Metabolic Impact of Increased NADH Availability in *Saccharomyces cerevisiae*[▽]

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Engineering the level of metabolic cofactors to manipulate metabolic flux is emerging as an attractive strategy for bioprocess applications. We present the metabolic consequences of increasing NADH in the cytosol and the mitochondria of *Saccharomyces cerevisiae*. In a strain that was disabled in formate metabolism, we either overexpressed the native NAD⁺-dependent formate dehydrogenase in the cytosol or directed it into the mitochondria by fusing it with the mitochondrial signal sequence encoded by the *CYB2* gene. Upon exposure to formate, the mutant strains readily consumed formate and induced fermentative metabolism even under conditions of glucose derepression. Cytosolic overexpression of formate dehydrogenase resulted in the production of glycerol, while when this enzyme was directed into the mitochondria, we observed glycerol and ethanol production. Clearly, these results point toward different patterns of compartmental regulation of redox homeostasis. When pulsed with formate, *S. cerevisiae* cells growing in a steady state on glucose immediately consumed formate. However, formate consumption ceased after 20 min. Our analysis revealed that metabolites at key branch points of metabolic pathways were affected the most by the genetic perturbations and that the intracellular concentrations of sugar phosphates were specifically affected by time. In conclusion, the results have implications for the design of metabolic networks in yeast for industrial applications.

The traditional use of baker's yeast, *Saccharomyces cerevisiae*, for ethanol production has resulted in the accumulation of substantial information about its genetics, metabolism, and process development. Consequently, the collection of compounds that are produced using *S. cerevisiae* has expanded to include organic acids and even secondary metabolites (1, 25, 28). Unlike ethanol, many of these products are not redox neutral relative to commonly used substrates such as glucose. Therefore, in addition to stoichiometry, redox constraints play an important role in the formation of the products. Additional reducing power has to be supplied to produce compounds whose degree of reduction is higher than that of the substrate. On the other hand, producing compounds with a degree of reduction lower than that of the substrate will force the synthesis of other compounds with higher degrees of reduction to compensate for excess reducing power generated from substrate oxidation. These constraints may decrease the product yield substantially.

The catabolic currency that balances the degree of reduction between the substrate and the products is usually NADH. In *S. cerevisiae*, NADH is produced in the cytosol by mainly glyceraldehyde-3-phosphate dehydrogenase and other assimilatory reaction enzymes (35). In the mitochondria, NADH is formed in the tricarboxylic acid (TCA) cycle and the reaction of the pyruvate dehydrogenase complex. Cytosolic NADH is oxidized by the glycerol-3-phosphate shuttle or the external cytosolic

NADH dehydrogenases, which are part of the electron transport chain (21). NADH can be transported across the outer mitochondrial membrane (18, 19) but not across the inner mitochondrial membrane (39). Therefore, a dedicated internal mitochondrial NADH dehydrogenase is required to oxidize mitochondrial NADH as part of the electron transport chain (22). The compartmental restriction of NADH oxidation has important ramifications for metabolism and electron transport. The electrons originating from cytosolic NADH are preferred over those originating from mitochondrial NADH (6) for entrance into the electron transport chain. The direct consequence of preferential utilization of cytosolic NADH is a higher redox potential (NADH/NAD⁺) in the mitochondria than in the cytosol. Consequently, during rapid NADH synthesis, as during exponential growth, the TCA cycle ceases to operate as a cycle and branches into oxidative and reducing pathways (12).

Metabolic consequences of the compartmentalization of NADH homeostasis were evident from the difference in the product formation profile upon lowering of cytosolic or mitochondrial NADH. Lowering cytosolic NADH by overexpressing bacterial NADH oxidase lowered the production of glycerol and biomass by *S. cerevisiae* (14, 36). On the other hand, decreasing the mitochondrial NADH level decreased ethanol production and increased the biomass yield (36). These results are likely to be a combination of effects from alleviating the feedback inhibition of the TCA cycle by mitochondrial NADH and increasing respiratory capacity due to improved efficiency of oxidative phosphorylation, as quantified by the P/O ratio (15). There are no reports that describe the effect of increasing NADH in *S. cerevisiae*, although formate has been used previously as a source of additional reducing power in *S. cerevisiae*

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TABLE 1. Primer sequences used to amplify DNA fragments introduced into *S. cerevisiae*^a

Fragment	Primer	Primer sequence (5' → 3')	Enzyme	Template
<i>FDH1</i>	Forward	TGCTACGGATCCATGTGCAAGGGAAAGGTT	BamHI	Genomic DNA of CEN.PK113-7D
	Reverse	TTGCTGG CTAACGGAATTCCTATTTCTTCTGTCCATAAG CTCTGGTGGC	EcoRI	
<i>GFP</i>	Forward	CTGATCGGATCCATGAGTAAAGGAGAAGAA CTTTTCACTGG	BamHI	In-house-developed plasmid
	Reverse	TTGAAGGAATTCCTATTTGTATAGTTCATCC ATGCCATGTG	EcoRI	
<i>CYB2-FDH1</i>	Forward	CTACGACTAGTATGCTAAAATACAAACCTTT ACTAAAAATCTCG	SpeI	Genomic DNA of CEN.PK113-7D
	Fusion	AGAACCAGCAAAACCTTTCCTTCGAGGATT TTGGAACGGTAGAACCG	BamHI	
	Reverse	CTACGGGATCCGAATTCCTATTTCTTCTGTCC ATAAGCTCTGGTG	EcoRI	

^a The sites for restriction enzyme digestion used for constructing the expression plasmids are underlined in the primer sequences. The mitochondrial signal sequence of *CYB2* corresponds to the first 39 amino acids (see the text for details).

(2, 4, 11, 23, 24, 27). Formate (HCOO^-) is efficiently oxidized to CO_2 by NAD^+ -dependent formate dehydrogenase (27) and, therefore, cannot be used as a carbon source for biomass synthesis. Thus, using formate as an auxiliary substrate for the generation of NADH to study the effect of increased NADH may be a feasible option. Given the compartment-dependent regulation of NADH homeostasis in *S. cerevisiae* (36), increasing the NADH level in the cytosol is likely to elicit a response different from that obtained by increasing the NADH level in the mitochondria.

