ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Understanding and Harnessing the Microaerobic Metabolism of Glycerol in *Escherichia coli*

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Received 18 September 2008; revision received 8 November 2008; accepted 24 November 2008

Published online 30 December 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.22246

ABSTRACT: Given its availability, low prices, and high degree of reduction, glycerol has become an ideal feedstock for the production of reduced compounds. The anaerobic fermentation of glycerol by *Escherichia coli* could be an excellent platform for this purpose but it requires expensive nutrients such as tryptone and yeast extract. In this work, microaerobic conditions were used as a means of eliminating the need for rich nutrients. Availability of low amounts of oxygen enabled redox balance while preserving the ability to synthesize reduced products. A fermentation balance analysis showed ~95% recovery of carbon and reducing equivalents. The pathways involved in glycerol dissimilation were identified using different genetic and biochemical approaches. Respiratory (GlpK-GlpD/GlpABC) and fermentative (GldA-DhaKLM) routes mediated the conversion of glycerol to glycolytic intermediates. Although pyruvate formate-lyase (PFL) and pyruvate dehydrogenase contributed to the synthesis of acetyl-CoA from pyruvate, most of the carbon flux proceeded through PFL. The pathways mediating the synthesis of acetate and ethanol were required for the efficient utilization of glycerol. The microaerobic metabolism of glycerol was harnessed by engineering strains for the co-production of ethanol and hydrogen (EH05 [pZSKLMgldA]), and ethanol and formate (EF06 [pZSKLMgldA]). High ethanol yields were achieved by genetic manipulations that reduced the synthesis of by-products succinate, acetate, and lactate. Co-production of hydrogen required the use of acidic pH while formate co-production was facilitated by inactivation of the enzyme formate-hydrogen lyase. High rates of product synthesis were realized by overexpressing glycerol dehydrogenase (GldA) and dihydroxyacetone kinase (DhaKLM). Engineered strains efficiently produced ethanol and hydrogen and ethanol and formate from glycerol in a minimal medium without rich supplements.

Biotechnol. Bioeng. 2009;103: 148–161.

KEYWORDS: glycerol metabolism; *Escherichia coli*; metabolic engineering; biofuels and biochemicals

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Contract grant sponsor: U.S. National Science Foundation
Contract grant number: CBET-0645188
Contract grant sponsor: National Research Initiative of the U.S. Department of Agriculture Cooperative State Research, Education and Extension Service
Contract grant number: 2005-35504-16698
Contract grant sponsor: Glycos Biotechnologies, Inc.

Introduction

Glycerol has become an inexpensive and abundant carbon source due to its generation as inevitable by-product of biodiesel fuel production. With every 100 lb of biodiesel produced by the transesterification of vegetable oils or animal fats, 10 lb of crude glycerol are generated. The tremendous growth of the biodiesel industry has created a large surplus of glycerol and a dramatic decrease in crude glycerol prices. The conversion of these low-priced glycerol streams to higher value products has been proposed as a path to economic viability for the biofuels industry (Yazdani and Gonzalez, 2007). Such technologies could be readily integrated into existing biodiesel facilities, thus establishing true biorefineries and revolutionizing the biodiesel industry by dramatically improving its economics.

Although availability and low prices make glycerol an attractive carbon source for fermentation processes, there is yet another advantage in using this compound: fuels and reduced chemicals could be produced from glycerol at yields higher than those obtained from common sugars (Yazdani and Gonzalez, 2007). This is possible because the average degree of reduction per carbon, κ (Nielsen et al., 2003), of glycerol ($C_3H_8O_3$: $\kappa = 4.67$) is significantly higher than that

of sugars such as glucose ($C_6H_{12}O_6$: $\kappa = 4$) or xylose ($C_5H_{10}O_5$: $\kappa = 4$). To fully realize the aforementioned advantages, the use of anaerobic fermentations is desirable.

While many microorganisms are able to metabolize glycerol in the presence of external electron acceptors (respiratory metabolism), few are able to do so fermentatively (i.e., in the absence of electron acceptors). Until recently, the fermentative metabolism of glycerol had been reported only in species of the genera *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*, *Lactobacillus*, *Bacillus*, *Propionibacterium*, and *Anaerobiospirillum* (Yazdani and Gonzalez, 2007 and references cited therein). The use of these organisms at the industrial level could be limited due to issues such as pathogenicity, need for strict anaerobic conditions, supplementation with rich nutrients, or lack of the genetic tools and physiological knowledge necessary for their effective manipulation. The aforementioned problems could be overcome by using microorganisms such as *Escherichia coli*, which are very amenable to industrial applications.

Although it was long thought that the metabolism of glycerol in *E. coli* required the presence of external electron acceptors (Booth, 2005; Bouvet et al., 1994, 1995; Lin, 1976; Quastel et al., 1925; Quastel and Stephenson, 1925) (Fig. 1A), we recently discovered that *E. coli* can metabolize glycerol in a fermentative manner (Dharmadi et al., 2006; Gonzalez et al., 2008; Murarka et al., 2008) (Fig. 1B). In a more recent report we used the knowledge base created by our previous studies and engineered *E. coli* for the efficient production of ethanol from glycerol (Yazdani and Gonzalez, 2008). A drawback of this process is the requirement of rich supplements in the form of tryptone or yeast extract. In the work reported here we use microaerobic conditions (i.e., limited amounts of oxygen) as a means of eliminating the need for rich nutrients while still taking advantage of the high degree of reduction of glycerol. We show that microaerobic utilization of glycerol results in the synthesis of highly reduced products and identify the pathways involved in this metabolic process. We also demonstrate that the microaerobic metabolism of glycerol can be harnessed for the production of ethanol and co-products hydrogen and formate at high yields and productivities in a medium containing mineral salts without rich supplements.

