ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Metabolic Engineering of *Escherichia coli* for the Production of 1,2-Propanediol From Glycerol

James M. Clomburg,[1] Ramon Gonzalez[1,2]

[1]Department of Chemical and Biomolecular Engineering, Rice University, 6100 Main Street, MS-362, Houston, Texas 77005; telephone: 713-348-4893; fax: 713-348-5478; e-mail: ramon.gonzalez@rice.edu

[2]Department of Bioengineering, Rice University, Houston, Texas

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ABSTRACT: Due to its availability, low-price, and high degree of reduction, glycerol has become an attractive carbon source for the production of fuels and reduced chemicals. Using the platform we have established from the identification of key pathways mediating fermentative metabolism of glycerol, this work reports the engineering of *Escherichia coli* for the conversion of glycerol into 1,2-propanediol (1,2-PDO). A functional 1,2-PDO pathway was engineered through a combination of overexpression of genes involved in its synthesis from the key intermediate dihydroxyacetone phosphate (DHAP) and the manipulation of the fermentative glycerol utilization pathway. The former included the overexpression of methylglyoxal synthase (*mgsA*), glycerol dehydrogenase (*gldA*), and aldehyde oxidoreductase (*yqhD*). Manipulation of the glycerol utilization pathway through the replacement of the native *E. coli* PEP-dependent dihydroxyacetone kinase (DHAK) with an ATP-dependent DHAK from *C. freundii* increased the availability of DHAP allowing for higher 1,2-PDO production. Analysis of the major fermentative pathways indentified ethanol as a required co-product while increases in 1,2-PDO titer and yield were achieved through the disruption of the pathways for acetate and lactate production. Combination of these key metabolic manipulations resulted in an engineered *E. coli* strain capable of producing 5.6 g/L 1,2-PDO, at a yield of 21.3% (*w*/*w*). This strain also performed well when crude glycerol, a by-product of biodiesel production, was used as the substrate. The titer and yield achieved in this study were favorable to those obtained with the use of *E. coli* for the production of 1,2-PDO from common sugars.

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KEYWORDS: 1,2-PDO; glycerol fermentation; metabolic engineering

Introduction

Glycerol, a byproduct of biodiesel, oleo-chemical, and bioethanol production processes, has recently become an inexpensive and abundant carbon source (Khanal et al., 2008; Rausch and Belyea, 2006; Yazdani and Gonzalez, 2007). Future opportunities for the availability of even larger amounts of glycerol exist due to the accumulation of high concentrations of glycerol by certain species of algae (Oren, 2005). Although *Escherichia coli* was long thought to require the presence of external electron acceptors for glycerol metabolism, it has recently been shown that this bacterium is able to metabolize glycerol in a fermentative manner (Dharmadi et al., 2006). Further identification of key pathways and environmental conditions affecting glycerol metabolism under anaerobic conditions (Gonzalez et al., 2008; Murarka et al., 2008) has provided a platform for the efficient conversion of glycerol into fuels and reduced chemicals (Hongbo and Wood, 2010; Trinh and Srienc, 2009; Yazdani and Gonzalez, 2008).

1,2-propanediol (1,2-PDO: $C_3H_8O_2$), a commodity chemical with global demand estimated around 3 billion lb/yr, has a major role in applications such as antifreeze and heat-transfer fluids, plasticizers and thermoset plastics, and cosmetics (Shelley, 2007). Recent processes for the microbial production of 1,2-PDO from sugars, predominantly glucose, have used microorganisms such as *Thermoanaerobacterium thermosaccharolyticum* (Altaras et al., 2001; Sanchez-Riera et al., 1987), *Clostridium sphenoides* (Trandin and Gottschalk, 1985), *Saccharomyces cerevisiae* (Jung et al., 2008; Lee and DaSilva, 2006), and *E. coli* (Altaras and Cameron, 1999, 2000; Huang et al., 1999). Utilizing batch cultures, 1,2-PDO titers from glucose ranged from 0.49 g/L with *E. coli* (Altaras and Cameron, 1999) to 1.11 g/L with *S. cerevisiae* (Jung et al., 2008) and 9.0 g/L with *T. thermosaccharolyticum* (Sanchez-Riera et al., 1987). An optimized fed-batch culture was used to achieve the highest titer of 1,2-PDO produced by *E. coli* from glucose (4.5 g/L) at a yield of 0.19 g/g (Altaras and Cameron, 2000).

In addition to its low cost and abundance, the higher degree of reduction of glycerol (compared to glucose) offers the opportunity for increased 1,2-PDO yields (Yazdani and Gonzalez, 2007). Under the assumption that no external

Correspondence to: R. Gonzalez

source of energy is available other than the given carbon source, the maximum theoretical yield of 1,2-PDO from glycerol is clearly higher than that from glucose, as shown in the corresponding reactions below.

Glucose :

$$C_6H_{12}O_6 \rightarrow \frac{3}{2}C_3H_8O_2 + \frac{3}{2}CO_2$$

Max Yield : 0.63 g/g

Glycerol :

$$C_3H_8O_3 \rightarrow \frac{7}{8}C_3H_8O_2 + \frac{3}{8}CO_2 + \frac{1}{2}H_2O$$

Max Yield : 0.72 g/g

In the work reported here, rational metabolic engineering strategies are used to design and engineer *E. coli* for the efficient production of 1,2-PDO from glycerol. The engineered *E. coli* strains produced 1,2-PDO at yields and titers higher than those reported with the use of this organism for the production of 1,2-PDO from other carbon sources.

Materials and Methods

Strains, Plasmids, and Genetic Methods

Wild-type K12 *Escherichia coli* strain MG1655 (Kang et al., 2004) was used as the host to implement metabolic engineering strategies. All resulting strains, along with primers and plasmids used in their construction are listed in Table I.

Gene knockouts were introduced in MG1655 and its derivatives by P1 phage transduction (Miller, 1972; Yazdani and Gonzalez, 2008). Single gene knockout mutants from the National BioResource Project (NIG, Japan) (Baba et al., 2006) were used as donors of specific mutations, except for the deletion of the *ackA-pta* operon, which was created by a previously reported method (Metcalf et al., 1996). All mutations were confirmed by polymerase chain reaction using the "verification" primers listed in Table I. The disruption of multiple genes in a common host was achieved as previously described (Yazdani and Gonzalez, 2008).

All primers used in the construction of plasmids are listed in Table I. The *E. coli* genes *yqhD* and *fucO* were PCR amplified with flanking *Xho*I and *Kpn*I sites and each cloned into pTrcHis2A (Invitrogen, Carlsbad, CA) doubly digested with *Xho*I and *Kpn*I to produce pTHyqhD and pTHfucO. To build the plasmid pTHKLcfgldAmgsAyqhD, the gene cluster KLcfgldAmgsA from pZSKLcfgldAmgsA (see below) was PCR amplified and cloned into pTHyqhD digested with *Xho*I, using the InFusion PCR cloning kit (Clontech Laboratories, Inc., Mountain View, CA). The same approach was used to build pTHKLcfgldAmgsAfucO, inserting the PCR product into pTHfucO, also linearized with *Xho*I. pZSKLcfgldAmgsA was constructed by PCR amplifying the *gldA* gene with flanking *Bam*HI and *Mlu*I sites and then cloning into pZSKLcf (Bachler et al., 2005) doubly digested with *Bam*HI and *Mlu*I to produce pZSKLcfgldA. The *mgsA* gene PCR amplified with *Mlu*I sites on both ends was then cloned into pZSKLcfgldA digested with *Mlu*I and treated with *Sap*I to produce pZSKLcfgldAmgsA. The *dhaKL* genes from *Citrobacter freundii* were removed from pTHKLcfgldAmgsAfucO and pTHKLcfgldAmgsAyqhD by using primers (containing a novel *Bgl*II site overhang) that flank the *KLcf* genes, thereby amplifying the entire plasmid except for *KLcf* region, followed by *Bgl*II digestion and self ligation. For construction of pZSadhE, pZSldhA and pZSackApta, the *adhE* gene, *ldhA* gene, and *ackA-pta* operon were PCR amplified from MG1655 genomic DNA using the primers listed in Table I. The resulting products were then cloned into pZSKLMgldA (Yazdani and Gonzalez, 2008) digested with *Kpn*I and *Mlu*I using the InFusion cloning kit.