The aim of the present study is to differentiate between the metabolic consequences of increasing NADH in the cytosol and those of increasing NADH in the mitochondria of *S. cerevisiae*. Toward this aim, we either overexpressed the native Fdh1 (NAD^+ -dependent formate dehydrogenase) in the cytosol or directed it into the mitochondria in a strain background that is otherwise devoid of formate metabolism. We present our understanding of the physiological characteristics of the mutant strains under steady-state or dynamic conditions in the presence of different levels of formate.

MATERIALS AND METHODS

Construction of the yeast strains. All the strains used in this study have the CEN.PK background with auxotrophy for uracil (34). The high-copy-number plasmid pRS426CT, containing the *URA3* gene as a marker, was used to complement the auxotrophy and overexpress the different heterologous genes with strong constitutive expression from the promoter of the *TEF1* gene and the terminator of the *CYC1* gene (40). The primers used for PCR, the sources of the corresponding templates for amplification, and the restriction sites the primers introduced are shown in Table 1. All PCRs were performed using the Expand high-fidelity PCR system (Roche Applied Science, Indianapolis, IN). The *FDH1* gene (YOR388c) was amplified from genomic DNA of *S. cerevisiae* CEN.PK113-7D, digested, and ligated into pRS426CT, and the resulting plasmid was designated pTEF-cFDH. The green fluorescent protein (GFP) gene was also amplified, digested, and ligated into pRS426CT by using the primers and restriction enzymes shown in Table 1. The resulting plasmid was named pTEF-GFP (Table 2). In order to direct the native formate dehydrogenase into the mitochondria, we fused the *FDH1* gene with a mitochondrial signal sequence. We screened 746 bona fide mitochondrial proteins from *S. cerevisiae* (32) to identify their leader peptides by using the Web-based software TargetP (10) and MitoP2 (9). Computational predictions of the mitochondrial signal sequences obtained by using the default settings of the two software packages agreed for only nine proteins. Among these proteins, only the product of the *CYB2* gene had a signal sequence demonstrated to direct a cytosolic protein into the mitochondria of *S. cerevisiae*.

TABLE 2. Strains and plasmids used in this study

Plasmid or strain	Relevant features	Reference or source
Plasmids		
pRS426CT	2 μ m expression vector with <i>TEF1</i> promoter, <i>CYC1</i> terminator, and <i>URA3</i> marker	40
pTEF-cFDH	2 μ m expression vector with <i>TEF1</i> promoter, <i>CYC1</i> terminator, <i>URA3</i> marker, and <i>FDH1</i> gene from <i>S. cerevisiae</i>	This study
pTEF-GFP	2 μ m expression vector with <i>TEF1</i> promoter, <i>CYC1</i> terminator, <i>URA3</i> marker, and <i>GFP</i> gene	This study
pTEF-CYB2-FDH-GFP	2 μ m expression vector with <i>TEF1</i> promoter, <i>CYC1</i> terminator, <i>URA3</i> marker, mitochondrial signal sequence from <i>CYB2</i> gene, <i>FDH1</i> gene from <i>S. cerevisiae</i> , and <i>GFP</i> gene	This study
pTEF-CYB2-FDH	2 μ m expression vector with <i>TEF1</i> promoter, <i>CYC1</i> terminator, <i>URA3</i> marker, mitochondrial signal sequence from <i>CYB2</i> gene, and <i>FDH1</i> gene from <i>S. cerevisiae</i>	This study
Strains		
CEN.PK113-7D	<i>MATa SUC2 MAL8</i>	P. Kötter
CEN.PK113-5D	<i>MATa SUC2 MAL8^c ura3-52</i>	P. Kötter
CEN.PK1001-71C	<i>MATa SUC2 MAL8^c ura3-52 FDH1(41,1091)::loxP-Kan-loxP FDH2(41,1091)::loxP-Kan-loxP</i>	P. Kötter
F-REF	CEN.PK1001-71C/pTEF	This study
cFDH	CEN.PK1001-71C/pTEF-cFDH	This study
GFP	CEN.PK1001-71C/pTEF-GFP	This study
mFDH-GFP	CEN.PK1001-71C/pTEF-CYB2-FDH-GFP	This study
mFDH	CEN.PK1001-71C/pTEF-CYB2-FDH	This study

(30). The sequence encoding the mitochondrial targeting signal, identified as the first 39 amino acids (corresponding to the first 117 bp), was fused to the 5' end of the *FDH1* gene without its start codon (Table 1). The *CYB2-FDH1* fusion fragment was then amplified using genomic DNA from *S. cerevisiae* CEN.PK113-7D as the template and was ligated into pRS426CT, and the resulting plasmid was named pTEF-CYB2-FDH. The *CYB2-FDH* fragment was also digested and ligated into pTEF-GFP, and the resulting plasmid was named pTEF-CYB2-FDH-GFP (Table 2).

S. cerevisiae CEN.PK1001-71C [*MATa SUC2 MAL8^c ura3-52 FDH1(41,1091)::loxP-Kan-loxP FDH2(41,1091)::loxP-kan-loxP*] was transformed with the plasmids pRS426CT, pTEF-cFDH, pTEF-GFP, pTEF-CYB2-FDH, and pTEF-CYB2-FDH-GFP, and the resulting strains were designated F-REF, cFDH, the GFP strain, mFDH, and mFDH-GFP, respectively. All the strains (Table 2) were maintained on synthetic complex medium agar plates lacking uracil. They were grown on synthetic complex medium lacking uracil, and an aliquot of each culture was mixed with glycerol to a final concentration of 20% and kept at -80°C for long-term storage.

Medium and growth conditions. For all cultivations, we used a previously described (38) mineral salts medium consisting of the following (per liter): $(\text{NH}_4)_2\text{SO}_4$, 5 g; KH_2PO_4 , 3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.50 g; Antifoam 289 (A-5551; Sigma-Aldrich), 0.050 ml; trace metals, 1 ml; and vitamins, 1.0 ml. The trace metal solution consisted of the following (per liter): EDTA (sodium salt), 15.0 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.45 g; $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 1 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.3 g; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.4 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.45 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g; H_3BO_3 , 0.1 g; and KI, 0.10 g. The pH of the trace metal solution was adjusted to 4.0 with 2 M NaOH prior to heat sterilization. The vitamin solution contained the following (per liter): biotin, 0.05 g; *p*-aminobenzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g; and *myo*-inositol, 25 g. The pH of the vitamin solution was adjusted to 6.5 with 2 M NaOH. The vitamin solution was filter sterilized and stored at 4°C . This medium was supplemented with 10 g/liter glucose and different amounts of formate.

