cytochrome *bd* oxidase (66). NDH-II-defective strains have been described; they oxidize NADH by the coupling of NAD-dependent lactate dehydrogenase with quinone-dependent lactate dehydrogenase LldD (48) or Dld (67) and/or by the coupling of NAD-dependent malate dehydrogenase and quinone-dependent malate oxidoreductase (68) to compensate for the lack of NDH-II in *C. glutamicum* (66). It is conceivable that suppressor mutations leading to NADP-dependent lactate dehydrogenase or NADP-dependent malate dehydrogenase activity might have relieved growth inhibition by excess NADPH; however, these have not been observed in this study. These mutations would have coupled the oxidation of excess NADPH indirectly to ATP generation, since ubiquinol oxidation generates ATP by ETP, as found for the suppressor mutation of non-proton-pumping NADH:ubiquinone oxidoreductase II identified here.

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