Manufacturer protocols and standard methods (Miller, 1972; Sambrook et al., 1989) were followed for DNA purification (Qiagen, Valencia, CA), restriction endonuclease digestion (New England Biolabs, Ipswich, MA), and DNA amplification (Stratagene, La Jolla, CA and Invitrogen, Carlsbad, CA). The strains were kept in 32.5% glycerol stocks at −80°C. Plates were prepared using LB medium containing 1.5% agar, and appropriate antibiotics were included at the following concentrations: ampicillin (100 μg/mL), kanamycin (50 μg/mL), and chloramphenicol (34 μg/mL).

Culture Medium and Cultivation Conditions

The minimal medium designed by Neidhardt et al. (1974) supplemented with 10 g/L glycerol, 10 g/L tryptone, 5 g/L yeast extract, and 1.32 mM Na_2HPO_4 in place of K_2HPO_4 was used unless otherwise stated. Antibiotics (see concentrations above) and inducer (0.1 mM Isopropyl β-D-1-thiogalactopyranoside) were included when appropriate. Chemicals were obtained from Fisher Scientific (Pittsburg, PA) and Sigma-Aldrich Co. (St. Louis, MO), except crude glycerol, which was provided by Renewable Energy Group, Inc. (Ames, IA). Crude glycerol had the following content (*w*/*w*%): glycerol (83.3), methanol (0.01), water (10.0), fatty acids (0.04), salt (6.63), and ash (6.6). The pH was 6.38 and the density was 1.26 g/mL.

Fermentations in tubes were conducted in modified 17-mL Hungate tubes (Bellco Glass, Inc., Vineland, NJ) as previously described (Murarka et al., 2008). Prior to inoculation, oxygen in the medium was removed by autoclaving and storage in an oxygen-free environment provided within a BACTRON I Anaerobic Chamber (Sheldon Manufacturing Inc., Cornelius, OR). A single colony was used to inoculate 10 mL of medium in each tube, which were then incubated (37°C) for 96 h in an Isotemp Incubator (Fisher Scientific, Pittsburgh, PA) and sparged with ultra-high purity argon (Matheson Tri-Gas, Houston, TX). To maintain sterile conditions, the inlet gas was passed through a 0.2 μm HEPA filter (Millipore, Billerica, CA) and the outlet gas line immersed in a 1 M $CuSO_4$ solution.

Additional fermentations were conducted in a SixFors multi-fermentation system (Infors HT, Bottmingen, Switzerland)

Table I. Strains, plasmids and primers used in this study.

Strain/Plasmid/Primer	Description/Genotype/Sequence	Source
Strains		
MG1655	F- λ- ilvG- rfb-50 rph-1	Kang et al. (2004)
Δ*dhaK*	MG1655, Δ*dhaK*::*FRT-Km-FRT*; deletion mutant for *dhaK* gene in MG1655	This study
Δ*gloA*	MG1655, Δ*gloA*::*FRT-Km-FRT*; deletion mutant for *gloA* gene in MG1655	This study
Δ*aldA*	MG1655, Δ*aldA*::*FRT-Km-FRT*; deletion mutant for *aldA* gene in MG1655	This study
Δ*frdA*	MG1655, Δ*frdA*::*FRT-Km-FRT*; deletion mutant for *frdA* gene in MG1655	This study
Δ*ackA-pta*	MG1655 Δ*ackA-pta*::*FRT*; deletion mutant for *ackA-pta* operon in MG1655	This study
Δ*ldhA*	MG1655, Δ*ldhA*::*FRT-Km-FRT*; deletion mutant for *ldhA* gene in MG1655	This study
Δ*adhE*	MG1655, Δ*adhE*::*FRT-Km-FRT*; deletion mutant for *adhE* gene in MG1655	This study
Δ*ackA-pta*Δ*ldhA*Δ*dhaK*	MG1655, *ΔackA-pta*::*FRT ΔldhA*::*FRT ΔdhaK*::*FRT-Kan-FRT*; sequential deletion of *ackA-pta*, *ldhA* and *dhaK* in MG1655	This study
Δ*ackA-pta*Δ*ldhA*Δ*dhaK*Δ*aceF*	MG1655, Δ*ackA-pta*::*FRT* Δ*ldhA*::*FRT* Δ*dhaK*::*FRT* Δ*aceF*::*FRT-Kan-FRT*; sequential deletion of *ackA-pta*, *ldhA*, *dhaK*, and *aceF* in MG1655	This study
Δ*ackA-pta*Δ*ldhA*Δ*dhaK*Δ*fdhF*	MG1655, Δ*ackA-pta*::*FRT* Δ*ldhA*::*FRT* Δ*dhaK*::*FRT* Δ*fdhF*::*FRT-Kan-FRT*; sequential deletion of *ackA-pta*, *ldhA*, *dhaK*, and *fdhF* in MG1655	This study
Δ*ackA-pta*Δ*ldhA*Δ*dhaK*Δ*aceF*Δ*fdhF*	MG1655, Δ*ackA-pta*::*FRT* Δ*ldhA*::*FRT* Δ*dhaK*::*FRT* Δ*aceF*::*FRT* Δ*fdhF*::*FRT-Kan-FRT*; sequential deletion of *ackA-pta*, *ldhA*, *dhaK*, *aceF*, and *fdhF* in MG1655	This study
pTrcHis2A	pTrcHis2A (pBR322-derived), oriR pMB1, *lacI*^{q}, *bla*	Invitrogen (Carlsbad, CA)
pTHgldAmgsAyqhD	*E. coli gldA, mgsA*, and *yqhD* genes under *trc* promoter and *lacI*^{q} control	This study
pTHgldAmgsAfucO	*E. coli gldA, mgsA*, and *fucO* genes under *trc* promoter and *lacI*^{q} control	This study
pTHKLcfgldAmgsAyqhD	*C. freundii dhaKL*, *E. coli gldA, mgsA*, and *yqhD* genes under *trc* promoter and *lacI*^{q} control	This study
pTHKLcfgldAmgsAfucO	*C. freundii dhaKL*, *E. coli gldA, mgsA*, and *fucO* genes under *trc* promoter and *lacI*^{q} control	This study
pTHyqhD	*E. coli yqhD* gene under *trc* promoter and *lacI*^{q} control	This study
pTHfucO	*E. coli fucO* gene under *trc* promoter and *lacI*^{q} control	This study
pZSKLcf	*C. freundii dhaKL* gene under control of $P_{LtetO-1}$ (tetR, oriR SC101*, cat)	Bachler et al. (2005)
pZSKLcfgldA	*C. freundii dhaKL* gene and *E. coli gldA* gene under control of $P_{LtetO-1}$ (tetR, oriR SC101*, cat)	This Study
pZSKLcfgldAmgsA	*C. freundii dhaKL* gene, *E. coli gldA* and *mgsA* genes under control of $P_{LtetO-1}$ (tetR, oriR SC101*, cat)	This study
pZSblank	oriR pSC101*, tetR, *cat*, contains $P_{LtetO-1}$	Yazdani and Gonzalez (2008)
pZSldhA	*E. coli ldhA* gene under control of $P_{LtetO-1}$(tetR, oriR SC101*, cat)	This Study
pZSadhE	*E. coli adhE* gene under control of $P_{LtetO-1}$(tetR, oriR SC101*, cat)	This Study
pZSackApta	*E. coli ackA* and *pta* genes under control of $P_{LtetO-1}$ (tetR, oriR SC101*, cat)	This Study
Primers[a,b]		
v-dhaK	CGTGTCGTTGAACATCATCC GAGTGAGCCAGTTAACAATTTG	This study
v-gloA	GAATCTGTTAGCCATTTTGAGG CTTGTCGATGCAGTAAAGATG	This study
v-aldA	TGTCCTTTCACGATTCCGTC TATGGCTTCGTCACACAGC	This study
v-frdA	TACCCTGAAGTACGTGGCTG AGGTAGTTGCGTCATAAGGC	This study
v-ackApta	CATAAAACGGATCGCATAACG CATGATGATGCCAACG	This study
v-adhE	CGAGCAGATGATTTACTAAAAAAG ATCGGCATTGCCCAGAAGG	This study
v-ldhA	GCTTAAATGTGATTCAACATCACTGG AGAATAGAGGATGAAAGGTCATTG	This study
v-aceF	CGTCTGGCGTAAGAGGTAAAAG CCGATTTGTCTATTCGCTAATAACC	This study
v-fdhF	TGCGTGATTTGATTAACTGGAGC CGAAAGGAGGCTGTAGAAAGG	This study
pZSKLcfgldA	GACAGGATCCAGGAGCAATTATGGACCGCA CAAGCTACGCGTTTATTCCCACTCTTGCAGGA	This study
pZSKLcfgldAmgsA	ACGTACGCGTAGGAGGGTACATTATGGAACTGACGACTC GATCACGCGTTTACTTCAGACGGTCCGCG	This study
pZSldhA	CATTAAAGAGGAGAAAGGTACCATGAAACTCGCCG GATGCCTCTAGCACGCGTTTAAACCAGTTCGTT	This study
pZSadhE	CATTAAAGAGGAGAAAGGTACCATGGCTGTTACTAATG GATGCCTCTAGCACGCGTTTAAGCGGATTTTTTCG	This study