The seed cultures for the cultivations were grown at 30°C in 500-ml shake flasks containing 100 ml of culture at a starting pH of 6.0 with agitation in an orbital shaker at 250 rpm. Seed cultures from the shake flasks were used to inoculate the fermentors to a final dry weight of 1 mg/liter. All cultivations were performed in triplicate. The chemostat cultures were grown in 1.0-liter stirrer-pro vessels (DasGip, Jülich, Germany) with a working volume of 0.5 liter. Agitation at 600 rpm was maintained using a magnetic stirrer integrated into the BioBlock module, which kept the temperature at 30°C . The rate of aeration was set to 0.5 liter/min. The pH of the medium was maintained at 5.0 by automatic addition of 2 N KOH. The temperature, agitation, gassing, pH, and composition of the off-gas were monitored and controlled using the DasGip monitoring and control system. Dissolved oxygen was monitored with an autoclavable polarographic oxygen electrode (Mettler Toledo, Columbus, OH). The effluent gas from the fermentation was analyzed for real-time determination of oxygen and CO_2 concentrations by DasGip feedback-pro gas analysis systems with the off-gas analyzer GA4 using the zirconium dioxide level and a two-beam infrared sensor (DasGip, Jülich, Germany). The chemostat cultures were initiated as batch cultures using 10 g/liter glucose, and feeding with fresh medium commenced at a constant rate of 0.05 liter/h (resulting in a dilution rate of 0.1 h^{-1}) only after the residual ethanol produced from the glucose consumption phase was completely depleted. The inlet medium contained 10 g/liter glucose and various amounts of potassium formate (0 to 13 g/liter) to result in different molar formate/glucose (F/G) ratios in the inlet feed. The working volumes of the cultures were kept constant by automatic removal of broth based on an electric level sensor measurement. Samples were taken after a steady state (defined by constant values of CO_2 and O_2 in the off-gas, as well as a constant biomass concentration for at least five residence periods) was achieved. Dynamic responses were studied by pulsing potassium formate to a final concentration of 5 g/liter.

Analytical methods. The dry weight of cells was measured by filtering known volumes of the cultures through predried and preweighed $0.45\text{-}\mu\text{m}$ -pore-size nitrocellulose filters (Supor-450 membrane filters; PALL Life Sciences, Ann Arbor, MI). The filters with the biomass were washed with water, dried for 15 min in a microwave oven at 150 W, and weighed again. The optical density at 600 nm was determined using a Hitachi U-1100 spectrophotometer.

Concentrations of glucose, formate, glycerol, ethanol, acetate, and pyruvate were analyzed by an isocratic high-performance liquid chromatograph with an Aminex HPX-87H ion-exchange column (Bio-Rad, Hercules, CA) at 65°C using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml min^{-1} . Glucose, glycerol, and ethanol were measured with a refractive index detector (a Waters 410 differential refractometer; Millipore, CA), and formate, acetate, and pyru-

vate were measured with a UV-visible light absorbance detector (a Waters 486 tunable absorbance detector set at 210 nm; Millipore, CA).

Biomass was rapidly quenched in cold (-40°C) methanol to freeze metabolism, and intracellular metabolites were extracted using chloroform according to a previously described protocol (33). Metabolites from glycolysis, the TCA cycle, and the pentose phosphate pathway (PPP) and the metabolic cofactors were measured by liquid chromatography coupled with tandem quadrupole mass spectrometry as described previously (20). The metabolite concentrations reported are the averages from at least two biological replicates and were normalized with respect to the biomass concentration.

Cell extracts from frozen 100-mg biomass samples from different cultivations were prepared by mechanical lysis with a FastPrep FP120 instrument (Savant Instruments, Farmingdale, NY) and resuspended in 50 mM phosphate buffer. The activities of formate dehydrogenase in the cell extracts were measured as described previously (5). The total protein was quantified by the Lowry method using bovine serum albumin as a standard. One unit of activity is defined as the amount of enzyme that will result in the formation of $1\text{ }\mu\text{mol}$ of NADH/min/mg of protein at 30°C .

Fluorescence imaging. Yeast strains containing the pTEF-CYB2-FDH-GFP plasmid were grown to mid-exponential phase in minimal medium in 500-ml shake flasks. The cells were observed on an inverted DMI4000B microscope connected to a DFC500 digital camera (Leica Microsystems, Sweden). The microscope was automatically controlled by the Leica application suite, which was also used to acquire and manage the images. The microscope was operated in differential interference contrast (DIC) mode with an exposure time of 0.1 s or in the fluorescence mode with a GFP filter and an exposure time of 0.90 s. The images acquired from the two phases were overlapped to determine the cellular localization. Yeast cells with the pTEF-GFP plasmid were used as a control to distinguish mitochondrial localization from cytosolic localization.

RESULTS

The primary objective of this study was to evaluate the metabolic response of *S. cerevisiae* to an increase in NADH availability in the mitochondria and compare it with the response to an increase in NADH availability in the cytosol. We achieved this by overexpressing Fdh1 in the cytosol (in the cFDH strain) or directing formate dehydrogenase to the mitochondria by tagging it with a mitochondrial signal peptide (in the mFDH strain) in a background that is otherwise devoid of formate assimilation (represented by the F-REF strain, which we used as the control).

Localization of recombinant formate dehydrogenase. The *FDH1* gene (encoding formate dehydrogenase) from the prototrophic *S. cerevisiae* CEN.PK113-7D strain was amplified and expressed in CEN.PK1001-71C (*fdh1 Δ fdh2 Δ ura3-52*) under the control of a strong constitutive promoter and a high-copy-number plasmid. Normally, Fdh1 is localized in the cytosol (27). In order to direct it to the mitochondria, we fused it to the mitochondrial signal peptide of Cyb2. The signal peptide from Cyb2 was tested previously for its efficacy in directing cytosolic proteins into the mitochondria (30). The mFDH-GFP strain (Table 2) was grown in 5 g/liter glucose supplemented with 5 g/liter formate in shake flasks. An aliquot of this culture was observed under the fluorescence microscope. We observed distinct localization of the GFP in the mitochondria (Fig. 1). As a control, we also expressed the pTEF-GFP plasmid in CEN.PK113-5D to distinguish cytosolic localization from mitochondrial localization. The localization patterns differed between the two strains, leading to the conclusion that the mitochondrial signal sequence of Cyb2 was extremely efficient in directing Fdh1 into the mitochondria. For all the subsequent studies, we used the plasmid pTEF-CYB2-FDH1 (without the GFP tag) in CEN.PK1001-71C to obtain strain mFDH.

