ARTICLE

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Improved Product-Per-Glucose Yields in P450-Dependent Propane Biotransformations Using Engineered *Escherichia coli*

Rudi Fasan,[1] Nathan C. Crook,[1] Matthew W. Peters,[2] Peter Meinhold,[2] Thomas Buelter,[2] Marco Landwehr,[2] Patrick C. Cirino,[3] Frances H. Arnold[1]

[1]Division of Chemistry and Chemical Engineering, California Institute of Technology, 1200 E. California Blvd. MC 210-41, Pasadena, California 91125; telephone: (626) 354 2565; fax: (626) 568 8743; e-mail: frances@cheme.caltech.edu

[2]Gevo, Inc., 345 Inverness Drive, Englewood, Colorado 80112

[3]Department of Chemical Engineering, Pennsylvania State University, University Park, Pennsylvania

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ABSTRACT: P450-dependent biotransformations in *Escherichia coli* are attractive for the selective oxidation of organic molecules using mild and sustainable procedures. The overall efficiency of these processes, however, relies on how effectively the NAD(P)H cofactors derived from oxidation of the carbon source are utilized inside the cell to support the heterologous P450-catalyzed reaction. In this work, we investigate the use of metabolic and protein engineering to enhance the product-per-glucose yield (Y_{PPG}) in whole-cell reactions involving a proficient NADPH-dependent P450 propane monooxygenase prepared by directed evolution [$P450_{PMO}R2$; Fasan et al. (2007); Angew Chem Int Ed 46:8414–8418]. Our studies revealed that the metabolism of *E. coli* (W3110) is able to support only a modest propanol:glucose molar ratio ($Y_{PPG} \sim 0.5$) under aerobic, non-growing conditions. By altering key processes involved in NAD(P)H metabolism of the host, considerable improvements of this ratio could be achieved. A metabolically engineered *E. coli* strain featuring partial inactivation of the endogenous respiratory chain (Δndh) combined with removal of two fermentation pathways (ΔadhE, Δldh) provided the highest Y_{PPG} (1.71) among the strains investigated, enabling a 230% more efficient utilization of the energy source (glucose) in the propane biotransformation compared to the native *E. coli* strain. Using an engineered $P450_{PMO}R2$ variant which can utilize NADPH and NADH with equal efficiency, we also established that dual cofactor specificity of the P450 enzyme can provide an appreciable improvement in Y_{PPG}. Kinetic analyses suggest, however, that much more favorable parameters (K_M, k_{cat}) for the NADH-driven reaction are required to effectively compete with the host's endogenous NADH-utilizing enzymes. Overall, the metabolic/protein engineering strategies described here can be of general value for improving the performance of NAD(P)H-dependent whole-cell biotransformations in *E. coli*.

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KEYWORDS: cytochrome P450 monooxygenase; alkane oxidation; BM-3; whole-cell biotransformations; protein engineering; *Escherichia coli*

Introduction

The selective oxidation of unactivated C–H bonds is a challenging chemical transformation of great synthetic and industrial value (Li et al., 2002; Schmid et al., 2001). Cytochrome P450 monooxygenases represent attractive biocatalysts to carry out this task due to their ability to catalyze the oxygenation of allylic, aromatic, and aliphatic C–H bonds under mild reaction conditions (Denisov et al., 2005; Sono et al., 1996). Natural P450s and engineered variants of these enzymes have proven useful for various applications, including the preparation of drug metabolites (Guengerich, 2002; Sawayama et al., 2009) and synthetic intermediates (Bell et al., 2006; Landwehr et al., 2006), the oxidation of natural products (Chang et al., 2007; Sowden et al., 2005) and alkanes (Fasan et al., 2007; Peters et al., 2003; Xu et al., 2005) and for chemo-enzymatic synthesis (Lewis et al., 2009; Rentmeister et al., 2009).

Rudi Fasan's present address is Department of Chemistry, University of Rochester, Rochester, New York 14627.

Correspondence to: Frances H. Arnold

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To support P450-catalyzed oxidations, a supply of reducing equivalents in the form of reduced nicotinamide cofactors NAD(P)H is generally required. While this can be achieved *in vitro* using auxiliary cofactor regeneration systems (Johannes et al., 2007; van der Donk and Zhao, 2003), the use of whole-cell systems offers important advantages (Duetz et al., 2001; Li et al., 2002) as NAD(P)H cofactors can be regenerated through the oxidation of inexpensive biomass-derived sugars within a metabolically active cell (Faber, 2004; Li et al., 2002; Schmid et al., 2001). A key aspect of these processes is the net balance between the energy input (glucose) and product output. This parameter, which can be expressed as the molar ratio between the product formed and the glucose consumed (Y_{PPG}), ultimately depends on the efficiency with which the reducing equivalents derived from glucose catabolism are channeled towards the heterologous NAD(P)H-dependent reaction in the presence of various cellular processes competing for the same cofactor(s). In previous studies, product-per-glucose yields (Y_{PPG}) have been analyzed in the context of biotransformations using heterologous dehydrogenases and reductases (Chin et al., 2009; Cirino et al., 2006; Walton and Stewart, 2004). Much scarcer is information about Y_{PPG} achievable in *Escherichia coli* for whole-cell reactions involving P450 enzymes and, especially, about strategies to enhance the efficiency of glucose utilization in these processes.

Mild oxidation catalysts for the selective oxidation of gaseous alkanes are highly sought-after because of their potential utility for the conversion of natural gases into liquid fuels (Olah et al., 2006). To this end, we have recently evolved a proficient P450 propane monooxygenase ($P450_{PMO}R2$) for the conversion of propane to propanol (Fasan et al., 2007). $P450_{PMO}R2$ was derived from $P450_{BM3}$, a self-sufficient P450 from *Bacillus megaterium* with high activity on long-chain (C_{12}–C_{20}) fatty acids (Narhi and Fulco, 1987). As a result of 23 amino acid substitutions and significant alteration of the active site configuration (Fasan et al., 2008), $P450_{PMO}R2$ was found to possess "native-like" proficiency in propane oxidation (370 turnovers/min, 98% coupling) supporting more than 45,000 turnovers (Fasan et al., 2007). $P450_{PMO}R2$ also showed promise as biocatalyst for whole-cell biotransformation of propane to isopropanol and 1-propanol in 9:1 ratio, providing in vivo activities up to 120 U g^{-1} cell dry weight (cdw) using resting *E. coli* (DH5α) cells (Fasan et al., 2007). Isopropanol could be useful for the production of isopropylene (Kibby and Hall, 1972), as monomer for polypropylene manufacturing, and for the preparation of fatty acid esters with reduced crystallization temperature in biodiesel production (Lee et al., 1995).

In this study, $P450_{PMO}R2$ was used as a model of biotechnologically useful P450 biocatalyst to identify metabolic engineering strategies to increase the efficiency of glucose utilization in supplying reducing equivalents to support a heterologous P450 reaction in *E. coli*. An experimental platform was set up for accurately measuring Y_{PPG} in the absence of potential limiting factors or biases. Using this system, the performance of engineered *E. coli* strains, prepared with the purpose of increasing the intracellular pool of NADPH available to the P450 enzyme, was investigated. A $P450_{PMO}R2$ derivative with dual NADPH/NADH cofactor specificity was also engineered in order to establish whether this property can increase the propanol-per-glucose yield by enabling the enzyme to recruit reducing equivalents from both the NADPH and NADH pools in the cell.

Materials and Methods

Strains, Plasmids, and Protein Purification

Strains and plasmids used in this study are listed in Table I. P450s were expressed from pCWori-based vectors containing the P450 gene under the control of a double *tac* promoter (*Bam*HI/*Eco*RI cassette). Amino acid mutation in $P450_{PMO}R2$ and 1–3 were reported previously (Fasan et al., 2007). The dual cofactor specificity dc1-12G was derived from 1-12G (Peters et al., 2003) by randomization of

Table I. Engineered *E. coli* strains and plasmids used in this study.