Table I. (*Continued*)

Strain/Plasmid/Primer	Description/Genotype/Sequence	Source
pZSackA pta	CATTAAAGAGGAGAAAGGTACCATGTCGAGTAAGTTAG GATGCCTCTAGCACGCGTTTACTGCTGCTGTGC	This study
pTHKLcfgldAmgsAyqhD	CCATGGATCCGAGCTCGAGAATGTCTCAATTCTTTTTTAACC GCAGATTAAAGTTGTTCATCTCGAGCCTCCTTACTTCAGACGGTCCG	This study
pTHKLcfgldAmgsAfucO	CCATGGATCCGAGCTCGAGAATGTCTCAATTCTTTTTTAACC CATTCTGTTAGCCATCATCTCGAGCCTCCTTACTTCAGACGGTCCG	This study
pTHgldAmgsAyqhD	GCTATCGAGATCTATGGACCGCATTATTCAATC GGCGGAGATCTTTATTCCTCCTTATTTAATCG	This study
pTHgldAmgsAfucO	GCTATCGAGATCTATGGACCGCATTATTCAATC GGCGGAGATCTTTATTCCTCCTTATTTAATCG	This study
pTHyqhD	CCGCTCGAGATGAACAACTTTAATCTGCAC GAGGTACCTTAGCGGGCGGC	This study
pTHfucO	GATCCTCGAGATGATGGCTAACAGAATGATTC GATCGGTACCTTACCAGGCGGTATGGTAAAGC	This study

[a]"v" indicates the primer sequences (5′ to 3′) that were used for verification purposes during the creation of disruption mutants. The reverse sequence follows the forward sequence in each case. Genes or operons deleted are apparent from primer names.

[ab]Primers listed for the construction of plasmids were utilized according to the procedures in Materials and Methods section. Genes cloned are apparent from primer names.

equipped with six 500 mL working volume vessels and independent control of temperature (37°C), pH (controlled to 7.5 with NaOH and H_2SO_4), and stirrer speed (200 rpm) operated as described previously (Dharmadi et al., 2006). Inocula were prepared as described elsewhere (Murarka et al., 2008) and used to inoculate 400 mL of medium in each fermentor, with a target initial optical density of 0.3 at 550 nm.

Analytical Methods

The concentrations of cell mass, glycerol, 1,2-propanediol, organic acids, and ethanol were measured as previously described (Dharmadi and Gonzalez, 2005; Dharmadi et al., 2006). Ethanol measurements for fermentation conducted in the SixFors multi-fermentation system (see above) were corrected for evaporation.

Enzyme Activities

Cell harvesting and preparation of crude cell extracts for enzyme assays was conducted as described elsewhere (Durnin et al., 2009; Murarka et al., 2008). Absorbance changes for all assays were monitored in a Biomate 5 spectrophotometer (Thermo Scientific, MA, USA). The linearity of reactions (protein concentration and time) was established for all assays and the nonenzymatic rates were subtracted from the observed initial reaction rates. Enzymatic activities are reported as micromole of substrate per minute per milligram of cell protein and represent averages for at least three cell preparations.

The activity of glycerol dehydrogenase on glycerol oxidation was measured as described previously (Gonzalez et al., 2008), with potassium carbonate (pH 9.5) as the buffer. ATP-dependent dihydroxyacetone kinase activity was followed in a coupled system in which the NADH-dependent reduction of the reaction product, dihydroxyacetone phosphate (DHAP), to glycerol 3-phosphate was measured at 340 nm (Barbirato et al., 1997). The activity of methylglyoxal synthase was determined using a colorimetric assay (Berrios-Rivera et al., 2003). The activity of pyruvate dehydrogenase was measured as previously reported (Murarka et al., 2010a).

Methylglyoxal reducing activities (i.e., its conversion to acetol or lacaldehyde) were assayed by measuring the absorbance change at 340 nm of a 1 mL reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 10 mM MG, 0.1 mM NAD(P)H, and 50 μL crude cell extract (Ko et al., 2005). 1,2-PDO reductase activities were measured in a 1 mL reaction mixture containing 100 mM Tris-HCl buffer (pH 9.0), 1 mM $MnCl_2$, 10 mM 1,2-PDO, 5 mM NAD^+, and 50 μL crude cell extract by monitoring the absorbance change at 340 nm (Oude Elferink et al., 2001). Reduction of acetol to 1,2-PDO was assayed by measuring the absorbance change at 340 nm of a 1 mL reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 10 mM acetol, 0.1 mM NADH, and 50 μL crude cell extract (Gupta et al., 2009).

Calculation of Fermentation Parameters

Growth and product yields (mmol/mmol glycerol or g/g glycerol) were calculated as the amount of cell mass or product synthesized per amount of glycerol consumed at the end of the fermentation time (96 h unless otherwise stated).

Results and Discussion

Proposed Metabolic Engineering Strategies for the Conversion of Glycerol into 1,2-PDO

Metabolic engineering strategies must be designed to enable 1,2-PDO synthesis while satisfying two key constraints governing fermentative metabolism, namely redox balance and ATP generation by substrate-level phosphorylation. The production of 1,2-PDO from glycerol results in the net consumption of reducing equivalents (Fig. 1). Consequently, the production of a more oxidized co-product,

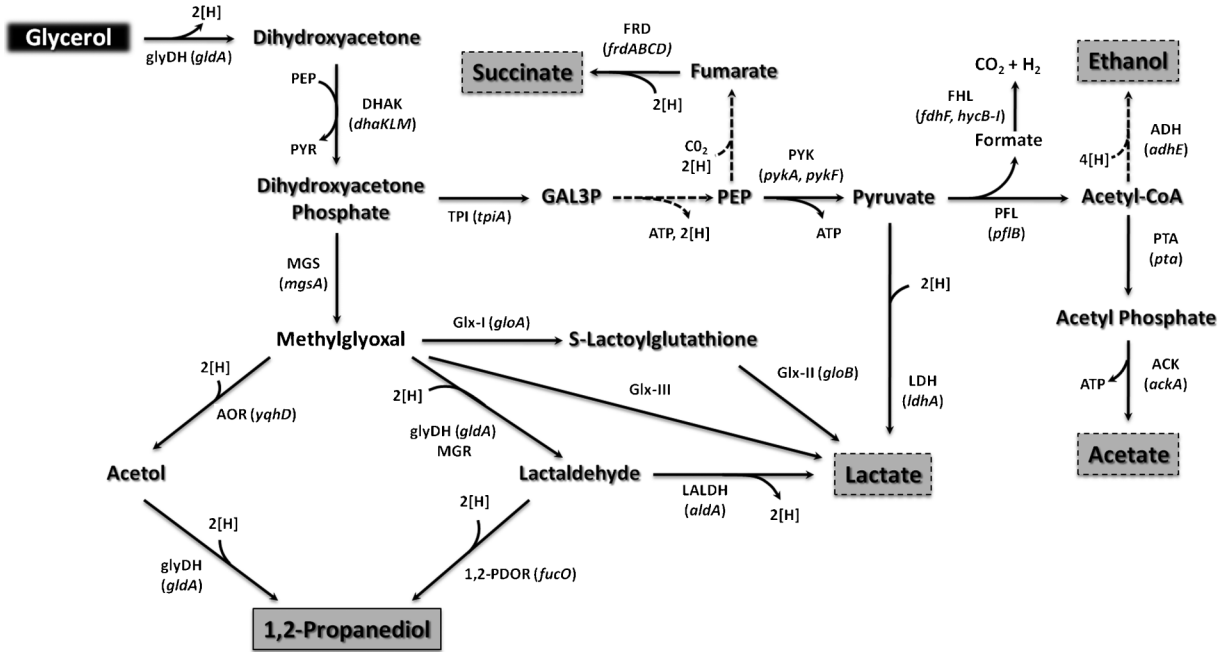

Figure 1. *E. coli* pathways involved in the synthesis of 1,2-PDO and other fermentation products during the fermentative metabolism of glycerol. Broken lines illustrate multiple steps. Relevant reactions are represented by the enzyme names with the gene(s) coding for the enzymes in parenthesis: ACK, acetate kinase; ADH, acetaldehyde/alcohol dehydrogenase; AOR, aldehyde oxidoreductase; DHAK, dihydroxyacetone kinase; FHL, fomate hydrogen lyase complex; FRD, fumarate reductase; Glx-1, Glyoxylase type I; Glx-II, Glyoxylase type II; Glx-III, Glyoxylase type III; glyDH, glycerol dehydrogenase; LALDH, lactaldehyde dehydrogenase; LDH, lactate dehydrogenase; MGR, methylglyoxal reductase; MGS, methylglyoxal synthase; PFL, pyruvate formate-lyase; PTA, phosphate acetyltransferase; PYK, pyruvate kinase; TPI, triose phosphate isomerase; 1,2-PDOR, 1,2-propanediol reductase. Abbreviations: GAL3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; 2[H], NADH/NADPH//$FADH_2$.