FIG. 1. Confirmation of the localization of GFP-tagged formate dehydrogenase fused with the mitochondrial signal sequence encoded by the *CYB2* gene. The corresponding strain was designated mFDH-GFP. As a control, the cytosolic localization of unmodified GFP is also shown. Overlaying of the bright-field image with that of GFP clearly indicates the localization of the Cyb2-Fdh1-GFP construct in the mitochondria.

Formate cometabolism in glucose-limited chemostats. Having confirmed the mitochondrial localization of formate dehydrogenase in mFDH, we studied formate cometabolism in this strain, cFDH, and F-REF. The reference strain (F-REF) cannot consume formate, as it lacks the two genes that encode formate dehydrogenase (mFDH, cFDH, and F-REF are otherwise isogenic). The differences observed between cFDH and mFDH were assumed to result from increased NADH formation in the cytosol and the mitochondria, respectively. We studied the impact of different levels of formate in the feed during steady-state growth of the three strains in glucose-limited chemostats. We varied formate levels such that the molar F/G ratios were in the range of 0 to 5, since higher ratios were reported to result in incomplete consumption of formate (27). The absence of any formate dehydrogenase activity in F-REF was confirmed by enzyme assays. We did not detect any formate dehydrogenase activity above the background level in F-REF, but it varied between 0.25 and 0.34 U in cFDH and mFDH grown with the presence of formate in the feed.

In the absence of formate, we did not observe any noticeable differences among the three strains. The differences among the strains became more prominent as the formate concentration in the feed increased. The biomass yield from F-REF remained constant (within 3%) for all F/G ratios studied. Constant biomass yields eliminate the possibility of any potential detrimental effect of formate in the medium. In concurrence with previous results (27) obtained using the same strain background, we also observed an increase in the biomass yield from cFDH as the formate concentration in the feed increased. The biomass yield from cFDH increased from 0.48 to 0.58 g/g glucose as the F/G ratio increased to 5 (Fig. 2A). In contrast, the biomass yield from mFDH remained constant (within 3%) until the F/G ratio increased to 1.3 before starting to decrease. The biomass yield decreased by more than 10% at an F/G ratio of 5 (Fig. 2A). The cells consumed all the available formate when the F/G ratio remained lower than 2 for cFDH and 1.3 for mFDH. Above these values, we observed small quantities of residual formate in the medium. The onset of formate accumulation coincides with the decrease in the biomass yield

from mFDH. We did not observe any difference in the specific rate of formate uptake (r_F) between cFDH and mFDH for all F/G ratios lower than 1.3. Above this value, r_F increased more rapidly for mFDH than for cFDH. As the rate of formate uptake increased, the corresponding rate of glucose uptake (r_G) decreased more than 20% for cFDH (Fig. 2B). For mFDH, r_G remained steady (with less than 3% variation) at 1.2 mmol g (dry weight)⁻¹ h⁻¹ until r_F increased to 2.8 mmol g (dry weight)⁻¹ h⁻¹ (corresponding to an F/G ratio of 1.3), when r_G started to increase to 1.3 mmol g (dry weight)⁻¹ h⁻¹. Under all the conditions studied, the variation in r_G for F-REF was 2% or less (Fig. 2B).

Steady-state flux redistribution during formate cometabolism. The differences in the specific glucose uptake rate and biomass yield between cFDH and mFDH in response to the presence of formate are the ramifications of the redistribution of the carbon fluxes to different products. In general, consumption of formate induced fermentative metabolism of glucose in cFDH and mFDH. We observed the accumulation of ethanol in only mFDH, even at low F/G ratios. The rate of ethanol

FIG. 2. Metabolic changes to formate supplementation. (A) Steady-state biomass yields as a function of the molar ratio of formate to glucose (F/G) in the feed medium. (B) Specific glucose uptake rates (r_G) as a function of the specific formate consumption rates (r_F) for F-REF (circles), cFDH (squares), and mFDH (triangles). The rates of consumption of glucose and formate were calculated from the data obtained from the chemostat cultures. gDW, grams (dry weight).

FIG. 3. Specific rates of ethanol production (r_{eth}) (A) and specific rates of glycerol production (r_{gly}) (B) for F-REF (circles), cFDH (squares), and mFDH (triangles) at different concentrations of formate in the inlet feed in glucose-limited chemostat cultures. The carbon content in the inlet feed is shown as the ratio of formate to glucose (F:G).

accumulation increased linearly with the F/G ratio to 1 mmol/g (dry weight)/h (Fig. 3A). This pattern corresponds to 0.88 g/liter of residual ethanol at the highest F/G ratio studied. We did not see any ethanol production when cytosolic NADH production was increased in cFDH. However, we observed the accumulation of glycerol when either cytosolic or mitochondrial NADH increased. In mFDH, the onset of partial fermentative metabolism commenced at F/G ratios of 0.8, while in cFDH, it was delayed until an F/G ratio of 2 was reached (Fig. 3B). The rate of glycerol accumulation was higher for cFDH than for mFDH and increased linearly to 0.05 mmol g (dry weight)⁻¹ h⁻¹ for mFDH and to 0.08 mmol g (dry weight)⁻¹ h⁻¹ for cFDH at the highest F/G ratio of 5, corresponding to 0.1 and 0.2 g/liter glycerol in mFDH and cFDH, respectively. Additionally, we also observed small quantities of pyruvate only in mFDH at F/G ratios higher than 1.3. Pyruvate accumulated linearly above this value to 0.11 mmol g (dry weight)⁻¹ h⁻¹. Interestingly, cFDH did not produce any pyruvate at any F/G ratio studied.