Strain/plasmids	Relevant features	Reference
Strains		
W3100	—	ATCC 27325
Zwf	W3110 Δ*zwf*:: FRT-*kan*-FRT (Kan^R)	This study
Pgi	W3110 Δ*pgi*:: FRT-*kan*-FRT (Kan^R)	This study
NdhNuo	W3110 Δ*nuoA-N*Δ*ndh*:: FRT-*kan*-FRT (Kan^R)	This study
NdhNuoAdhLdh	W3110 Δ*nuoA-N* Δ*ndh* Δ*adhE* Δ*ldh*:: FRT-*kan*-FRT (Kan^R)	This study
NdhAdhLdh	W3110 Δ*ndh* Δ*adhE* Δ*ldh*:: FRT-*kan*-FRT (Kan^R)	This study
NdhAdhLdhPtaAckA	W3110 Δ*ndh* Δ*adhE* Δ*ldh* Δ*pta-ackA*:: FRT-*kan*-FRT (Kan^R)	This study
NdhAdhLdhPgi	W3110 Δ*ndh* Δ*adhE* Δ*ldh* Δ*pgi*:: FRT-*kan*-FRT (Kan^R)	This study
Plasmids		
pCWori	*bla*, pBR322-origin vector for expression of P450 variants under control of double *tac* promoter, contains $lacI^q$ gene	Barnes et al. (1991)
$pP450_{PMO}R2$	pCWori encoding for $P450_{PMO}R2$ variant	Fasan et al. (2007)
p1-3	pCWori encoding for 1–3 variant	Fasan et al. (2007)
$pcsP450_{PMO}R2$	pCWori encoding for $csP450_{PMO}R2$ variant	This study

position R966, K972, Y974, and W1046 followed by screening for increased activity in the presence of NADPH and NADH on hexylmethylether (Peters et al., 2003). To prepare dcP450$_{PMO}$R2, the C-terminal portion (772–1049) of dc1-12G was fused to P450$_{PMO}$R2(1-771) by overlap extension PCR using primers BMfor_2315 (5′-CGGTCTGCCCGCCGCATAAAG-3′) and BM3RedRev (5′-CCGGGCTCAGATCTGCTCATGTTTGACAGC-3′). P450$_{BM3}$ variants were expressed in Terrific Broth medium (ampicillin, 100 μg L^{-1}) and purified by ion exchange chromatography as described previously (Fasan et al., 2007). P450 concentration was determined from CO-binding difference spectra ($\varepsilon_{450-500} = 91{,}000\ M^{-1}\,cm^{-1}$).

Engineered Strains

The engineered *E. coli* strains were prepared using W3110 as parent strain. Pgi and Zwf were prepared by P1 phage transduction using single gene deletion K-12 strains from the Keio collection (Baba et al., 2006). The other strains were prepared using the recombination method with phage λ-red recombinase (Datsenko and Wanner, 2000). Plasmid pKD46 was used to express the recombinase. To prepare NdhNuo, W3110(Δndh) and W3110(ΔnuoA-N) were first prepared by transforming W3110(pKD46) with the PCR product obtained using pKD13 template (Datsenko and Wanner, 2000) with primers ndhF and ndhR for W3110(Δndh) and primers nuoA_NF and nuoA_NR for W3110(ΔnuoA-N). After removing the FRT-flanked *kan* gene in W3110(Δndh) with FLP recombinase (Causey et al., 2003), the ΔnuoA-N deletion was transferred into this strain using P1 phage transduction, resulting in NdhNuo. W3110(ΔldhA) was constructed by transforming W3110(pKD46) with the PCR product obtained using primers ldhA_ko_f and ldhA_ko_r and pKD13 as template. W3110(ΔadhE) was constructed by transforming W3110(pKD46) with the PCR product obtained using primers adhE_ko_f and adhE_ko_r and pKD13 as template. NdhNuoAdhE was prepared by first removing the *kan* gene from NdhNuo and then transducing ΔadhE mutation from W3110(ΔadhE). NdhNuoAdhE(Kan^R) was treated with FLP recombinase and then transduced with W3110(ΔldhA) P1 phage lysate, resulting in NdhNuoAdhLdh. Similarly, NdhAdhLdh was prepared starting from Ndh strain. NdhAdhLdhPgi and NdhAdhLdhPtaAckA were prepared transforming NdhAdhLdh(pKD46) cells with the PCR product obtained using primers pgi_KO_for/pgi_KO_rev and ackA-pta_KO_for/ackA-pta_KO_rev, respectively. All chromosomal deletions were verified by colony PCR and sequencing. Sequences of the primers are provided in the Supplementary Information.

Media and Cell Cultures

Freshly transformed *E. coli* cells were grown (37°C, 220 rpm) in 50 mL M9Y medium (1 L: 31 g Na_2HPO_4, 15 g KH_2PO_4, 2.5 g NaCl, 5.0 g NH_4Cl, 0.24 g $MgSO_4$, 0.01 g $CaCl_2$, 1.5% yeast extract, 1 mL micronutrients) and induced with IPTG (0.25 mM) and δ-aminolevulinic acid (0.25 mM) at $OD_{600} = 1.2$. Cultures were harvested after 12 h (27°C, 200 rpm) and resuspended ($OD_{600} = 2.0$) in nitrogen-free M9 medium (1 L: 31 g Na_2HPO_4, 15 g KH_2PO_4, 2.5 g NaCl, 0.24 g $MgSO_4$, 0.01 g $CaCl_2$, 1 mL micronutrients). The micronutrient solution contains 0.15 mM $(NH_4)_6Mo_7O_{24}$, 20.0 mM H_3BO_3, 1.5 mM $CoCl_2$, 0.5 mM $CuSO_4$, 4.0 mM $MnCl_2$, and 0.5 mM $ZnSO_4$. Aliquots of the cell suspension were used for determination of the cdw (6 mL) and P450 expression level (10 mL) and the remaining (80 mL) used for the whole-cell reactions.

Whole-Cell Reactions

Biotransformations were carried out in a 100 mL-fermentor at 25°C using a 1:1 mixture of propane and air at a flow rate of 5 L h^{-1}, and a glucose concentration of 2 mM maintained through feeding at 30-min intervals. Total propanol (1- and 2-propanol) produced was determined by GC according to described analytical procedures (Fasan et al., 2007). The amount of glucose consumed was determined using the QuantiChrom Glucose Assay Kit (BioAssay Systems, Hayward, CA). Experiments were carried out at least in duplicate. Excreted metabolites (α-ketoglutarate, pyruvate, succinate, lactate, fumarate, and acetate) were quantified by HPLC using a Rezex ROA column (Phenomenex) pre-equilibrated at 40°C and isocratic elution with 0.008 N H_2SO_4. Quantification was carried out using calibration curves prepared with authentic standards. P450 concentration was determined by CO binding analysis of the cell lysates. The expression level of the P450 variants in the W3110-derived strains was comparable to that observed in W3110 and ranged between 1.5% and 2.5% of the total cell mass. No significant changes in the Y_{PPG} of the catalyst/strain system were observed within two-fold variation in the P450 expression level.

Catalytic and Kinetic Properties of the P450 Variants

The catalytic properties of the P450 variants for NADPH- and NADH-driven propane oxidation were measured according to described methods (Fasan et al., 2007). Kinetic parameters for NAD(P)H oxidation were determined by steady-state kinetic analysis using the cytochrome c reduction assay (Neeli et al., 2005). Reactions were carried using 20 nM P450 and 50 μg mL^{-1} bovine cytochrome *c*. Initial cytochrome *c* reduction rates were measured over 20 s using an $\varepsilon_{550} = 22{,}640\ M^{-1}\,cm^{-1}$. K_M and k_{cat} values were estimated from least-squares nonlinear regression to the Michaelis–Menten equation from saturation plots of v_o (initial rate) versus NAD(P)H concentration. All activity and kinetic measurements were performed at least in triplicate.

Results

Experimental System for Accurate Measurement of Y_{PPG}

Various factors may affect Y_{PPG} analysis in whole-cell reactions involving oxygenases. In addition to potential substrate- or product-dependent toxicity and substrate availability limitations (Buhler et al., 2008; Walton and Stewart, 2004), the need for molecular oxygen for activity, cofactor uncoupling, and potential instability of these biocatalysts could be problematic (Li et al., 2002; van Beilen et al., 2003). Initial efforts were directed at developing an experimental platform for measuring Y_{PPG} where potentially limiting factors are minimized.

Whole-cell reactions were carried out using suspensions of P450-expressing *E. coli* cells (wild-type W3110 and derivatives thereof) exposed to a 1:1 mixture of propane and air using nitrogen-free minimal medium. The nitrogen-free medium helped maintain the cells in a metabolically active, non-growing status where NAD(P)H-utilizing processes required for cellular growth are suppressed and a larger fraction of NAD(P)H becomes available to drive heterologous NAD(P)H-dependent reactions (Walton and Stewart, 2002). Under these conditions, the effects of gene deletions can be studied in the context of a simplified model of *E. coli* metabolism (Fig. 1). The use of non-growing cells also eliminated potential biases in Y_{PPG} analysis due to differences in growth rate (and thus background energy demand) among the engineered *E. coli* strains.