such as lactate ($C_3H_6O_3$) or acetate ($C_2H_4O_2$), would maintain an overall redox balance as seen from the following balances.

$$1,2\text{-PDO/Acetate}: \quad 3C_3H_8O_3 \rightarrow 2C_3H_8O_2 + C_2H_4O_2 + CH_2O_2 + H_2O$$

$$1,2\text{-PDO/Lactate}: \quad 2C_3H_8O_3 \rightarrow C_3H_8O_2 + C_3H_6O_3 + H_2O$$

The synthesis of acetate could support higher 1,2-PDO yields as the molar ratio of 1,2-PDO to acetate required to achieve redox balance is 2:1 while that of 1,2-PDO to lactate is 1:1. It is important to note however, that the molar ratio of either acetate or lactate to 1,2-PDO required to enable redox poise would result in a net ATP balance of zero, placing a significant metabolic constraint on the system due to the ATP required for cell growth and maintenance. Therefore, the production of ethanol, whose synthesis along with formate (or CO_2 and H_2) from glycerol represents a redox balanced pathway that generates ATP, may also be vital for 1,2-PDO production by providing the ability to generate ATP without disturbing redox poise: $\text{Glycerol} \rightarrow \text{Ethanol} + \text{Formate}/(CO_2 + H_2) + \text{ATP}$ (see Fig. 1 and Murarka et al., 2008).

The proposed pathways mediating the conversion of glycerol into 1,2-PDO are shown in Figure 1. Glycerol dehydrogenase (glyDH) and dihydroxyacetone kinase (DHAK) catalyze the conversion of glycerol into the glycolytic intermediate dihydroxyacetone phosphate (DHAP) (Gonzalez et al., 2008). DHAP is converted to methylglyoxal (MG) by the action of methylglyoxal synthase (MGS, *mgsA*) (Hopper and Cooper, 1972). The conversion of MG into 1,2-PDO takes place through two alternate pathways, one with acetol as an intermediate through the action of aldehyde oxidoreductase (AOR, *yqhD*) (Lee et al., 2010; Soucaille et al., 2008) and glycerol dehydrogenase (glyDH, *gldA*) (Subedi et al., 2008; Tang et al., 1979), and the other with lactaldehyde as an intermediate, mediated by glyDH (Altaras and Cameron, 1999; Subedi et al., 2008) and 1,2-PDO reductase (1,2-PDOR, *fucO*) (Zhu and Lin, 1989) (Fig. 1). Based on these pathways, the overexpression of *mgsA* in combination with either alternative 1,2-PDO pathway will be investigated as a means of increasing 1,2-PDO production. The availability of DHAP for conversion into methylglyoxal (MG) will also be investigated, as it represents a critical node where partitioning of carbon flux between 1,2-PDO synthesis and glycolysis occurs.

Methylglyoxal, a key intermediate in the 1,2-PDO pathway, is a very toxic metabolite whose accumulation at sub-millimolar concentrations can lead to significant growth inhibition and even cell death (Booth et al., 2003; MacLean et al., 1998). Several detoxification pathways exist, the most important of which is glutathione (GSH)-

dependent GlxI/GlxII (Glyoxylase type I/II, encoded by *gloA*/*gloB*) pathway, whose net result is the conversion of MG into lactate (MacLean et al., 1998) (Fig. 1). In addition, the glyoxylase III pathway (Misra et al., 1995) and the conversion of lactaldehyde into lactate through the action of lactaldehyde dehydrogenase (encoded by *aldA*) (Caballero et al., 1983) are other non-1,2-PDO forming MG detoxification pathways found in *E. coli* (Fig. 1). Since these pathways shuttle carbon away from the production of 1,2-PDO, their disruption could provide higher 1,2-PDO production.

Due to the fact that the 1,2-PDO synthesis pathway is both redox and ATP consuming, it is likely that in addition to 1,2-PDO production, other fermentation products will also be produced. Therefore, the effect of the disruption and amplification of the fermentative pathways responsible for the production of lactate (*ldhA*), succinate (*frdABCD*), ethanol (*adhE*) and acetate (*ackA-pta*), in combination with the engineered 1,2-PDO pathway, will be investigated.

Engineering of 1,2-Propanediol Synthesis Pathways

Two plasmids were constructed to overexpress the genes involved in the synthesis of 1,2-PDO from the intermediate DHAP (Fig. 1): pTHgldAmgsAyqhD, expressing *gldA*, *mgsA*, and *yqhD* and pTHgldAmgsAfucO expressing *gldA*, *mgsA*, and *fucO*. While the expression of either pathway in wild-type MG1655 resulted in a similar increase in 1,2-PDO production, the product titers and yields were still low (less than 0.1 g/L) (Fig. 2). In addition to the overexpression of a 1,2-PDO pathway, the disruption of genes involved in MG detoxification pathways could potentially increase the titers and yields of 1,2-PDO by minimizing the conversion of MG into other products (see previous section). This was investigated by deleting the *gloA* and *aldA* genes in combination with expression of the 1,2-PDO pathway. However, the disruption of either gene did not result in significant increases in 1,2-PDO production or changes in the distribution of fermentation products (Fig. 2), which suggests that the carbon flux through the glyoxylase systems might be negligible in this strain.

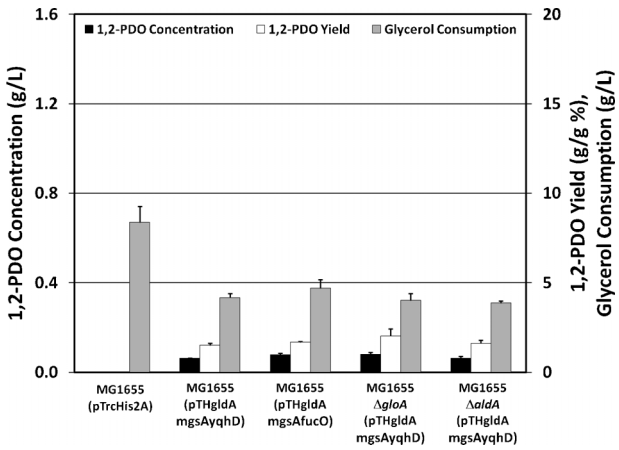

Figure 2. Effect of overexpression of 1,2-PDO-synthesis pathways on 1,2-PDO production and glycerol consumption in 96-h sparged tube experiments. Gene overexpressions are indicated by the plasmid name in parenthesis (i.e., pTrcHis2A: control vector; pTHgldAmgsAyqhD expressing glycerol dehydrogenase, methylglyoxal synthase and aldehyde oxidoreductase; pTHgldAmgsAfucO expressing glycerol dehydrogenase, methylglyoxal synthase and 1,2-PDO reductase). Δ"*gene*" represents gene deletion. Error bars represent standard deviations for a minimum of triplicate measurements.