The consumption of formate will result in equimolar production of CO₂. The concentration of CO₂ in the off-gas from F-REF remained constant at all F/G ratios. However, this value increased linearly for cFDH and mFDH with increasing F/G ratios in the feed. For both strains, the specific rate of CO₂ evolution (r_{CO_2}) increased from 2.8 mmol g (dry weight)⁻¹ h⁻¹ when there was no formate present to 7.8 mmol g (dry weight)⁻¹ h⁻¹ when the F/G ratio in the feed was 2.6. Beyond this F/G ratio, r_{CO_2} reached a plateau at 10 mmol g (dry weight)⁻¹ h⁻¹ for cFDH. For mFDH, r_{CO_2} continued to increase linearly to 14 mmol g (dry weight)⁻¹ h⁻¹ (Fig. 4A). Although we detected residual formate in the effluent at higher F/G ratios for both the strains, the amount of CO₂ evolved was in stoichiometric proportion to the formate consumed, except for cFDH at the highest F/G ratio. The profiles of CO₂ for cFDH and mFDH were also reflected in the respiratory quotient (RQ). The RQ for F-REF remained constant at unity, indicating complete oxidation of glucose at all F/G ratios. The RQ increased with increasing proportions of formate in the feed similarly for cFDH and mFDH for all F/G ratios below 1.3. It was the F/G ratio of 1.3 in the feed at which we observed a small decrease in the biomass yield for mFDH. Above this value, the RQ for mFDH increased more rapidly than that for

FIG. 4. Specific rates of CO₂ evolution (r_{CO_2}) (A) and RQs (B) for F-REF (circles), cFDH (squares), and mFDH (triangles) at different concentrations of formate in the inlet feed in glucose-limited chemostat cultures. The carbon content in the inlet feed is shown as the ratio of formate to glucose (F:G).

cFDH (Fig. 4B). For cFDH, the RQ reached a maximum of 1.9, while for mFDH, the RQ reached 2.2 at an F/G ratio of 5 in the feed. Taking these products into account, we were able to close the carbon and degree of reduction balances for cFDH and mFDH for all F/G ratios with at least 94% accuracy.

Dynamic responses to formate pulse. Increasing the formate concentration in the feed for chemostat cultures clearly revealed differences associated with cytosolic and mitochondrial localization patterns of Fdh1. These differences signify the net result of all the metabolic adaptations of the cell to increased NADH in either the cytosol or the mitochondria. However, they do not capture the intracellular dynamics of these adaptations. In order to study the dynamics, we pulsed glucose-limited chemostat cultures of F-REF, cFDH, and mFDH with potassium formate and monitored its consumption and related changes in the concentrations of intracellular metabolites. Since F-REF lacks formate dehydrogenase, it could not metabolize any formate. However, cFDH and mFDH started to consume formate almost instantaneously after the pulse (Fig. 5, inset). The consumption of formate ceased about 15 min after the pulse, even though there was ample formate available. The concen-

FIG. 5. Consumption of formate by F-REF (circles), cFDH (squares), and mFDH (triangles) after pulsing of steady-state, glucose-limited cultures with formate. The inset shows the raw data on the residual concentration of formate. The specific formate uptake rate was calculated from the raw data.

FIG. 6. (A) Hierarchical clustering of the strains and the 23 intracellular metabolites that exhibited significantly different profiles ($P < 0.05$) as determined by ANOVA. Also shown are the time points (in minutes) at which the different samples were quantified. Numbers at the bottom are amounts of change (n -fold). AKG, α -ketoglutarate; acetyl-CoA, acetyl coenzyme A; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; cAco, *cis*-aconitate; E4P, erythrose-4-phosphate; 2-/3-PG, 2-phosphoglycerate/3-phosphoglycerate; PEP, phosphoenolpyruvate; S7P, sedoheptulose-7-phosphate; R5P, ribose-5-phosphate; 6PG, 6-phosphogluconate; Ru-/Xy5P, ribulose-5-phosphate/xylulose-5-phosphate. (B) The data presented in panel A were used to illustrate the effect of time using PC analysis for F-REF (data points clustered together), cFDH (dark gray data points), and mFDH (light gray data points). The inset shows the amount of variance that is explained by the different PCs. The data are projected only on the first two PCs, which captured 83% of the variance.

tration of residual formate decreased in similar manners for cFDH and mFDH. The specific rate of formate uptake (r_F) rapidly peaked at $5.5 \text{ mmol g (dry weight)}^{-1} \text{ h}^{-1}$ for cFDH and $5 \text{ mmol g (dry weight)}^{-1} \text{ h}^{-1}$ for mFDH within the first 10 min, before it started to decrease. We did not observe any striking differences in the formate consumption patterns between cFDH and mFDH (Fig. 5). Consumption of formate did not have any observable impact on the glucose uptake rate. As during steady-state growth, we observed ethanol production only by mFDH, while glycerol was produced by both cFDH and mFDH. The rates of ethanol and glycerol production peaked at 30 min after the formate pulse before decreasing (data not shown). Stoichiometric analysis indicated that the amount of NADH consumed during the production of glycerol and ethanol was much lower (40%) than the amount of NADH that would have been produced by complete conversion of formate to CO_2 .

We also determined the concentrations of central carbon metabolites and associated cofactors following the formate pulse. Despite the presence of formate, the concentrations of metabolites and cofactors in F-REF did not change with time. Strain-specific changes in the metabolite concentrations were identified by analysis of variance (ANOVA). At a significance cutoff (P value) of 0.05, we identified 23 metabolites whose concentrations in the three strains changed with time. Hierarchical clustering of these metabolites clearly separated cFDH and mFDH strains from F-REF (Fig. 6A). Upon formate pulsing, the concentrations of the cofactors (NADH, NAD⁺,

NADPH, and ATP) and key branch point metabolites such as dihydroxyacetone phosphate (DHAP), pyruvate, and succinate in cFDH and mFDH increased compared to those in F-REF. On the other hand, the concentrations of the pentose phosphates and some intermediates of the TCA cycle (isocitrate and *cis*-aconitate) decreased after pulsing of the cultures with formate. Interestingly, the concentrations of α -ketoglutarate, succinate, and ATP were significantly higher in mFDH than in cFDH. On the other hand, the concentrations of DHAP and AMP were higher in cFDH than in mFDH (Fig. 6A). The differences between the strains based on the metabolite concentrations were also identified by multivariate analysis, by projecting the concentrations onto the principal components (PCs). Using the 23 metabolites identified by ANOVA, the first two PCs captured about 83% of the variance (Fig. 6B, inset). The concentrations of the metabolites in F-REF were relatively unchanged, which is also indicated by the segregation of all the loadings from PC1 and PC2 for F-REF (Fig. 6B). The separation between cFDH and mFDH was clearly captured by PC1 and PC2. While PC1 clearly separated the strains (F-REF from cFDH and mFDH), PC2 was more indicative of the dynamic profiles of the metabolites. The metabolites that contributed to PC1 were acetyl coenzyme A, phosphoenolpyruvate, pentose phosphates, and NADP (positive correlation) and ATP, pyruvate, succinate, NAD, and NADPH (negative correlation). NADPH, ATP, pyruvate, NAD, and succinate were positively correlated with PC2, while the sugar phosphates were negatively correlated with PC2. PC1 (which cap-

tured 68% of the total variance) reflected the effect of genetic perturbation, while PC2 (which captured 15% of the total variation) reflected the effect of time.