Analysis of the in vivo activity of $P450_{PMO}R2$ expressed in W3110 cells revealed a sharp decrease in the biocatalyst/strain performance at cell densities higher than 1.1 g cdw L^{-1} ($OD_{600} > 5$) (Fig. 2A), presumably as a result of a sub-optimal supply of oxygen to the biocatalyst and/or the cell. A cell density of ~0.4 g cdw L^{-1} ($OD_{600} \sim 2$) was thus utilized to minimize the effect of oxygen transfer on the Y_{PPG}. Substrate transfer rate was not expected to be limiting because of the eight-fold higher solubility of propane in water (~1.6 mM) compared to oxygen, the lack of endogenous processes competing for this substrate, and the low amount of propane consumed per unit of time under the applied conditions (up to 0.02 mM min^{-1}). Saturating levels of propane in the reaction medium also allowed for near-saturation of $P450_{PMO}R2$ and the other $P450_{BM3}$ variants investigated, whose K_M for propane was previously determined to be between 150 and 300 μM (Fasan et al., 2008).

In previous studies, the intracellular concentration of folded $P450_{PMO}R2$ in whole-cell biotransformations was found to decrease over time (Fasan et al., 2007), which was attributed to oxidative self-inactivation of the biocatalyst, a phenomenon observed in whole-cell reactions with other oxygenases as well (van Beilen et al., 2003; Walton and Stewart, 2004). Since the biocatalyst cannot be replenished under non-growing conditions, Y_{PPG} were calculated over

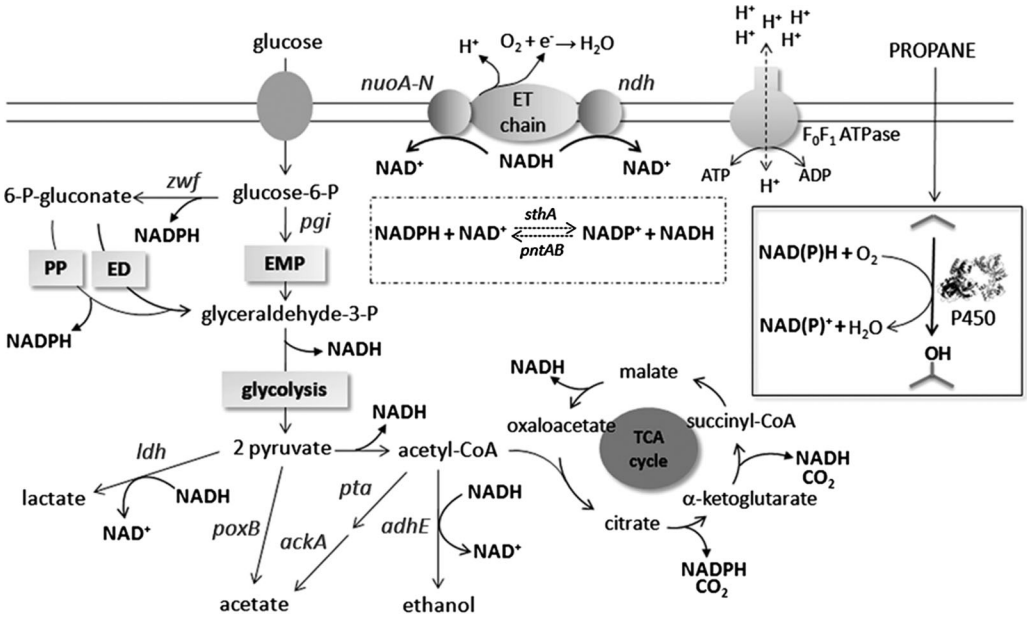

Figure 1. Schematic representation of *E. coli* central metabolism illustrating the major pathways implicated in the metabolism of NAD(P)H under aerobic, non-growing conditions. The heterologous P450-dependent reaction investigated in this work is highlighted (box). The genes targeted for deletion are noted in italics: *ackA* = acetate kinase; *adhE* = acetaldehyde dehydrogenase; *ldh* = lactate dehydrogenase; *ndh* = NADH dehydrogenase II; *nuoA-N* = NADH dehydrogenase I; *pgi* = phosphoglucose isomerase; *pta* = phosphotransacetylase; *zwf* = glucose-6-phosphate-1-dehydrogenase. EMP: Embden-Meyerhof-Parnas pathway, PP: pentose phosphate pathway, ED: Entner-Doudoroff pathway, TCA: tricarboxylic acid. Endogenous THD activity in *E. coli* is dependent on a membrane-bound (*pntAB*) and a soluble pyridine nucleotide THD (*sthA*) (dashed box). In the scheme, the reaction catalyzed by pyruvate oxidase (*poxB*) is also indicated.

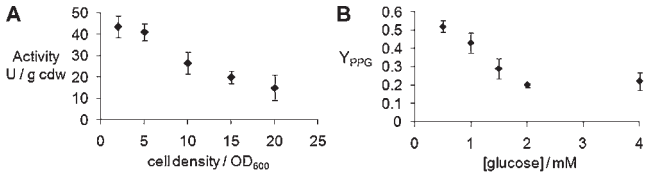

Figure 2. Factors influencing enzyme activity and Y_{PPG} in P450-dependent whole-cell propane biotransformation as measured using $P450_{PMO}R2$ in wild-type *E. coli* (W3110) under non-growing conditions. **A**: Dependence of the enzyme in vivo activity on the cell density. Values refer to activities measured over 30 min at 25°C in whole-cell reactions using air/propane (1:1) feed and minimal M9 medium pH 7.2 supplemented with 20 mM glucose. One unit (U) = 1 μmol product (propanol) per minute; cdw = cell dry weight. **B**: Dependence of Y_{PPG} on the glucose concentration as determined by the molar ratio between propanol produced and glucose consumed after 2 h at a cell density of 0.4–0.45 g cdw L^{-1} (corresponding to $OD_{600} \sim 2.0$ in the reaction vessel).

2 h. Within this time, the concentration of catalytically active P450 remained >70% of the initial value (prior to the reaction) as indicated by CO-binding assay on the cell lysate.

Under our experimental conditions, a progressive decrease in Y_{PPG} and accumulation of acetate up to 2 mM was observed as the glucose concentration was increased from 1 to 4 mM (Fig. 2B). This was consistent with less efficient consumption of the energy source at higher glucose concentrations due to "metabolic overflow" (Akesson et al., 2001). A glucose feeding system was thus implemented to maintain glucose around 0.5 (±0.2) mM throughout the biotransformation, thereby minimizing the effect of metabolic overflow on the Y_{PPG}.

Higher Propanol-Per-Glucose Yields Through Modification of the Glycolytic Pathway

Using the protocols described above, the Y_{PPG} with $P450_{PMO}R2$ in wild-type *E. coli* (W3110) was found to be 0.52 ± 0.5 (Fig. 3A). While much higher product:glucose stoichiometries (2.3–4.2) were reported for NAD(P)H-dependent dehydrogenases and reductases (Chin et al., 2009; Cirino et al., 2006; Walton and Stewart, 2004), previous studies with flavin-dependent monooxygenases indicate that oxygenases may have higher "cofactor requirements" compared to dehydrogenases (Buhler et al., 2008) possibly due to cofactor uncoupling (Blank et al., 2008).

To improve the efficiency of *E. coli* in supporting P450 catalysis, W3110 variants were constructed (Table I), where the major pathways implicated in NAD(P)H metabolism were altered in order to increase the amount of intracellular NADPH available to the P450 enzyme. In *E. coli*, the pentose phosphate (PP) pathway and the NADPH-dependent isocitrate dehydrogenase in the TCA cycle are considered to be the major sources of NADPH during glucose oxidation (Gottschalk, 1986). To analyze the role of the former in providing NADPH for $P450_{PMO}R2$ catalysis, a strain featuring a non-functional PP pathway (Zwf) was prepared. This strain was found to provide a Y_{PPG} of 0.49 ± 0.07, a value only slightly lower than that observed with W3110. This suggested that the relative carbon flux passing through this pathway in resting W3110 cells is small (≤10%), probably reflecting the metabolic pattern of *E. coli* when grown under nitrogen-limited conditions (Hua et al., 2003) rather than in rich media (where this fraction is 20% and 45% (Csonka and Fraenkel, 1977; Sauer et al., 2004)).