In order to ensure that inappropriate expression of the genes contained in each vector was not the cause of the low 1,2-PDO production, a functional characterization of the plasmids through enzyme assays was conducted. MGS activity (conversion of MG from DHAP), MG reducing activities (both NADPH and NADH dependent for the reduction of MG into either acetol or lactaldehyde), acetol reduction activity (for 1,2-PDO production from acetol), and 1,2-PDOR activity (for 1,2-PDO production from lactaldehyde) were all assayed (Table II). The MGS, MG reducing, and acetol reducing activities (encoded by the pTHgldAmgsAyqhD plasmid) are all more than 10-fold higher than those of the control vector (pTrcHis2A) indicating the amplification of this 1,2-PDO pathway. In the case of MG reducing activities, an increase in both NADH- and NADPH-dependent reduction of MG was observed. While the AOR encoded by *yqhD* is believed utilize NADPH as a co-factor (Lee et al., 2010), the fact that the MG reducing assay cannot distinguish between the reduction of MG to acetol and that to lactaldehyde opens the possibility that the high activity of the NADH-dependent MG reducing assay is a result of the overexpression of glyDH, which can reduce MG to lactaldehyde. In addition, *E. coli* has a number of native aldo-keto reductases (both NADH and NADPH dependent) (Ko et al., 2005; Misra et al., 1996) that have been shown to reduce MG, which may be highly active in the presence of the larger MG pool likely seen with the overexpression of MGS. Similar results were obtained for pTHgldAmgsAfucO, as the MGS, MG reducing, and 1,2-PDO reductase activities are all more than nine-fold higher than those of pTrsHis2A. Taken together, the results from these enzyme assays indicate that the low 1,2-PDO production was not the result of poor gene expression or pathway functionality and further analysis is required to ascertain the reasons behind the low titers (see next section).

Manipulation of Glycerol Utilization Pathways

The fact that the *E. coli* DHAK utilizes phosphoenolpyruvate (PEP) as the phosphate group donor in the phosphorylation of dihydroxyacetone (DHA) has the effect of coupling the native fermentative pathway for glycerol dissimilation with the requirement for the synthesis of PEP. Therefore, every mole of DHA converted to DHAP utilizes one mole of PEP resulting in the requirement for the conversion of DHAP

Table II. Functional characterization of constructs used in the overexpression of 1,2-PDO synthesis and glycerol utilization pathways. Reported values are from 96-h sparged tube cultures.

	Activity (μmol/mg protein/min)[a,b]			
Enzyme assayed	pTrcHis2A	pTHgldAmgsAyqhD	pTHgldAmgsAfucO	pTHKLcfgldAmgsAyqhD
1,2-PDO Synthesis Pathways				
Methylglyoxal synthase	0.116 ± 0.009	2.96 ± 0.09	3.18 ± 0.08	2.61 ± 0.07
Methylglyoxal reducing (NADH)	0.020 ± 0.001	1.43 ± 0.04	1.8 ± 0.1	0.60 ± 0.02
Methylglyoxal reducing (NADPH)	0.51 ± 0.03	5.62 ± 0.04	4.69 ± 0.08	1.90 ± 0.09
Glycerol dehydrogenase (Acetol reducing)	0.010 ± 0.001	0.56 ± 0.02	Not Measured	0.173 ± 0.003
1,2-PDO Reductase	0.170 ± 0.009	4.0 ± 0.2	4.5 ± 0.2	Not Measured
Glycerol Utilization Pathways				
Glycerol dehydrogenase (Glycerol oxidation)	0.075 ± 0.003	Not Measured	Not Measured	1.47 ± 0.03
ATP-dependent Dihydroxyacetone Kinase	0.178 ± 0.004	Not Measured	Not Measured	0.586 ± 0.007

[a]All activities were measured as described in Materials and Methods section and values are reported as average ± standard deviation for triplicate assays.
[cb]Activities measured in wild-type strain MG1655 containing the listed plasmids overexpressing the specified enzyme(s) (native *E. coli* enzymes unless otherwise specified): i.e., pTrcHis2A: control vector; pTHgldAmgsAyqhD expressing glycerol dehydrogenase, methylglyoxal synthase and aldehyde oxidoreductase; pTHgldAmgsAfucO expressing glycerol dehydrogenase, methylglyoxal synthase and 1,2-PDO reductase; pTHKLcfgldAmgsAyqhD expressing *C. freundii* ATP-dependent dihydroxyacetone kinase, glycerol dehydrogenase, methylglyoxal synthase, and aldehyde oxidoreductase.

into PEP (i.e., re-generation of the PEP pool) for continued glycerol dissimilation (Fig. 1). This may limit the amount of DHAP (an intermediate upstream of PEP synthesis) available for the conversion into MG and subsequently 1,2-PDO. While we believed that the required co-production of fermentation products downstream of PEP along with 1,2-PDO would reduce this requirement, the low levels of 1,2-PDO seen as a result of the engineered 1,2-PDO pathway (Fig. 2) show that this may still be a major issue.

In order to increase the availability of DHAP for 1,2-PDO synthesis, we decoupled glycerol utilization from the requirement for PEP synthesis by expressing an ATP-dependent DHAK from *Citrobacter freundii* (*dhaKL*), which utilizes ATP as the phosphate group donor instead of PEP (Daniel et al., 1995). A vector was constructed containing the previously engineered 1,2-PDO pathway (i.e., *gldA*, *mgsA*, and *yqhD* from *E. coli*) in addition to *C. freundii dhaKL* (pTHKLcfgldAmgsAyqhD). Once transformed with this vector, MG1655 produced nearly 12-fold higher levels of 1,2-PDO (over eight-fold increase in 1,2-PDO yield) and consumed approximately 33% more glycerol than that seen from the expression of only the engineered 1,2-PDO pathway (Fig. 3). This dramatic increase in 1,2-PDO production and rise in the amount of glycerol consumption shows the combined advantages of increasing both DHAP availability for 1,2-PDO production and the rates of glycerol utilization. The latter results from the simultaneous overexpression of glyDH and DHAK, as previously reported (Yazdani and Gonzalez, 2008).

Proper gene expression was investigated by the functional characterization of pTHKLcfgldAmgsAyqhD through the use of enzyme assays. The activity of the steps within the 1,2-PDO pathway was significantly higher with the expression of pTHKLcfgldAmgsAyqhD when compared to the control vector (Table II). In addition, the increased ATP-dependent DHAK activity shows proper expression and when combined with the increase in glycerol oxidation activity, indicates the functionality of both activities required for increased glycerol utilization rates. The ATP-dependent DHAK activity found in the control strain is due to the action of glycerol kinase (GK, *glpK*), an enzyme involved in the respiratory glycerol utilization pathway, and which acts on both glycerol and DHA (Garcia-Alles et al., 2004; Jin et al., 1982).

While the overexpression of the ATP-dependent DHAK from *C. freundii* helps alleviate the requirement of PEP

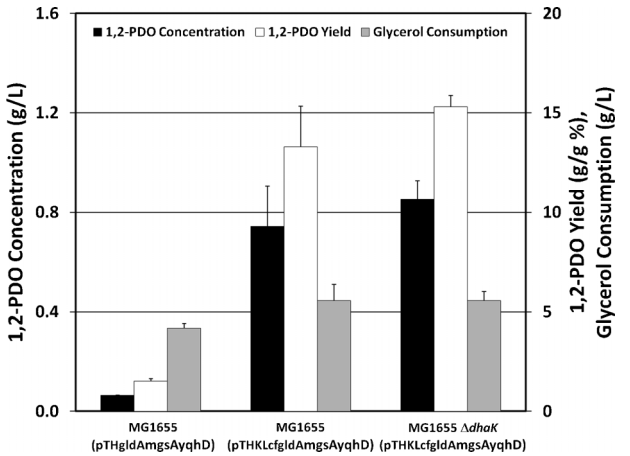

Figure 3. Manipulation of glycerol utilization pathways for increased 1,2-PDO production. Fermentations conducted in 96-h sparged-tube experiments. Gene overexpressions are indicated by the plasmid name in parenthesis (i.e., pTHgldAmgsAyqhD expressing *E. coli* glycerol dehydrogenase, methylglyoxal synthase and aldehyde oxidoreductase; pTHKLcfgldAmgsAyqhD expressing *C. freundii* ATP-dependent DHA kinase, and *E. coli* glycerol dehydrogenase, methylglyoxal synthase and aldehyde oxidoreductase). Δ"*gene*" represents gene deletion. Error bars represent standard deviations for a minimum of triplicate measurements.

synthesis for glycerol dissimilation and significantly increases 1,2-PDO production, the additional disruption of the native *E. coli* DHAK (through the deletion of *dhaK*) will completely decouple the two pathways. An *E. coli* mutant lacking a functional PEP-dependent DHAK (Δ*dhaK*) was constructed and transformed with the pTHKLcfgldAmgsAyqhD. This strain showed a slight increase (~15%) in both 1,2-PDO concentration and yield when compared to wild-type *E. coli* MG1655 expressing the same plasmid (Fig. 3). Based on these results, it is apparent that the complete decoupling of glycerol dissimilation and PEP formation through the replacement of *E. coli* PEP-dependent DHAK with the *C. freundii* ATP-dependent DHAK provides the most efficient route to 1,2-PDO synthesis. While this represents a promising result, it is important to note that significant amounts of ethanol, succinate, acetate, and lactate were also synthesized (Table III).