Cofactor variation following formate pulse. In the three strains, under steady-state conditions (just prior to pulsing with formate), the concentration of NAD^+ was two orders of magnitude higher than that of NADH. We observed linear increases in the concentrations of NAD^+ in cFDH (from 2.6 to 2.9 $\mu\text{mol/g}$ [dry weight]) and mFDH (from 2.2 to 2.84 $\mu\text{mol/g}$ [dry weight]) in 60 min after pulsing of the cultures with formate. On the contrary, NADH increased quite rapidly (in the first 5 min) in cFDH and more slowly (in the first 15 min) in mFDH after pulsing with formate. These changes are reflected in the NADH/NAD^+ ratios, which increased rapidly in the two strains after the pulse, before gradually decreasing to the steady-state values (Fig. 6A). Although we did not directly affect the $\text{NADPH}/\text{NADP}^+$ redox couple, the formate pulse appeared to have an indirect effect on NADPH and NADP^+ in cFDH and mFDH. Unlike the intracellular levels of NADH and NAD^+ , which showed a large difference, the concentrations of NADP^+ and NADPH were comparable in all our measurements. After the formate pulse, the concentrations of NADP^+ in both cFDH and mFDH decreased gradually by about 15% in 60 min. On the other hand, the concentrations of NADPH in both cFDH and mFDH increased from 0.36 $\mu\text{mol/g}$ (dry weight) at the steady-state level and reached a plateau at about 0.90 $\mu\text{mol/g}$ (dry weight) at the end of 60 min. The overall ratio of the $\text{NADPH}/\text{NADP}^+$ redox couple increased from 0.3 and leveled out at about 1.4 (Fig. 7B) for the two strains. The concentration of ATP also increased with formate pulsing, from 5.8 $\mu\text{mol/g}$ (dry weight) in the steady state to 6.9 $\mu\text{mol/g}$ (dry weight) in cFDH and to 7.04 $\mu\text{mol/g}$ (dry weight) in mFDH within 60 min after the formate pulse. The concentration of ADP decreased only marginally. These changes resulted in an overall increase in the ATP/ADP ratio from approximately 1.3 in the steady state to 2 at the end of 60 min after the formate pulse (Fig. 7C).

DISCUSSION

Redirecting metabolic fluxes by altering the level of redox cofactors is emerging as a promising way to increase product yields using microbial processes. This study provides strong evidence that compartment-dependent NADH homeostasis has an impact on metabolic flux distribution. The results have high relevance in designing the metabolism of yeasts for metabolic engineering applications. Lowering cytosolic NADH decreases glycerol production (14, 36), while ethanol production is dependent on the mitochondrial redox (36). The reverse process of increasing NADH has been implemented previously by using formate as an auxiliary substrate that can provide additional reducing power in the form of NADH (2, 4, 11). As this approach increases only the cytosolic NADH level, the effect of increasing mitochondrial NADH was not evaluated. In the present study, we increased the NADH content in either the cytosol or the mitochondria by overexpressing formate dehydrogenase (in cFDH or mFDH, respectively) in the respective compartment and supplementing the growth medium with formate. The perturbations resulted in the formation of fermentative products (such as glycerol and/or ethanol) under

FIG. 7. Variation in the NADH/NAD^+ (A), $\text{NADPH}/\text{NADP}^+$ (B), and ATP/ADP (C) ratios in F-REF (open circles), cFDH (squares), and mFDH (triangles) after pulsing of steady-state, glucose-limited cultures with formate.

conditions that are normally conducive to complete respiratory metabolism of glucose.

Energetics of formate uptake and metabolic consequences. Formate metabolism by formate dehydrogenase results in equimolar amounts of NADH and CO_2 . Since CO_2 is a benign by-product that could be easily removed, the net effect of formate addition to the medium is increased NADH in the respective compartment in which the formate dehydrogenase reaction occurs. Whether formate uptake occurs by passive diffusion or by energy-dependent transport is not conclusive (7, 37). In the strain background used in this study, indirect evidence from meticulous stoichiometric modeling suggests the prevalence of both mechanisms (27). Although a transport stoichiometry of one proton for every ATP molecule hydrolyzed was considered in the model, there is strong evidence that the stoichiometry may vary and that, in fact, ATP hydrolysis may be decoupled from proton transport (26, 37). Additionally, *S. cerevisiae* possesses efflux pumps, such as Pdr12, to resist the accumulation of weak organic acids (17, 29, 31). Interestingly, the specific rate of formate consumption was higher in steady-state cultures than in cultures pulsed with formate. It appears that the defense mechanism against for-

mate metabolism is a slow process, allowing enough time for full activation only during chemostat cultivation. The absence of any noticeable change in the biomass yield from F-REF, which can take up formate but cannot metabolize it, suggests only that the energetic changes emanating from stress due to the transport of formate or its accumulation are insignificant compared to the total generation of ATP by glucose metabolism. Moreover, respiratory complex I of *S. cerevisiae* does not translocate protons (8). A direct consequence of this is that electrons transported from NADH, whether generated from glucose dissimilation or from formate oxidation, cannot contribute to the proton motive force. Therefore, formate addition is not expected to increase the efficiency of oxidative phosphorylation (the P/O ratio) but merely to increase the rate of electron transport. Interestingly, *S. cerevisiae* could tolerate a higher specific rate of formate consumption during steady-state growth than after a sudden pulse.

We observed glycerol production in response to increasing NADH. Glycerol production in *S. cerevisiae* is a consequence of an imbalance in cytosolic NADH (3). However, ethanol production was observed only when mitochondrial NADH was increased. Increasing the NADH level in the mitochondria is likely to have forced the TCA cycle into operating as two distinct branches. The TCA cycle operates as two branches during glucose repression, due to the suppression of the succinate dehydrogenase (SDH) complex (12). Inhibition of the SDH complex by excess mitochondrial NADH, leading to the branching out of the TCA cycle, does not seem to be a remote possibility. Consequently, glycolytic carbon was shunted to ethanol. The results from our study, supported by previous reports from our lab (15, 36), conclusively demonstrate that ethanol overflow in *S. cerevisiae* is a ramification of redox imbalance in the mitochondria.