Increasing glucose oxidation through the PP pathway was thus expected to increase significantly the overall amount of NADPH available to the P450 reaction. Strain Pgi (W3110 Δpgi), which lacks the enzyme catalyzing the committed step of the glycolytic pathway (phosphoglucose isomerase), was prepared and found to support an Y_{PPG} of 1.33 ± 0.21 (Fig. 3A), which indicated that the single gene deletion enabled a 155% more efficient utilization of glucose for the production of propanol as compared to wild-type *E. coli*.

Removal of Intracellular NADH-Oxidizing Processes Increases Y_{PPG}

Another potential source of NADPH in *E. coli* is through the endogenous transhydrogenase (THD) activity and, in particular, through the membrane-bound PntAB THD, which is believed to catalyze the reversible reduction of $NADP^+$ by NADH using the membrane proton motive force (Hua et al., 2003; Sauer et al., 2004). We reasoned that removing some of the endogenous NADH-utilizing processes would favor transfer of reducing equivalents from NADH to NADPH through the endogenous PntAB-dependent THD activity of the host. To this end, we targeted the major cellular processes contributing to re-oxidation of this cofactor, namely the reactions catalyzed by the NADH dehydrogenases (NDH-I and NDH-II) in the respiratory chain and the fermentation pathways responsible for the production of ethanol and lactate (Fig. 1). First, we evaluated the effect of eliminating both membrane-bound NADH dehydrogenases (strain NdhNuo). Deletion of these enzymes was expected to reduce *E. coli* respiration, thereby reducing competition with the P450 biocatalyst for both reducing power and for oxygen. The Y_{PPG} obtained with strain NdhNuo was almost identical to that of the reference strain (0.55 vs. 0.52, respectively). Despite similar Y_{PPG}, a drastic increase in the amount of secreted fermentation products (primarily lactate and ethanol) was observed with NdhNuo (Fig. 3B), suggesting that the surplus of NADH equivalents is re-directed towards the fermentative pathways rather than towards the production of additional NADPH, perhaps because of the more favorable kinetic parameters of lactate and alcohol dehydrogenases (Shone and Fromm, 1981; Yin and Kirsch, 2007) compared to PtnAB (Hu et al., 1995). Based on this result, another strain was tested where in addition to ΔnuoA-N and Δndh deletions, the genes responsible for lactate and ethanol production had also been removed (strain NdhNuoAdhLdh). This genetic modification resulted in

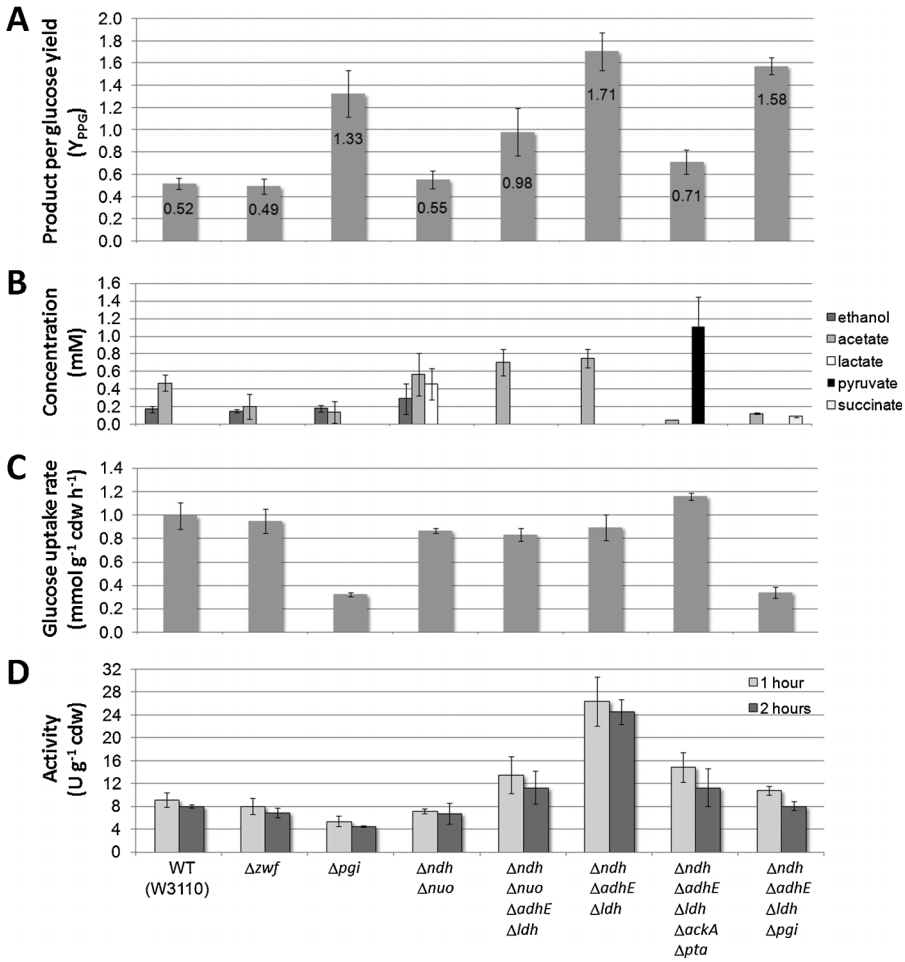

Figure 3. Results from $P450_{PM0}R2$-dependent propane biotransformations with engineered *E. coli* strains. The reference strain is wild-type *E. coli* W3110 and the enzymes corresponding to the deleted genes are listed in Figure 1 legend. **A**: Propanol per glucose yields (Y_{PPG}) as calculated from the molar ratio between the amount of propanol produced (sum of 1- and 2-propanol) and the glucose consumed during the reaction. **B**: Concentration of metabolites accumulated in the reaction medium at the end of the biotransformation as determined by HPLC analysis. **C**: Glucose uptake rate of the different strains as measured during the biotransformations. Values are reported as mmol glucose per gram cdw per hour. **D**: In vivo propane oxidation activity of $P450_{PM0}R2$ as measured after 1 and 2 h. 1 U = 1 μmol propanol min^{-1}.

about a two-fold increase in Y_{PPG} (0.98 ± 0.21) and, consistent with the genotype, only acetate accumulated in the medium as the major secreted byproduct during biotransformation.

While elimination of respiratory and fermentative reactions did result in improved propanol yield, significant quantities of acetate were also produced, which is indicative of overflow metabolism. Effects resulting from insufficient NAD^+ regeneration in glucose-driven metabolism include inhibition of pyruvate dehydrogenase and citrate synthase (Gottschalk, 1986). We therefore tested whether NAD^+ regeneration rates could be elevated to an intermediate level to help alleviate NADH-dependent regulation. Since NDH-II (encoded by *ndh*) is the predominant form of NADH dehydrogenase under aerobic conditions and its NADH oxidation activity tends to be highly uncoupled to proton translocation (Calhoun et al., 1993; Unden and Bongaerts, 1997), a strain lacking *ndh* in combination with Δldh and ΔadhE was prepared (strain NdhAdhLdh). This resulted in a considerable improvement in Y_{PPG} (1.71 ± 0.17), indicating that the altered metabolism of NdhAdhLdh enables a 230% more efficient utilization of glucose to drive the heterologous P450-dependent reaction than possible with wild-type *E. coli*.

In an attempt to further increase the Y_{PPG} by recovering the glucose-derived energy dissipated through acetate secretion by NdhAdhLdh (Fig. 3B), the genes encoding for phosphotransacetylase (*pta*) and acetate kinase (*ackA*) were also removed, resulting in strain NdhAdhLdhPtaAckA. The Y_{PPG} obtained with this strain however was only 0.71 ± 0.11, and large quantities of pyruvate were secreted as a result of the new deletions (Fig. 3B), which had not been detected with any of the other strains. The accumulation of pyruvate in cultures of *pta-ackA*-deficient strains has been observed before (Yang et al., 1999).

As the best performing strain among the NADH dehydrogenase-deficient variants, NdhAdhLdh was then used as parent strain to introduce the Δpgi mutation

Table II. Catalytic and kinetic properties of the $P450_{BM3}$ variants.