Engineering of Major Fermentation Pathways

Based on the significant amounts of ethanol, lactate, acetate, and succinate produced along with 1,2-PDO upon overexpression of the ATP-dependent DHAK and 1,2-PDO pathway (Table III), the minimization of these by-products may provide the opportunity for increased 1,2-PDO titer and yield. For this purpose, *E. coli* mutants with deletions of key genes in the synthesis of fermentation products lactate (Δ*ldhA*), succinate (Δ*frdA*), ethanol (Δ*adhE*), and acetate (Δ*ackA-pta*) were transformed with the pTHKLcfgldAmgsAyqhD plasmid (Fig. 4). Disruption of fumarate reductase (Δ*frdA*) eliminated almost all succinate production but had a negative impact on 1,2-PDO synthesis, the latter apparently due to a reduction in hydrogen recycling (see next section for details). In addition, the deletion of the *adhE* gene, resulting in the inability to produce ethanol, nearly eliminated glycerol consumption and 1,2-PDO synthesis (Fig. 4), in agreement with the requirement of ethanol synthesis for glycerol fermentation (Gonzalez et al., 2008; Murarka et al., 2008).

Deletion of the *ldhA* gene encoding lactate dehydrogense, the enzyme responsible for the conversion of pyruvate into lactate, resulted in a 1.17-fold increase in 1,2-PDO concentration, although the 1,2-PDO yield slightly decreased (Fig. 4). It is important to note that this deletion would only eliminate the synthesis of lactate from pyruvate (fermentative route) and the activity of several methylglyoxal detoxification pathways could also result in lactate production (Fig. 1). While minimal amounts of lactate were seen with the overexpression of the 1,2-PDO pathway, the increase in lactate production when DHAP availability is increased may be a result of these methylglyoxal detoxification pathways (Table III). Specifically, the overexpression of both AOR and glyDH may result in competition for MG between these enzymes which could result in significant lactate production through lactaldehyde (Fig. 1). However, the fact only minimal amounts of lactate were produced upon deletion of *ldhA* indicates that the majority of lactate production was a result of the fermentative route through pyruvate (Table III). Therefore, it is likely that the lactate production accompanying the increased 1,2-PDO synthesis is a result of the need to achieve redox poise as the conversion of glycerol to lactate through pyruvate provides a means of generating reducing equivalents consumed in the synthesis of 1,2-PDO from glycerol (Fig. 1: note that glycerol conversion to lactate through this route also generates ATP). Interestingly, the deletion of *ldhA* and the subsequent decrease in lactate production was not accompanied by an increase in the yield of acetate produced, as one would expect in order to achieve redox poise (Table III). In fact, the disruption of the fermentative lactate pathway actually led to an increase in the yield of ethanol produced (Table III).

Another unexpected finding is the fact that blocking the route for fermentative acetate synthesis from acetyl-CoA (*ackA-pta* operon) resulted in the highest 1,2-PDO titers achieved by any deletion (1.2 g/L at a yield of 14.6% *w*/*w*) (Fig. 4). The deletion of the acetate synthesis pathway also resulted in nearly 50% increase in glycerol consumption and an increase in ethanol concentration and yield (2.5 g/L at a yield of 29.7% *w*/*w*) (Table III). Overall, these findings indicate that, as found in previous studies with wild-type *E. coli* (Murarka et al., 2008), ethanol synthesis is required for fermentative glycerol utilization despite the expression of a functional 1,2-PDO pathway. This dependence is further shown by the increases in ethanol production that result from the disruption of fermentative acetate and lactate production pathways. Both the requirement for and shift toward ethanol synthesis are likely the result of the aforementioned redox neutral and ATP producing nature of the glycerol to ethanol and formate/($CO_2 + H_2$) pathway, which unlike the fermentative acetate and lactate pathways, enables ATP generation without any stoichiometric constraints required for redox poise: $\text{Glycerol} \rightarrow \text{Ethanol} + \text{Formate}/(CO_2 + H_2) + \text{ATP}$ (see Fig. 1 and Murarka et al., 2008).

Given the redox- and ATP-consuming nature of 1,2-PDO synthesis from glycerol (Fig. 1), overexpression of fermentative pathways facilitating increased rates of co-product formation could aid in the generation of ATP and/or reducing equivalents which may favor 1,2-PDO production. A series of vectors were constructed to overexpress *E. coli ackA-pta* (pZSackApta), *ldhA* (pZSldhA), and *adhE* (pZSadhE) genes. However, the co-expression of either of these vectors with the 1,2-PDO pathway (i.e., pTHKLcfgldAmgsAyqhD) in MG1655 did not result in improved 1,2-PDO production over MG1655 expressing the 1,2-PDO pathway vector and the control vector pZSblank (data not shown). To ensure that the results from the overexpression of the acetate synthesis pathway were not affected by any kinetic limitation of phosphate acetyltransferase (*pta*) in the competition with acetaldehyde/alcohol dehydrogenase (*adhE*) for acetyl-CoA, the *adhE* deletion strain expressing the 1,2-PDO pathway

Table III. Product yields and carbon recovery in *E. coli* strains engineered for the production of 1,2-PDO.

Strain[a]	Glycerol consumed (g/L)	Product mass yields (% g/g)							Carbon[b] recovery
		1,2-PDO	Ethanol	Acetate	Lactate	Succinate	Formate	Pyruvate	
Engineering of 1,2-PDO synthesis pathways									
MG1655 (pTrcHis2A)	8.4 (0.9)	0.0 (0.0)	45.5 (1.3)	7.5 (1.2)	0.0 (0.0)	11.7 (3.5)	2.0 (1.1)	0 (0)	1.12
MG1655 (pTHgldAmgsAyqhD)	4.2 (0.2)	1.5 (0.1)	36.7 (1.1)	17.6 (0.4)	0.0 (0.0)	12.0 (0.1)	9.2 (0.5)	0 (0)	1.11
MG1655 (pTHgldAmgsAfucO)	4.7 (0.5)	1.7 (0.0)	38.0 (0.7)	14.0 (0.4)	0.0 (0.0)	11.5 (0.4)	7.3 (1.0)	0 (0)	1.08
MG1655 Δ*gloA* (pTHgldAmgsAyqhD)	4.0 (0.4)	2.0 (0.4)	44.3 (1.3)	14.2 (1.4)	0.0 (0.0)	10.3 (1.0)	11.0 (1.7)	0 (0)	1.21
MG1655 Δ*aldA* (pTHgldAmgsAyqhD)	3.9 (0.1)	1.6 (0.2)	45.5 (1.0)	14.1 (1.3)	0.0 (0.0)	10.8 (0.8)	11.5 (0.7)	0 (0)	1.23
Manipulation of glycerol utilization pathways									
MG1655 (pTHKLcfgldAmgsAyqhD)	5.6 (0.8)	13.3 (2.1)	25.6 (3.3)	8.6 (2.3)	19.7 (4.9)	5.8 (1.9)	0.3 (0.8)	0 (0)	1.05
MG1655 Δ*dhaK* (pTHKLcfgldAmgsAyqhD)	5.6 (0.4)	15.3 (0.6)	25.9 (1.0)	8.3 (1.2)	21.1 (2.0)	7.4 (0.4)	0.0 (0.0)	0 (0)	1.10
Engineering of major fermentation pathways									
MG1655 Δ*frdA* (pTHKLcfgldAmgsAyqhD)	3.9 (0.5)	11.5 (0.6)	24.7 (1.8)	15.3 (2.2)	21.0 (1.6)	1.0 (2.4)	0.0 (0.0)	0 (0)	1.08
MG1655 Δ*ldhA* (pTHKLcfgldAmgsAyqhD)	7.5 (1.7)	11.5 (4.6)	35.1 (4.0)	9.1 (3.0)	3.1 (1.1)	6.1 (1.4)	0.0 (0.0)	0 (0)	1.06
MG1655 Δ*ackA*Δ*pta* (pTHKLcfgldAmgsAyqhD)	8.3 (1.2)	14.6 (0.8)	29.7 (2.8)	1.2 (0.5)	20.9 (2.9)	3.2 (0.8)	0.2 (0.4)	0 (0)	1.03
MG1655 Δ*adhE* (pTHKLcfgldAmgsAyqhD)	1.1 (0.0)	2.1 (0.3)	0.0 (0.0)	61.0 (0.6)	0.0 (0.0)	14.4 (2.1)	0.0 (0.0)	0 (0)	1.07
Kinetics of glycerol consumption and product formation in *E. coli* strain engineered for 1,2-PDO production									
MG1655 Δ*ackA*Δ*pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD)	26.2 (0.1)	21.3 (0.1)	33.0 (0.1)	0.3 (0.1)	0.0 (0.0)	4.5 (0.1)	15.1 (0.2)	5.2 (0.1)	1.02
MG1655 Δ*ackA*Δ*pta*Δ*ldhA*Δ*dhaK*Δ*aceF* (pTHKLcfgldAmgsAyqhD)	19.9 (0.2)	21.2 (0.1)	32.9 (0.3)	0.3 (0.1)	0.0 (0.0)	5.2 (0.2)	17.3 (0.2)	7.3 (0.2)	1.05
MG1655 Δ*ackA*Δ*pta*Δ*ldhA*Δ*dhaK*Δ*fdhF* (pTHKLcfgldAmgsAyqhD)	17.9 (0.2)	21.6 (0.5)	27.2 (0.2)	0.3 (0.1)	0.0 (0.0)	4.7 (0.1)	29.1 (0.4)	12.6 (0.3)	1.04
MG1655 Δ*ackA*Δ*pta*Δ*ldhA*Δ*dhaK*Δ*aceF*Δ*fdhF* (pTHKLcfgldAmgsAyqhD)	13.7 (0.1)	19.8 (0.2)	31.6 (0.1)	0.4 (0.1)	0.0 (0.0)	5.4 (0.2)	29.7 (0.3)	11.5 (0.2)	1.04
MG1655 Δ*ackA*Δ*pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD) (Crude Glycerol)	18.9 (0.5)	23.9 (1.7)	32.7 (1.5)	0.4 (0.1)	0.0 (0.0)	4.6 (1.2)	6.1 (0.3)	6.2 (1.3)	1.05