Dynamic response to formate addition. Formate pulsing of glucose-limited chemostat cultures of *S. cerevisiae* resulted in different responses in cFDH and mFDH, compared with F-REF. The primary difference we observed was the cessation of formate consumption, 15 min after the formate pulse. This is likely due to the pH effect generated by the dissociation of the salt as a free acid inside the cell. Decreased biomass from both cFDH and mFDH upon formate pulsing provides further evidence for the onset of severe stress following the formate pulse. This is likely to be a consequence of decreased intracellular pH, triggered by the accumulation of protons resulting from the oxidation of formate. Since we did not observe any detrimental effect of formate in F-REF, the differences we observed in cFDH and mFDH are clearly related to the metabolism of formate in these strains. The absence of such effects in glucose-limited chemostat cultures supplemented with formate indicates the robust operation of pH homeostasis.

Analysis of intracellular metabolite dynamics indicated that the responses in cFDH and mFDH were different. Concentrations of key branch point metabolites (such as glucose-6-phosphate, DHAP, pyruvate, phosphoenolpyruvate, and acetyl coenzyme A) and cofactors (ATP, ADP, NADH, NAD⁺, NADPH, and NADP⁺) in cFDH and mFDH emerged as statistically different from those in F-REF. This finding is an indication that regulatory changes manifest at the branch points of the pathways that lead to the formation of glycerol and ethanol. Furthermore, the sugar phosphates emerged as

being particularly sensitive to time and appeared to follow similar profiles in cFDH and mFDH. In general, the metabolism shifted to producing compounds that are more reduced. A major setback in our analysis was that we were unable to distinguish between mitochondrial and cytosolic NAD(H) concentrations. Nevertheless, the NADH/NAD ratios in cFDH and mFDH increased in response to the formate pulse before decreasing within 15 min, following the trajectory of the specific formate consumption rate. The redistribution of metabolic flux to produce glycerol (in cFDH and mFDH) and ethanol (in mFDH) is clearly a consequence of NADH homeostasis in the cytosol and the mitochondria. Although we did not directly affect the NADPH/NADP⁺ redox couple, the NADPH/NADP⁺ ratios in cFDH and mFDH also increased upon pulsing of the cultures with formate. Since *S. cerevisiae* does not have a transhydrogenase to interconvert NADH and NADPH (35), NADPH could have been produced in the PPP. It remains to be seen whether the increase in the concentration of NADPH is due to the inhibition of the NADH-generating steps in the later stages of the Embden-Meyerhof-Parnas pathway resulting in shunting of carbon into the PPP or due to an active regulatory mechanism to maintain the NADH/NADPH ratio in the cell.

In conclusion, we demonstrate that the compartment-dependent regulation of NADH homeostasis has important consequences for metabolic flux distribution. Increasing mitochondrial NADH triggers ethanol production by restricting the entry of glycolytic carbon into the TCA cycle. Increasing cytosolic NADH results in glycerol production. Indeed, such information could be used to manipulate carbon flux in the central carbon metabolism, as demonstrated recently (13, 16). We believe that the results presented here may have important implications in the rational design of eukaryotic strains for metabolic engineering applications.

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REFERENCES

- Abbott, D. A., R. M. Zelle, J. T. Pronk, and A. J. van Maris. 2009. Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: current status and challenges. *FEMS Yeast Res.* 9:1123–1136.
- Babel, W., R. H. Muller, and K. D. Markus. 1983. Improvement of growth yield of yeast on glucose to the maximum by using an additional energy source. *Arch. Microbiol.* 136:203–208.
- Bakker, B. M., K. M. Overkamp, A. J. van Maris, P. Kötter, M. A. Luttik, J. P. Van Dijken, and J. T. Pronk. 2001. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 25:15–37.
- Bruinenberg, P. M., R. Jonker, J. P. van Dijken, and W. A. Scheffers. 1985. Utilization of formate as an additional energy source by glucose-limited chemostat cultures of *Candida utilis* CBS 621 and *Saccharomyces cerevisiae* CBS 8066. *Arch. Microbiol.* 142:302–306.
- Bruinenberg, P. M., J. P. Van Dijken, and W. A. Scheffers. 1983. An enzymic analysis of NADPH production and consumption in *Candida utilis*. *J. Gen. Microbiol.* 129:965–971.
- Bunoust, O., A. Devin, N. Averet, N. Camougrand, and M. Rigoulet. 2005. Competition of electrons to enter the respiratory chain: a new regulatory