	$P450_{PMO}R2$	$dcP450_{PMO}R2$	1–3	$P450_{BM3}$
NADPH				
Propanol rate (min^{-1})[a]	370 ± 35	122 ± 5	320 ± 15	0
% Coupling[b]	98%	76%	72%	0
K_M (μM)[c]	6.6 ± 0.7	28.2 ± 5.8	12.8 ± 0.5	6.6 ± 0.4
k_{cat} (min^{-1})[c]	2,460 ± 175	1,440 ± 25	6,050 ± 115	6,010 ± 180
NADH				
Propanol rate (min^{-1})[a]	0	144 ± 12	0	0
% Coupling[b]	0	62%	0	0
K_M (μM)[c]	8,350 ± 380	52.5 ± 3.0	14,900 ± 650	6,230 ± 260
k_{cat} (min^{-1})[c]	4,390 ± 340	1,650 ± 200	3,870 ± 70	2,130 ± 120

[a]Initial (20 s) propanol formation rates as determined by GC.
[b]Coupling efficiency as given by the ratio between propanol formation rate and cofactor (either NADPH or NADH) consumption rate in propane-saturated buffer.
[c]Kinetic parameters for NAD(P)H cofactors as determined from kinetic analyses using cytochrome *c* reduction assay. K_M and k_{cat} values for $P450_{BM3}$ are consistent with those reported in literature by Munro and co-workers (K_M(NADPH) = 7.2 μM; k_{cat}(NADPH) = 6,720 min^{-1}; K_M(NADH) = 12,800 μM; k_{cat}(NADH) = 1,032 min^{-1} (Neeli et al., 2005)).

previously found to be beneficial. However the Y_{PPG} obtained with the resulting strain (NdhAdhLdhPgi) did not improve compared to NdhAdhLdh (1.58 ± 0.08 compared to 1.71 ± 0.17). Despite similar Y_{PPG}, the metabolite profiles and the glucose uptake rates of the two strains showed notable differences. The amount of acetate accumulated in the reaction with NdhAdhLdhPgi was <20% of that observed with the NdhAdhLdh strain. Also, detectable quantities of the TCA cycle intermediate succinate were observed with NdhAdhLdhPgi. Compared to NdhAdhLdh, NdhAdhLdhPgi showed a considerably reduced (−60%) glucose uptake rate (0.34 vs. 0.89 mmol g^{-1} cdw h^{-1}). A similar effect (−67%) was observed after deletion of the *pgi* gene in the wild-type strain (0.32 mmol g^{-1} cdw h^{-1} for Pgi compared to 0.99 mmol g^{-1} cdw h^{-1} for W3110, Fig. 3C). The negative effect of Δpgi on the *E. coli* glucose uptake rate has been observed previously (Canonaco et al., 2001; Chin et al., 2009; Sauer et al., 2004), and it is enhanced when cells are grown under nitrogen-limited conditions (Hua et al., 2003).

While the in vivo activity of $P450_{PMO}R2$ in W3110 cells at low glucose concentration (0.5 mM) is lower than that measured at high glucose concentration (20 mM) (9.2 ± 1.3 (1 h) vs. 43.6 ± 5.0 U g^{-1} cdw (0.5 h)), the measured Y_{PPG} under the former conditions is about 2.5-fold higher (0.5 vs. 0.2) indicating that glucose is utilized more efficiently. Across the strains (Fig. 3D), the increase in Y_{PPG} resulting from the gene deletions was reflected by an increase in the biocatalyst in vivo activity with the exception of the strains carrying the Δpgi deletion, whose glucose uptake rates are reduced compared to the others.

P450 Propane Monooxygenase With Dual NADH/NADPH Cofactor Specificity

Next, we reasoned that a P450 enzyme which can utilize both NADPH and NADH would harvest reducing equivalents more readily than a strictly NADPH-dependent isoform, possibly eliminating potential energy losses and overcoming thermodynamic limitations associated with THD activity (Sauer et al., 2004). To test this hypothesis, we prepared a $P450_{PMO}R2$-derived variant with dual cofactor specificity. First, studies were conducted to confirm that $P450_{PMO}R2$ is exclusively driven by NADPH as $P450_{BM3}$ is. Despite two mutations near the NADPH-binding site (D698G and I710T), $P450_{PMO}R2$ was found to share with $P450_{BM3}$ similar K_M for NADPH and NADH (Table II). Also, $P450_{PMO}R2$ produced no propanol in the presence of 4 mM NADH, which far exceeds the physiological levels of this cofactor in *E. coli* (Bennett et al., 2009). To engineer a $P450_{PMO}R2$ variant with dual cofactor specificity, the amino acid residues primarily involved in NADPH recognition were targeted for mutagenesis (Fig. 4). Switching of $P450_{BM3}$ cofactor specificity has been reported (Maurer et al., 2005; Neeli et al., 2005). Our goal however was to obtain a P450 variant capable of utilizing both NADPH and NADH at physiological relevant conditions. A set of amino acid substitutions (R966N, K972H, Y974F, and W1046D) were found to confer dual cofactor specificity to an alkane-oxidizing variant of $P450_{BM3}$ (J.A. Rodriguez and F.H. Arnold, unpublished results). The introduction of these mutations into $P450_{PMO}R2$ led to $dcP450_{PMO}R2$, which catalyzes either NADPH- or NADH-driven propane oxidation with good catalytic rates (122 and 144 turnovers/min, respectively) and coupling efficiency (76% and 62%, respectively) (Table II). Importantly, the K_M of $dcP450_{PMO}R2$ for NADPH and NADH were determined to be between 3- and 10-fold lower than the expected concentration of these cofactors in *E. coli* (Bennett et al., 2009), indicating that this enzyme can support both NADPH- and NADH-dependent propane oxidation in vivo (Table II).

Y_{PPG} values were measured with $dcP450_{PMO}R2$ in W3110 and the other strains, resulting in values from 30% to 50% lower than those obtained with $P450_{PMO}R2$. This difference is likely due to the lower coupling efficiency and less favorable catalytic rate of $dcP450_{PMO}R2$ compared to $P450_{PMO}R2$. To better evaluate the impact of cofactor

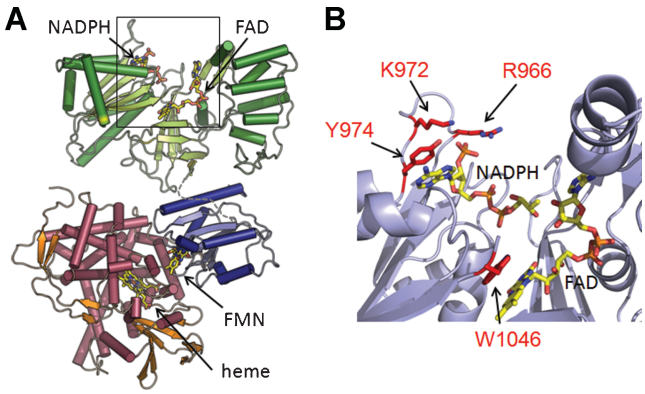

Figure 4. Engineering of $P450_{PM0}R2$ NADPH-binding site to produce a propane monooxygenase with dual cofactor specificity. **A**: Three-dimensional representation of $P450_{BM3}$ structure prepared using the crystal structure of the heme and FMN domain (Li and Poulos, 1997) and a homology model of $P450_{BM3}$ FAD-binding domain (Fasan et al., 2007). Arrows indicate the prosthetic groups and cofactors of the enzyme: FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate. **B**: Enlarged view of the region of $P450_{BM3}$ targeted for mutagenesis (corresponding to the boxed area in panel A). Dual cofactor specificity was achieved by mutating R966, K972, Y974, and W1046, which are primarily involved in NADPH binding as indicated by sequence alignment (Neeli et al., 2005) and inspection of $P450_{BM3}$ reductase domain model (Fasan et al., 2007). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

specificity on Y_{PPG} without biases due to differences in coupling properties, the performance of $dcP450_{PMO}R2$ in W3110, Zwf and Pgi strains was compared with that of variant 1–3 (Fasan et al., 2007), which shares with $dcP450_{PMO}R2$ similar coupling efficiency in propane oxidation and K_M for NADPH (Table II). The Y_{PPG} values obtained with $dcP450_{PMO}R2$-expressing cells were found to be higher than those obtained with 1–3 (Fig. 5A). These results indicate that the NADH/NADPH-utilizing variant is indeed able to retrieve extra reducing equivalents compared to the strictly NADPH-dependent isoform. Interestingly, the increase in Y_{PPG} varied with the genotype of the host, being relatively large in Zwf (+90%), moderate in W3110 (+40%), and small in Pgi (+20%). This likely reflects a difference in the relative levels of NADPH and NADH between these strains. The much lower performance of 1–3 compared to $P450_{PMO}R2$ also highlighted the impact of the coupling efficiency on the Y_{PPG}, considering that these P450 variants have comparable propane turnover rates and K_M for NADPH (Table II).