[a]Data represent the average of a minimum of three independent samples (standard deviations shown in parenthesis) taken from 96-h sparged tube cultures, except those in "*Kinetics of glycerol consumption and product formation in E. coli strain engineered for 1,2-PDO production*" section which represent experiments in controlled fermentors with samples taken at 72 h.

[b]Carbon recovery is expressed as the ratio of mol of carbon in products per mol of carbon in glycerol consumed, assuming that moles of acetate *plus* moles of ethanol *equals* moles of 1-C compounds (formate *plus* CO_2) generated by the dissimilation of pyruvate (Dharmadi et al., 2006; Murarka et al., 2008).

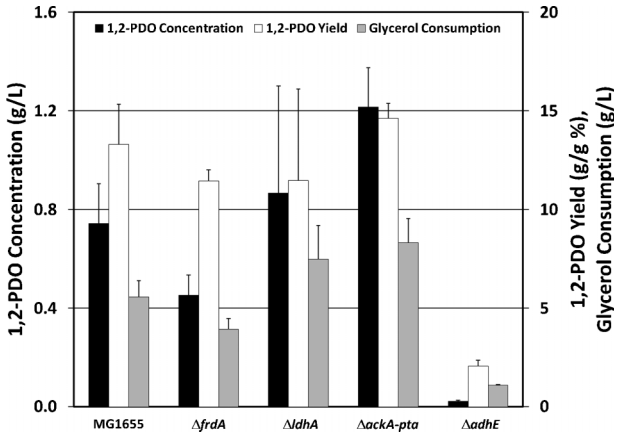

Figure 4. Effect of the disruption of major fermentative pathways on 1,2-PDO production and glycerol consumption in 96-h sparged tube experiments. Δ"*gene*" represents gene deletion. All strains containing pTHKLcfgldAmgsAyqhD expressing *C. freundii* ATP-dependent DHA kinase and *E. coli* glycerol dehydrogenase, methylglyoxal synthase and aldehyde oxidoreductase. Error bars represent standard deviations for a minimum of triplicate measurements.

(MG1655 Δ*adhE* (pTHKLcfgldAmgsAyqhD)) was transformed with pZSackApta. However, this strain consumed only small amounts of glycerol with no improvement to 1,2-PDO yield or titer (data not shown), further showing the importance of ethanol synthesis.

Kinetics of Glycerol Consumption and Product Formation in *E. coli* Strain Engineered for 1,2-PDO Production

Based on the results presented in previous sections, there are several genetic manipulations that warrant combination in an attempt to engineer *E. coli* for efficient 1,2-PDO production from glycerol. These manipulations include the expression of the engineered 1,2-PDO pathway (*E. coli gldA*, *mgsA*, and *yqhD* genes), the expression of ATP-dependent DHAK (*C. freundii dhaKL*) in place of the native *E. coli* PEP-dependent DHAK (Δ*dhaK*), and blocking the fermentative pathways for the production of acetate (Δ*ackA-pta*) and lactate (Δ*ldhA*). While the disruption of the fermentative pathways for both acetate and lactate production is a deviation from the theoretical design of co-production of 1,2-PDO and an oxidized co-product, the dependence of 1,2-PDO synthesis on ethanol production as well as the increased ethanol production when either fermentative lactate or acetate production is blocked point to the fact that co-production of 1,2-PDO and ethanol may be the best strategy. This appears to result from the additional ATP needed for cell growth and maintenance, as the amounts of acetate (or lactate) and 1,2-PDO co-produced to achieve redox balance would result in no net ATP generation. On the other hand, the production of ethanol would enable ATP generation without any constraints on its production due to the stoichiometric proportions needed for redox poise (Fig. 1).

An *E. coli* strain containing gene deletions blocking the synthesis of acetate and lactate, and including a deletion for the disruption of the native *E. coli* DHAK (MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK*) was transformed with pTHKLcfgldAmgsAyqhD expressing the engineered 1,2-PDO pathway and ATP-dependent DHAK. The performance of this strain was characterized in controlled fermentors (SixFors multi-fermentation system, see Materials and Methods section) and using a higher initial glycerol concentration (~30 g/L). Glycerol fermentation with MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD) resulted in excellent 1,2-PDO production throughout the time-course of the fermentation, with a final 1,2-PDO concentration of 5.6 g/L after 72 h (yield of 21.3% g/g) (Fig. 5A). As expected, the production of 1,2-PDO was accompanied with the co-production of ethanol (Fig. 5B). In addition, formate,

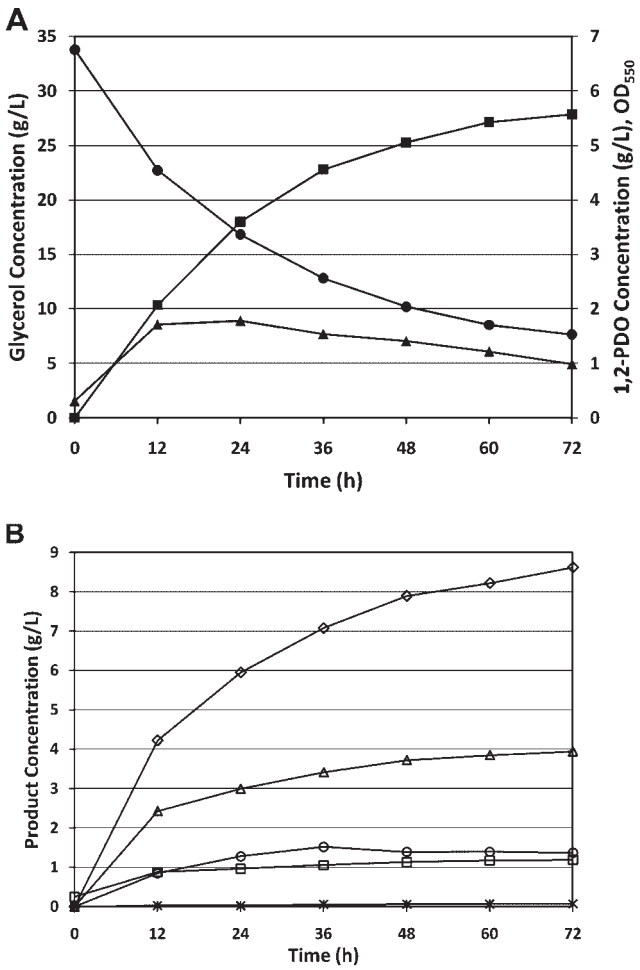

Figure 5. Kinetics of 1,2-PDO production by strain MG1655 Δ*ackA-pta*Δ*ldhA*Δ"'*dhaK* (pTHKLcfgldAmgsAyqhD). Experiments conducted in fully controlled fermentor at pH 7.5. (**A**) Fermentation profile with concentration of cells (▲), glycerol (●), and 1,2-PDO (■). (**B**) Concentration of by-products ethanol (◇), formate (Δ), succinate (□), acetate (*), and pyruvate (○). The coefficient of variation (standard deviation/mean × 100) was below 7% in all cases.

pyruvate, succinate, and minor amounts of acetate (which could be synthesized through pyruvate oxidase, encoded by *poxB*) were also produced (Fig. 5B, Table III).