- mechanism of oxidative metabolism in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**:3407–3413.
7. Casal, M., S. Paiva, O. Queiros, and I. Soares-Silva. 2008. Transport of carboxylic acids in yeasts. *FEMS Microbiol. Rev.* **32**:974–994.
 8. de Vries, S., and C. A. Marres. 1987. The mitochondrial respiratory chain of yeast. Structure and biosynthesis and the role in cellular metabolism. *Biochim. Biophys. Acta* **895**:205–239.
 9. Elstner, M., C. Andreoli, U. Ahting, I. Tetko, T. Klopstock, T. Meitinger, and H. Prokisch. 2008. MitoP2: an integrative tool for the analysis of the mitochondrial proteome. *Mol. Biotechnol.* **40**:306–315.
 10. Emanuelsson, O., S. Brunak, G. von Heijne, and H. Nielsen. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protoc.* **2**:953–971.
 11. Geertman, J. M., J. P. Van Dijken, and J. T. Pronk. 2006. Engineering NADH metabolism in *Saccharomyces cerevisiae*: formate as an electron donor for glycerol production by anaerobic, glucose-limited chemostat cultures. *FEMS Yeast Res.* **6**:1193–1203.
 12. Gombert, A. K., M. Moreira dos Santos, B. Christensen, and J. Nielsen. 2001. Network identification and flux quantification in the central metabolism of *Saccharomyces cerevisiae* under different conditions of glucose repression. *J. Bacteriol.* **183**:1441–1451.
 13. Guadalupe Medina, V., M. J. H. Almering, A. J. A. van Maris, and J. T. Pronk. 2010. Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. *Appl. Environ. Microbiol.* **76**:190–195.
 14. Heux, S., R. Cachon, and S. Dequin. 2006. Cofactor engineering in *Saccharomyces cerevisiae*: expression of a H₂O-forming NADH oxidase and impact on redox metabolism. *Metab. Eng.* **8**:303–314.
 15. Hou, J., N. F. Lages, M. Oldiges, and G. N. Vemuri. 2009. Metabolic impact of redox cofactor perturbations in *Saccharomyces cerevisiae*. *Metab. Eng.* **11**:253–261.
 16. Hou, J., and G. N. Vemuri. 2010. Using regulatory information to manipulate glycerol metabolism in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **85**:1123–1130.
 17. Jungwirth, H., and K. Kuchler. 2006. Yeast ABC transporters—a tale of sex, stress, drugs and aging. *FEBS Lett.* **580**:1131–1138.
 18. Lee, A. C., X. Xu, E. Blachly-Dyson, M. Forte, and M. Colombini. 1998. The role of yeast VDAC genes on the permeability of the mitochondrial outer membrane. *J. Membr. Biol.* **161**:173–181.
 19. Lee, A. C., X. Xu, and M. Colombini. 1996. The role of pyridine dinucleotides in regulating the permeability of the mitochondrial outer membrane. *J. Biol. Chem.* **271**:26724–26731.
 20. Luo, B., K. Groenke, R. Takors, C. Wandrey, and M. Oldiges. 2007. Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography-mass spectrometry. *J. Chromatogr. A* **1147**:153–164.
 21. Luttk, M. A., K. M. Overkamp, P. Kotter, S. de Vries, J. P. van Dijken, and J. T. Pronk. 1998. The *Saccharomyces cerevisiae* NDE1 and NDE2 genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. *J. Biol. Chem.* **273**:24529–24534.
 22. Marres, C. A., S. de Vries, and L. A. Grivell. 1991. Isolation and inactivation of the nuclear gene encoding the rotenone-insensitive internal NADH: ubiquinone oxidoreductase of mitochondria from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **195**:857–862.
 23. Muller, R. H., K. D. Markuske, and W. Babel. 1985. Formate gradients as a means for detecting the maximum carbon conversion efficiency of heterotrophic substrates: correlation between formate utilization and biomass increase. *Biotechnol. Bioeng.* **27**:1599–1602.
 24. Neuhauser, W., M. Steininger, D. Haltrich, K. D. Kulbe, and B. Nidetzky. 1998. A pH-controlled fed-batch process can overcome inhibition by formate in NADH-dependent enzymatic reductions using formate dehydrogenase-catalyzed coenzyme regeneration. *Biotechnol. Bioeng.* **60**:277–282.
 25. Nevoigt, E. 2008. Progress in metabolic engineering of *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **72**:379–412.
 26. Niggli, V., and E. Sigel. 2008. Anticipating antiport in P-type ATPases. *Trends Biochem. Sci.* **33**:156–160.
 27. Overkamp, K. M., P. Kotter, R. van der Hoek, S. Schoondermark-Stolk, M. A. Luttk, J. P. Van Dijken, and J. T. Pronk. 2002. Functional analysis of structural genes for NAD⁺-dependent formate dehydrogenase in *Saccharomyces cerevisiae*. *Yeast* **19**:509–520.
 28. Petranovic, D., and G. N. Vemuri. 2009. Impact of yeast systems biology on industrial biotechnology. *J. Biotechnol.* **144**:204–211.
 29. Piper, P., Y. Mahe, S. Thompson, R. Pandjaitan, C. Holyoak, R. Egner, M. Muhlbauer, P. Coote, and K. Kuchler. 1998. The pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. *EMBO J.* **17**:4257–4265.
 30. Sass, E., E. Blachinsky, S. Karniely, and O. Pines. 2001. Mitochondrial and cytosolic isoforms of yeast fumarase are derivatives of a single translation product and have identical amino termini. *J. Biol. Chem.* **276**:46111–46117.
 31. Schuller, C., Y. M. Mammun, M. Mollapour, G. Krapf, M. Schuster, B. E. Bauer, P. W. Piper, and K. Kuchler. 2004. Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **15**:706–720.
 32. Sickmann, A., J. Reinders, Y. Wagner, C. Joppich, R. Zahedi, H. E. Meyer, B. Schonfisch, I. Perschil, A. Chacinska, B. Guiard, P. Rehling, N. Pfanner, and C. Meisinger. 2003. The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* **100**:13207–13212.
 33. Smits, H. P., A. Cohen, T. Buttler, J. Nielsen, and L. Olsson. 1998. Cleanup and analysis of sugar phosphates in biological extracts by using solid-phase extraction and anion-exchange chromatography with pulsed amperometric detection. *Anal. Biochem.* **261**:36–42.
 34. Van Dijken, J. P., J. Bauer, L. Brambilla, P. Duboc, J. M. Francois, C. Gancedo, M. L. Giuseppin, J. J. Heijnen, M. Hoare, H. C. Lange, E. A. Madden, P. Niederberger, J. Nielsen, J. L. Parrou, T. Petit, D. Porro, M. Reuss, N. van Riel, M. Rizzi, H. Y. Steensma, C. T. Verrips, J. Vindelov, and J. T. Pronk. 2000. An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme Microb. Technol.* **26**:706–714.
 35. Van Dijken, J. P., and W. A. Scheffers. 1986. Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol. Rev.* **32**:199–224.
 36. Vemuri, G. N., M. A. Eiteman, J. E. McEwen, L. Olsson, and J. Nielsen. 2007. Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A.* **104**:2402–2407.
 37. Venema, K., and M. G. Palmgren. 1995. Metabolic modulation of transport coupling ratio in yeast plasma membrane H⁺-ATPase. *J. Biol. Chem.* **270**:19659–19667.
 38. Verduyn, C., E. Postma, W. A. Scheffers, and J. P. Van Dijken. 1992. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**:501–517.
 39. von Jagow, G., and M. Klingenberg. 1970. Pathways of hydrogen in mitochondria of *Saccharomyces carlsbergensis*. *Eur. J. Biochem.* **12**:583–592.
 40. Wattanachaisaereekul, S., A. E. Lantz, M. L. Nielsen, and J. Nielsen. 2008. Production of the polyketide 6-MSA in yeast engineered for increased malonyl-CoA supply. *Metab. Eng.* **10**:246–254.