As evinced from the data in Figures 3C and 5B, the glucose uptake rate of the strains did not appear to be significantly affected (±20%) by the type of P450 variant expressed in the cell. Importantly, the metabolite profiles observed in the whole-cell reactions with $dcP450_{PMO}R2$ and $P450_{PMO}R2$ were virtually identical. With NdhNuo, acetate, lactate, and ethanol concentrations were 0.56 (±0.20), 0.45 (±0.18), and 0.25 (±0.14) mM for $dcP450_{PMO}R2$ and 0.58 (±0.14), 0.48 (± 0.21), and 0.23 (± 0.09) mM for $P450_{PMO}R2$, respectively. The negligible differences in the amount of secreted metabolites even in the presence of excess NADH (as in NdhNuo) suggested that $dcP450_{PMO}R2$ competes only poorly with the endogenous lactate dehydrogenase and acetaldehyde/alcohol dehydrogenase for NADH oxidation.

Discussion

P450-dependent biotransformations have attracted much interest as environmentally friendly strategies for carrying out oxidations of synthetic relevance (Chang et al., 2007; Pandey et al., 2010; Sowden et al., 2005; van Beilen et al., 2005). Analyzing the propanol-per-glucose yield in $P450_{PMO}R2$-dependent whole-cell reactions, we determined that the native metabolic network of *E. coli* is fairly inefficient in supporting the activity of the heterologous P450 enzyme through glucose oxidation ($Y_{PPG} \sim 0.5$). Since $P450_{PMO}R2$ features "native-like" catalytic properties (high coupling, turnover rate and total turnover numbers (Fasan et al., 2007) and a k_{cat}/K_M for propane oxidation of $\sim 5 \times 10^4\ M^{-1}\ s^{-1}$ (Fasan et al., 2008)), this yield is probably representative of those achievable in biotransformations involving natural P450 monooxygenases and possibly other NAD(P)H-dependent oxygenases (Buhler et al., 2008; Walton and Stewart, 2004).

In this study, the three strategies investigated to improve this yield were: (a) *directly* increasing NADPH production in the cell by increasing flux through NADPH-producing reactions (PP pathway); (b) *indirectly* increasing NADPH production (via endogenous THD activity) by eliminating competition for NADH (Δndh, ΔnuoA-N, Δldh, and ΔadhE); and (c) using a dual-cofactor-specificity mutant of the P450 propane monooxygenase. While all these approaches led to appreciable increases in the Y_{PPG}, the use of a NADPH-dependent propane monooxygenase and an engineered *E. coli* strain with the triple knockout Δndh, ΔadhE, and Δldh (strategy "b") provided the best solution among those tested, enabling a 3.3-fold more efficient utilization of glucose to drive the P450 reaction compared to W3110.

Increasing the carbon flux through the PP pathway is often pursued (Chin et al., 2009; Lim et al., 2002; Poulsen et al., 2005; Skorupa Parachin et al., 2009) or proposed (Walton and Stewart, 2004) as a strategy to increase the efficiency of NADPH-dependent reactions in whole-cell systems. Our results indicate that while the Δpgi mutation improves the Y_{PPG} (2.6-fold), this benefit is counterbalanced by a decrease in the specific in vivo activity of the biocatalyst (Fig. 3D), which in the case of $P450_{PMO}R2$ drops from $8.0 \pm 0.3\ U\ g^{-1}$ cdw in W3110 to $4.5 \pm 0.1\ U\ g^{-1}$ cdw in Pgi as a result of the much reduced glucose uptake rate of the latter strain (Fig. 3C). In contrast, the altered metabolism of strain NdhLdhAdhE resulted in a 3.3-fold higher Y_{PPG} without altering significantly the glucose uptake rate of the host, which thus translated into a proportionally large increase in the in vivo activity of the biocatalyst ($24.6 \pm 2.2\ U\ g^{-1}$ cdw). The effect of Δpgi mutation on the

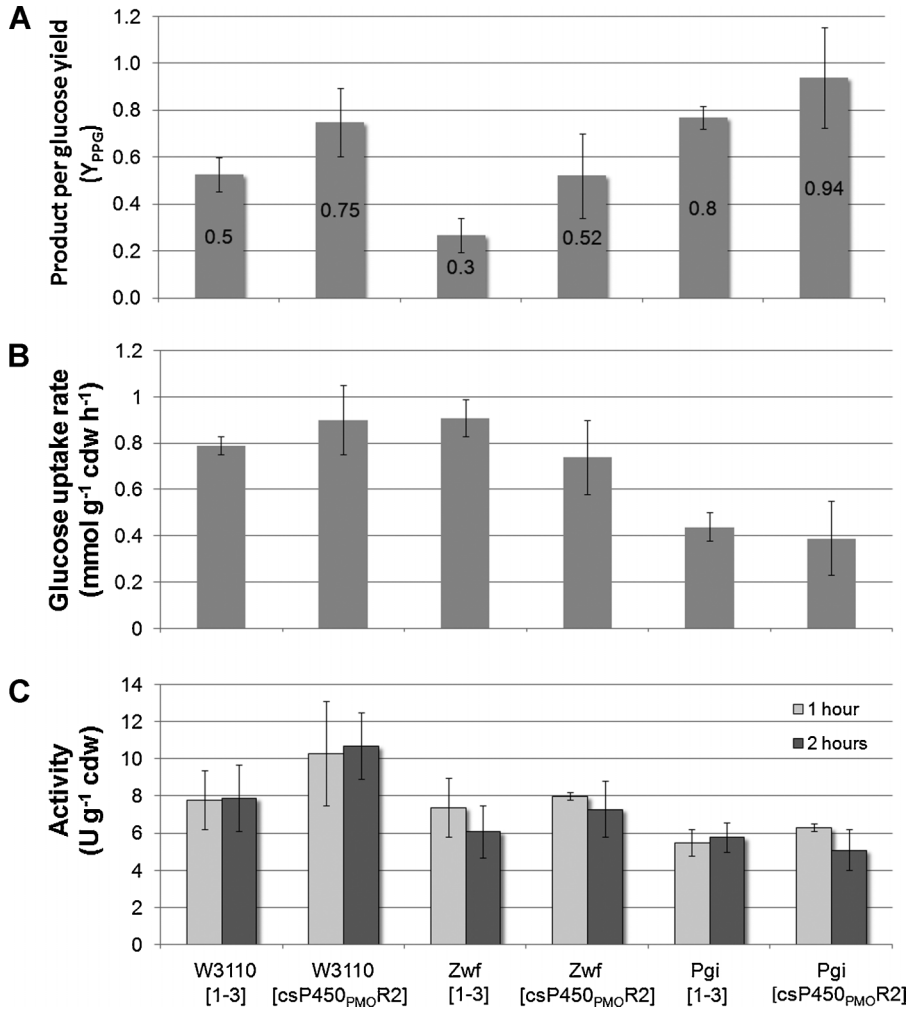

Figure 5. Side-by-side comparison of the performance of W3110, Zwf, and Pgi strains expressing either the NADPH-utilizing P450 propane monooxygenase 1–3 or the NADPH/NADH-utilizing variant dcP450$_{PMO}$R2. **A**: Propanol-per-glucose yields as measured after 2 h. **B**: Glucose uptake rates of the strains expressed in mmol glucose per gram cdw per hour. **C**: In vivo propane oxidation activity of the P450 variants as measured after 1 and 2 h. 1 U = 1 μmol propanol min^{-1}.

glucose uptake rate of the strain and on the catalyst in vivo activity is also apparent from the studies with 1–3 and csP450$_{PMO}$R2 (Fig. 5).

Removing respiratory NADH dehydrogenases proved effective in increasing the Y_{PPG} of the propane biotransformations. In particular, eliminating only the *ndh* gene (strain NdhAdhLdh) resulted in a yield that was 70% higher than that of the strain having deletions in both *ndh* and *nuo* (NdhNuoAdhLdh). It is interesting to note that despite the difference in Y_{PPG}, the level of acetate produced by these two strains is similar, which suggests that more reducing power is made available for propanol production in NdhAdhLdh, even though glucose oxidation was not increased. One possible explanation is that in NdhNuoAdhLdh a greater portion of acetate production proceeds through pyruvate oxidase (PoxB) (Fig. 1), in which reducing equivalents are delivered directly to the electron transport chain, rather than via pyruvate dehydrogenase which generates an additional NADH. Another explanation may be that the relative PP pathway and glycolytic fluxes differ between the two strains, resulting in different NADPH versus NADH production ratios.