With the disruption of fermentative pathways for the production of acetate and lactate in strain MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD), there is no obvious oxidized product that could be synthesized to enable redox balance. Other than the increased 1,2-PDO titers and yields, the most obvious difference in the product profile is the synthesis of pyruvate, which was not produced by any other strain tested in this study (Table III). While pyruvate does represent an oxidized product whose synthesis from glycerol generates reducing equivalents, the amount of pyruvate synthesized does not enable redox balance closure as the overall balance remains on the reduced side (i.e., negative value) (Table IV). The generation of the additional reducing equivalents required for redox poise can be accounted for by two possible scenarios, namely the dissimilation of pyruvate through the pyruvate dehydrogenase complex (PDH) and/or the recycling of hydrogen evolved from formate hydrogen lyase (FHL). PDH, which converts pyruvate to acetyl-CoA with the generation of CO_2 and NADH, is typically active under respiratory conditions and the absence of activity under anaerobic conditions has been explained by its sensitivity to NADH inhibition (de Graef et al., 1999; Sawers and Clark, 2004). However, it has recently been shown that PDH plays a significant role in fermentative metabolism of glucose and glucuronate indicating that it can function under anaerobic conditions (Murarka et al., 2010a, 2010b). In addition, the highly reduced nature of the product distribution in the engineered *E. coli* strain (Table IV) may result in a lower NADH/NAD ratio, which could relieve some of the NADH inhibition on PDH. The latter scenario involves the recycling of hydrogen evolved by FHL through the hydrogenase isoenzyme 1 or 2, a process which has been shown to play a role in the fermentative metabolism of *E. coli* (Alam and Clark, 1989; Murarka et al., 2008). Either scenario, which is not accounted for in the redox balances shown in Table IV, would result in the generation of additional reducing equivalents possibly enabling redox poise.

In order to test the importance of the aforementioned processes during 1,2-PDO production by the engineered strain, additional mutations to disrupt PDH activity (Δ*aceF*) and the evolution of hydrogen by FHL (Δ*fdhF*) were added both separately and in combination to the engineered strain (see Table I for complete genotypes). Fermentations with these strains were then conducted in identical conditions to the engineered strain. Based on the results seen in Table III, both PDH and hydrogen recycling appear to play a role in

Table IV. Analysis of redox balance during 1,2-PDO production.

		Strain							
		MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldA mgsAyqhD)		MG1655 Δ*ackA-pta*Δ*ldhA* Δ*dhaK*Δ*aceF* (pTHKLcfgldA mgsAyqhD)		MG1655 Δ*ackA-pta*Δ*ldhA* Δ*dhaK*Δ*fdhF* (pTHKLcfgldA mgsAyqhD)		MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK* Δ*aceF*Δ*fdhF* (pTHKLcfgldA mgsAyqhD)	
Substrate consumed and products formed[a]	Oxidation state[b]	Redox balance[c]	Carbon recovery[d]	Redox balance	Carbon recovery	Redox balance	Carbon recovery	Redox balance	Carbon recovery
Substrate									
Glycerol	−2	−2.0	3.0	−2.0	3.0	−2.0	3.0	−2.0	3.0
Products									
1,2-PDO	−4	−1.030	0.773	−1.028	0.771	−1.048	0.786	−0.960	0.720
Ethanol	−4	−2.636	1.318	−2.629	1.314	−2.396	1.198	−2.526	1.263
Acetic Acid	0	0	0.008	0	0.008	0	0.010	0	0.012
Pyruvic Acid	2	0.109	0.164	0.152	0.228	0.264	0.396	0.241	0.361
Succinic Acid	2	0.071	0.141	0.081	0.162	0.073	0.146	0.084	0.168
Formic Acid	2	0.603	0.302	0.693	0.346	1.162	0.581	1.189	0.594
Carbon dioxide[e]	4	1.446	0.361	1.259	0.315	0	0	0	0
Hydrogen[f]	−2	−0.723	0	−0.630	0	0	0	0	0
Subtotal-products	—	−2.160	3.067	−2.102	3.144	−1.944	3.116	−1.974	3.118
Total	—	−0.160	0.067	−0.102	0.144	0.056	0.116	0.026	0.118

[a]Substrate consumption and product formation data taken from 72 h samples shown in the "*Kinetics of glycerol consumption and product formation in E. coli strain engineered for 1,2-PDO production*" section of Table III.

[b]The oxidation state of carbon atoms was calculated assuming oxidation states of −2 and +1 for oxygen and hydrogen, respectively (Sawers and Clark, 2004).

[c]Redox balance values obtained by multiplying the number of moles of product per mole of glycerol by the oxidation state of carbon atoms.

[d]Carbon recovery calculated by multiplying the number of moles of product per mole of glycerol by the number of carbon atoms in the molecule.

[e]Carbon dioxide was calculated assuming that moles of acetate *plus* moles of ethanol *equals* moles of 1-C compounds (formate *plus* CO_2) generated by the dissimilation of pyruvate (Dharmadi et al., 2006; Murarka et al., 2008).

[f]Hydrogen values were obtained assuming that all pyruvate dissimilation to acetyl-CoA takes place through pyruvate formate lyase (i.e., no PDH activity) (de Graef et al., 1999; Sawers and Clark, 2004) and hence moles of hydrogen equals moles of carbon dioxide evolved through formate hydrogen lyase (Sawers et al., 2004).

1,2-PDO production as 1,2-PDO titers were significantly lower with PDH (4.2 g/L) or FHL (3.9 g/L) disruption when compared to the engineered strain (Fig. 5). In addition, the disruption of both PDH and FHL in the engineered strain (i.e., MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK*Δ*aceF*Δ*fdhF* (pTHKLcfgldAmgsAyqhD)) resulted in the production of only 2.7 g/L 1,2-PDO, appreciably lower than the 1,2-PDO titers seen with only one of PDH or FHL disrupted (Table III). The redox balances with these fermentations were significantly improved, with the strains containing the *fdhF* deletion shifting to a redox balance on the oxidative side (i.e., positive value) (Table IV). The closure of the redox balance in the case of PDH and FHL disruption is a direct consequence of forcing the assumptions used for the calculation of the redox balance (no PDH activity and hydrogen levels equal to the amount evolved by FHL) to hold true. The drastic difference in the redox balance of the engineered strain reflects the fact that these assumptions do not hold true with this strain, i.e., significant PDH activity and overestimation of hydrogen levels (due to hydrogen recycling) result in the calculation of a highly reduced redox balance. The measured PDH activity was 0.0125 ± 0.007 μmol/mg protein/min in the engineered strain and undetectable in the derivatives containing the *aceF* deletion. Since fumarate reductase (*frdABCD*) has been implicated in hydrogen recycling during glycerol fermentation (Murarka et al., 2008), the above results also support our observation that an *frdA* mutation had a negative impact on 1,2-PDO production (Fig. 3 and previous section). Overall, these results show that a combination of both PDH activity and hydrogen recycling is the most likely scenario enabling redox poise to be achieved during 1,2-PDO production with the engineered strain. Both processes appear to be beneficial for 1,2-PDO production.

Additional fermentations with strain MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD) were carried out using crude glycerol generated from the production of biodiesel. The performance of the engineered strain was comparable to the fermentation with pure glycerol (Table III), illustrating the potential for the industrial scale conversion of glycerol waste streams into 1,2-PDO.

Conclusions

The design and implementation of rational metabolic engineering strategies enabled the construction of an *E. coli* strain engineered to produce 1,2-PDO from glycerol. The overexpression of a pathway for 1,2-PDO synthesis from DHAP, the replacement of the native *E. coli* PEP-dependent DHAK with the ATP-dependent DHAK from *C. freundii* to ensure DHAP availability, and the disruption of the fermentative lactate and acetate pathways all contributed to the efficient production of 1,2-PDO. Since the synthesis of 1,2-PDO from glycerol is both redox- and ATP-consuming, the co-production of other fermentation products was required to ensure redox balance and ATP generation. The final strain produced 5.6 g/L 1,2-PDO (at a yield of 21.3% *w*/*w*), surpassing the performance of *E. coli* strains engineered to produce 1,2-PDO from glucose and was able to use crude glycerol, a by-product of biodiesel production.

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