The higher yields of strains NdhNuoAdhLdh and NdhAdhLdh compared to NdhNuo also indicate that eliminating fermentative routes is important for improving NADPH supply, either by reducing competition with the THD for NADH or by directly re-routing carbon flux through NADPH-producing reactions (e.g., in the PP pathway). The latter possibility is supported by the observation that removal of *pgi* in NdhAdhLdh does not further improve the Y_{PPG}. Activation of the glyoxylate shunt, and thus reduced NADPH output from the TCA cycle, has been observed in phosphoglucose isomerase-deficient *E. coli* and this may contribute to the lower yields observed with NdhAdhLdhPgi (Hua et al., 2003). It is also possible that as a result of the Δpgi mutation the THD reaction becomes

thermodynamically limited from further increasing NADPH levels in the cell. While calculation of the exact number of reducing equivalents channeled to the P450 reaction in these strains is not possible due to uncertainty with respect to the coupling efficiency of the monoxygenase in vivo, we can conclude that the additional amount of NADPH made available for the P450 reaction as a result of the triple gene deletion in NdhAdhLdh corresponds to more than two times that available in the wild-type strain.

With dcP450$_{PMO}$R2, we determined that a P450 with dual cofactor specificity can lead to relative Y_{PPG} improvements of up to 90% compared to a strictly NADPH-dependent isoform. The benefit provided by the relaxed cofactor specificity, however, appears to be off-set by the less favorable catalytic properties of this variant compared to P450$_{PMO}$R2, making *E. coli* cells expressing dcP450$_{PMO}$R2 a comparatively less efficient biocatalytic system. The relatively poor affinity of dcP450$_{PMO}$R2 for NADH also likely limits its NADH-dependent activity in vivo. A NADH/NADPH-utilizing P450 variant with more favorable kinetic and coupling properties is therefore expected to provide even higher yields than currently achieved.

Although a glucose feeding strategy was implemented to minimize overflow metabolism, Y_{PPG} values in this study could be significantly improved by reducing secretion of glucose byproducts and more completely oxidizing glucose. For example, quantification of secreted metabolites (ethanol and acetate) for strains W3110, Pgi, and NdhAdhLdh reveals that about 50%, 10%, and 25% of glucose carbon, respectively, is lost through fermentation rather than being further oxidized through the TCA cycle. With fermentative pathways deleted in strain NdhAdhLdhPtaAckA, large amounts of pyruvate were instead secreted. Even when correcting for the unobtainable reducing equivalents present in secreted byproducts, however, the resulting Y_{PPG} calculations appear to be lower than theoretically achievable. Similar results were reported for the case of NADPH-dependent xylose reduction to xylitol during glucose oxidation in the absence of acetate secretion (Cirino et al., 2006) or when correcting for secreted reducing power (Chin et al., 2009). Reducing metabolic competition for reducing equivalents, overcoming NADH-dependent metabolic regulation, and eliminating metabolic inefficiencies leading to "wasted" energy are all strategies expected to further improve reduced cofactor regeneration for whole-cell biocatalysis.

In summary, we report gene knock-out combinations which can significantly increase the product-per-glucose productivity of *E. coli* for P450$_{PMO}$R2-dependent reactions. Insights were gained with respect to features of the host and biocatalyst that could be modified to achieve further improvements in the Y_{PPG}. These analyses will help guide metabolic and protein engineering strategies for improving the performance of this and other biotechnologically important P450-dependent biotransformations in *E. coli*.

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References

Akesson M, Hagander P, Axelsson JP. 2001. Avoiding acetate accumulation in *Escherichia coli* cultures using feedback control of glucose feeding. Biotechnol Bioeng 73(3):223–230.

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. Mol Syst Biol 2:2006.0008.

Barnes HJ, Arlotto MP, Waterman MR. 1991. Expression and enzymatic activity of recombinant cytochrome P450 17 alpha-hydroxylase in *Escherichia coli*. Proc Natl Acad Sci USA 88(13):5597–5601.

Bell SG, Hoskins N, Xu F, Caprotti D, Rao Z, Wong LL. 2006. Cytochrome P450 enzymes from the metabolically diverse bacterium *Rhodopseudomonas palustris*. Biochem Biophys Res Commun 342(1):191–196.

Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD. 2009. Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. Nat Chem Biol 5(8):593–599.

Blank LM, Ebert BE, Buhler B, Schmid A. 2008. Metabolic capacity estimation of *Escherichia coli* as a platform for redox biocatalysis: Constraint-based modeling and experimental verification. Biotechnol Bioeng 100(6):1050–1065.

Buhler B, Park JB, Blank LM, Schmid A. 2008. NADH availability limits asymmetric biocatalytic epoxidation in a growing recombinant *Escherichia coli* strain. Appl Environ Microbiol 74(5):1436–1446.

Calhoun MW, Oden KL, Gennis RB, de Mattos MJ, Neijssel OM. 1993. Energetic efficiency of *Escherichia coli*: Effects of mutations in components of the aerobic respiratory chain. J Bacteriol 175(10):3020–3025.

Canonaco F, Hess TA, Heri S, Wang T, Szyperski T, Sauer U. 2001. Metabolic flux response to phosphoglucose isomerase knock-out in *Escherichia coli* and impact of overexpression of the soluble transhydrogenase UdhA. FEMS Microbiol Lett 204(2):247–252.

Causey TB, Zhou S, Shanmugam KT, Ingram LO. 2003. Engineering the metabolism of *Escherichia coli* W3110 for the conversion of sugar to redox-neutral and oxidized products: Homoacetate production. Proc Natl Acad Sci USA 100(3):825–832.

Chang MC, Eachus RA, Trieu W, Ro DK, Keasling JD. 2007. Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. Nat Chem Biol 3(5):274–277.

Chin JW, Khankal R, Monroe CA, Maranas CD, Cirino PC. 2009. Analysis of NADPH supply during xylitol production by engineered *Escherichia coli*. Biotechnol Bioeng 102(1):209–220.

Cirino PC, Chin JW, Ingram LO. 2006. Engineering *Escherichia coli* for xylitol production from glucose-xylose mixtures. Biotechnol Bioeng 95(6):1167–1176.

Csonka LN, Fraenkel DG. 1977. Pathways of NADPH formation in *Escherichia coli*. J Biol Chem 252(10):3382–3391.

Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97(12):6640–6645.

Denisov IG, Makris TM, Sligar SG, Schlichting I. 2005. Structure and chemistry of cytochrome P450. Chem Rev 105(6):2253–2277.

Duetz WA, van Beilen JB, Witholt B. 2001. Using proteins in their natural environment: Potential and limitations of microbial whole-cell hydroxylations in applied biosynthesis. Curr Opin Biotechnol 12:419–425.

Faber K. 2004. Biotransformations in organic chemistry. Berlin: Springer-Verlag.

Fasan R, Chen MM, Crook NC, Arnold FH. 2007. Engineered alkane-hydroxylating cytochrome P450(BM3) exhibiting nativelike catalytic properties. Angew Chem Int Ed Engl 46(44):8414–8418.

Fasan R, Meharenna YT, Snow CD, Poulos TL, Arnold FH. 2008. Evolutionary history of a specialized P450 propane monooxygenase. J Mol Biol 383(5):1069–1080.

Gottschalk G. 1986. Bacterial metabolism. New York, NY: Springer-Verlag.
Guengerich FP. 2002. Cytochrome P450 enzymes in the generation of commercial products. Nat Rev Drug Discov 1(5):359–366.
Hu X, Zhang JW, Persson A, Rydstrom J. 1995. Characterization of the interaction of NADH with proton pumping *E. coli* transhydrogenase reconstituted in the absence and in the presence of bacteriorhodopsin. Biochim Biophys Acta 1229(1):64–72.
Hua Q, Yang C, Baba T, Mori H, Shimizu K. 2003. Responses of the central metabolism in *Escherichia coli* to phosphoglucose isomerase and glucose-6-phosphate dehydrogenase knockouts. J Bacteriol 185(24):7053–7067.
Johannes TW, Woodyer RD, Zhao H. 2007. Efficient regeneration of NADPH using an engineered phosphite dehydrogenase. Biotechnol Bioeng 96(1):18–26.
Kibby CL, Hall WK. 1972. Studies of acid catalyzed reactions. XII. Alcohol decomposition over hydroxyapatite catalysis. J Catal 29:144–159.
Landwehr M, Hochrein L, Otey CR, Kasrayan A, Backvall JE, Arnold FH. 2006. Enantioselective alpha-hydroxylation of 2-arylacetic acid derivatives and buspirone catalyzed by engineered cytochrome P450 BM-3. J Am Chem Soc 128(18):6058–6059.
Lee I, Johnson LA, Hammond EG. 1995. Used of branched chain esters to reduce the crystallization temperature of biodiesel. J Am Oil Chem Soc 72:1155–1160.
Lewis JC, Bastian S, Bennett CS, Fu Y, Mitsuda Y, Chen MM, Greenberg WA, Wong CH, Arnold FH. 2009. Chemoenzymatic elaboration of monosaccharides using engineered cytochrome P450BM3 demethylases. Proc Natl Acad Sci USA 106(39):16550–16555.
Li H, Poulos TL. 1997. The structure of the cytochrome p450BM-3 haem domain complexed with the fatty acid substrate, palmitoleic acid. Nat Struct Biol 4(2):140–146.
Li Z, van Beilen JB, Duetz WA, Schmid A, de Raadt A, Griengl H, Witholt B. 2002. Oxidative biotransformations using oxygenases. Curr Opin Chem Biol 6(2):136–144.
Lim SJ, Jung YM, Shin HD, Lee YH. 2002. Amplification of the NADPH-related genes zwf and gnd for the oddball biosynthesis of PHB in an *E. coli* transformant harboring a cloned phbCAB operon. J Biosci Bioeng 93(6):543–549.
Maurer SC, Kuhnel K, Kaysser LA, Eiben S, Schmid RD, Urlacher VB. 2005. Catalytic hydroxylation in biphasic systems using CYP102A1 variants. Adv Synth Catal 347:1090–1098.
Narhi LO, Fulco AJ. 1987. Identification and characterization of two functional domains in cytochrome P-450BM-3, a catalytically self-sufficient monooxygenase induced by barbiturates in *Bacillus megaterium*. J Biol Chem 262(14):6683–6690.
Neeli R, Roitel O, Scrutton NS, Munro AW. 2005. Switching pyridine nucleotide specificity in P450 BM3: Mechanistic analysis of the W1046H and W1046A enzymes. J Biol Chem 280(18):17634–17644.
Olah GA, Goeppert A, Prakash GKS. 2006. Beyond oil and gas: The methanol economy. Weinheim: Wiley-VCH.
Pandey BP, Roh C, Choi KY, Lee N, Kim EJ, Ko S, Kim T, Yun H, Kim BG. 2010. Regioselective hydroxylation of daidzein using P450 (CYP105D7) from *Streptomyces avermitilis* MA4680. Biotechnol Bioeng 105(4):697–704.
Peters MW, Meinhold P, Glieder A, Arnold FH. 2003. Regio- and enantioselective alkane hydroxylation with engineered cytochromes P450 BM-3. J Am Chem Soc 125(44):13442–13450.
Poulsen BR, Nohr J, Douthwaite S, Hansen LV, Iversen JJ, Visser J, Ruijter GJ. 2005. Increased NADPH concentration obtained by metabolic engineering of the pentose phosphate pathway in *Aspergillus niger*. FEBS J 272(6):1313–1325.
Rentmeister A, Arnold FH, Fasan R. 2009. Chemo-enzymatic fluorination of unactivated organic compounds. Nat Chem Biol 5(1):26–28.
Sauer U, Canonaco F, Heri S, Perrenoud A, Fischer E. 2004. The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. J Biol Chem 279(8):6613–6619.
Sawayama AM, Chen MM, Kulanthaivel P, Kuo MS, Hemmerle H, Arnold FH. 2009. A panel of cytochrome P450 BM3 variants to produce drug metabolites and diversify lead compounds. Chemistry 15(43):11723–11729.
Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B. 2001. Industrial biocatalysis today and tomorrow. Nature 409(6817):258–268.
Shone CC, Fromm HJ. 1981. Steady-state and pre-steady-state kinetics of coenzyme A linked aldehyde dehydrogenase from *Escherichia coli*. Biochemistry 20(26):7494–7501.
Skorupa Parachin N, Carlquist M, Gorwa-Grauslund MF. 2009. Comparison of engineered *Saccharomyces cerevisiae* and engineered *Escherichia coli* for the production of an optically pure keto alcohol. Appl Microbiol Biotechnol 84(3):487–497.
Sono M, Roach MP, Coulter ED, Dawson JH. 1996. Heme-containing oxygenases. Chem Rev 96(7):2841–2888.
Sowden RJ, Yasmin S, Rees NH, Bell SG, Wong LL. 2005. Biotransformation of the sesquiterpene (+)-valencene by cytochrome P450cam and P450BM-3. Org Biomol Chem 3(1):57–64.
Unden G, Bongaerts J. 1997. Alternative respiratory pathways of *Escherichia coli*: Energetics and transcriptional regulation in response to electron acceptors. Biochim Biophys Acta 1320(3):217–234.
van Beilen JB, Duetz WA, Schmid A, Witholt B. 2003. Practical issues in the application of oxygenases. Trends Biotechnol 21(4):170–177.
van Beilen JB, Holtackers R, Luscher D, Bauer U, Witholt B, Duetz WA. 2005. Biocatalytic production of perillyl alcohol from limonene by using a novel *Mycobacterium sp*. cytochrome P450 alkane hydroxylase expressed in *Pseudomonas putida*. Appl Environ Microbiol 71(4):1737–1744.
van der Donk WA, Zhao HM. 2003. Recent developments in pyridine nucleotide regeneration. Curr Opin Biotechnol 14:421–426.
Walton AZ, Stewart JD. 2002. An efficient enzymatic Baeyer-Villiger oxidation by engineered *Escherichia coli* cells under non-growing conditions. Biotechnol Prog 18(2):262–268.
Walton AZ, Stewart JD. 2004. Understanding and improving NADPH-dependent reactions by nongrowing *Escherichia coli* cells. Biotechnol Prog 20(2):403–411.
Xu F, Bell SG, Lednik J, Insley A, Rao Z, Wong LL. 2005. The heme monooxygenase cytochrome P450cam can be engineered to oxidize ethane to ethanol. Angew Chem Int Ed Engl 44(26):4029–4032.
Yang YT, Bennett GN, San KY. 1999. Effect of inactivation of nuo and ackA-pta on redistribution of metabolic fluxes in *Escherichia coli*. Biotechnol Bioeng 65(3):291–297.
Yin Y, Kirsch JF. 2007. Identification of functional paralog shift mutations: Conversion of *Escherichia coli* malate dehydrogenase to a lactate dehydrogenase. Proc Natl Acad Sci USA 104(44):17353–17357.

Supplemental information

Sequences of the primers used for the chromosomal gene deletions:

ndhF
5’-ATACACCCCTCACTCTATATCACTCTCACAAATTCGCTCAGTGTAGGCTGGAGCTGCTTC-3’

ndhR
5’-ATGCAACTTCAAACGCGGACGGATAACGCGGTTAATACTCATTCCGGGGATCCGTCGACC-3’

nuoA_NF
5’-TTCATCGCATCGGACGATAGATAATTCCTGAGACAATAGTGTGTAGGCTGGAGCTGCTTC-3’

nuoA_NR
5’-CATCAGCGGCATTGCCAAACGCACAATGCTAATCAGCGGTATTCCGGGGATCCGTCGACC-3’

ldhA_ko_f
5’-TGTGATTCAACATCACTGGAGAAAGTCTTATGAAACTCGCGTGTAGGCTGGAGCTGCTTC-3’

ldhA_ko_r
5’-TTGCAGCGTAGTCTGAGAAATACTGGTCAGAGCTTCTGCTATTCCGGGGATCCGTCGACC-3’

adhE_ko_f
5’-GTTATCTAGTTGTGCAAAACATGCTAATGTAGCCACCAAATCGTGTAGGCTGGAGCTGCTTC-3’

adhE_ko_r
5’-GCAGTTTCACCTTCTACATAATCACGACCGTAGTAGGTATCATTCCGGGGATCCGTCGACC-3’

pgi_KO_for
5’-GCGCTAAGGGTTTACACTCAACATTACGCTAACGGCACTAAAACCATCACAATTAACCCTCACTAA
AGGGCG-3’

pgi_KO_rev
5’-CACGCTTTATAGCGGTTAATCAGACCATTGGTCGAGCTATCGTGGCTGCTTAATACGACTCACTATA
GGGCTC-3’

ackA-pta_KO_for
5’-CCATACCCACTATCAGGTATCCTTTAGCAGCCTGAAGGCCTAAGTAGTACAATTAACCCTCACTAAA
GGGCG-3’

ackA-pta_KO_rev
5’-CTGCTGCTGTGCAGACTGAATCGCAGTCAGCGCGATGGTGTAGACGATATCGTAATACGACTCACT
ATAGGGCTC-3’