Materials and Methods

Strains, Plasmids, and Genetic Methods

Strain BW25113, a derivative of the F-, λ-, *E. coli* K-12 strain BD792 (Datsenko and Wanner, 2000), along with its

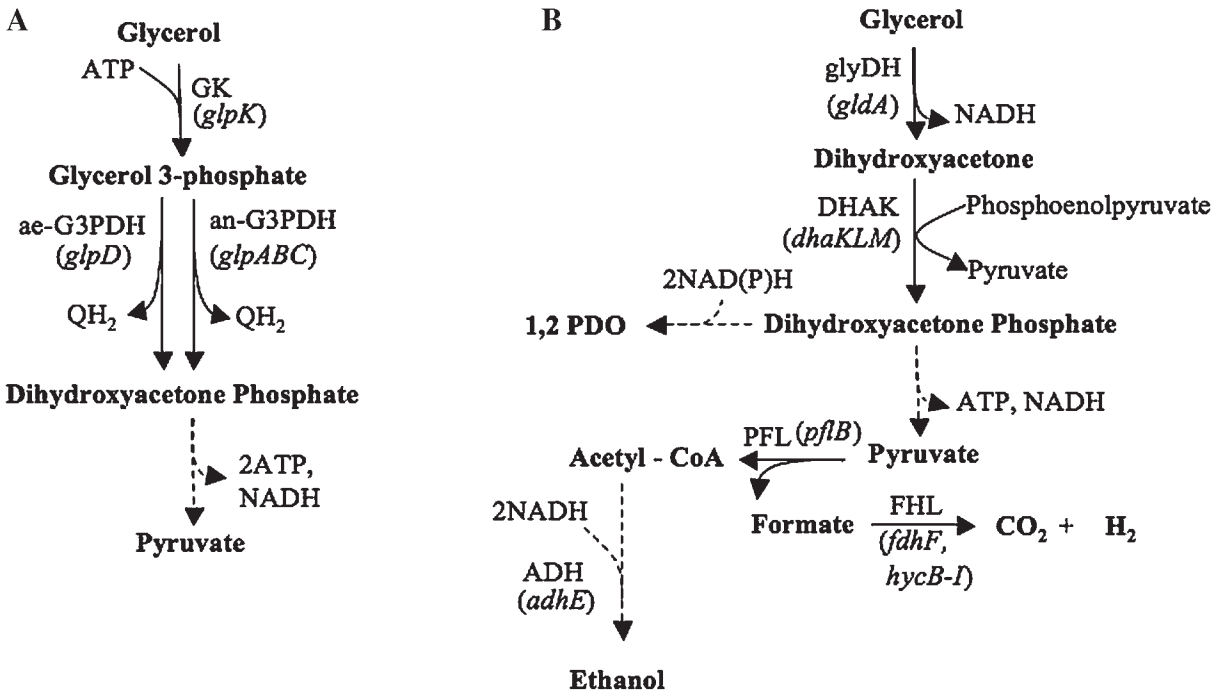

Figure 1. Pathways involved in the respiratory and fermentative utilization of glycerol in *E. coli*. **A**: Glycerol dissimilation in the presence of electron acceptors (i.e., respiratory metabolism) is mediated by an ATP-dependent glycerol kinase and two respiratory glycerol-3-phosphate dehydrogenases. **B**: Glycerol utilization in the absence of electron acceptors (i.e., fermentative metabolism) is mediated by a type II glycerol dehydrogenase and a phosphoenolpyruvate-dependent dihydroxyacetone kinase. Relevant enzymes and genes are shown. Broken lines represent multiple steps. ADH, acetaldehyde/alcohol dehydrogenase; ae-G3PDH, aerobic glycerol-3-phosphate dehydrogenase; an-G3PDH, anaerobic glycerol-3-phosphate dehydrogenase; DHAK, dihydroxyacetone kinase; FHL, formate hydrogen-lyase; GK, glycerol kinase; glyDH, glycerol dehydrogenase; PFL, pyruvate formate-lyase; QH_2, reduced quinones; 1,2 PDO, 1,2-propanediol.

otherwise isogenic single-gene knock out derivatives were obtained from the National BioResource Project (NIG, Japan) (Baba et al., 2006). For simplicity, these single-gene knockout mutants are referred to throughout the article using the following nomenclature: Δ*gene_name*, where "*gene_name*" is the disrupted gene. Wild-type K12 *E. coli* strain MG1655 was obtained from the University of Wisconsin *E. coli* Genome Project (www.genome.wisc.edu) (Kang et al., 2004) and used as the host to implement metabolic engineering strategies. Gene knockouts were introduced in MG1655 and its derivatives by P1 phage transduction as described elsewhere (Miller, 1972), using single gene knock out mutants from the National BioResource Project as donors. Details of the specific protocol used have been described elsewhere (Yazdani and Gonzalez, 2008). All strains, along with primers and plasmids used in their construction, are listed in Table I. Standard recombinant DNA procedures were used for gene cloning, plasmid isolation, and electroporation. 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 Luria-Bertani (LB) medium containing 1.5% agar and appropriate antibiotics were included at the following concentrations: 34 μg/mL chloramphenicol and 50 μg/mL kanamycin.

Culture Medium and Cultivation Conditions

The minimal medium designed by Neidhardt et al. (1974) with Na_2HPO_4 in place of K_2HPO_4 and supplemented with 20 g/L glycerol, 5 μM sodium selenite, 3.96 mM Na_2HPO_4, 5 mM $(NH_4)_2SO_4$, and 30 mM NH_4Cl was used unless otherwise specified. Chemicals were obtained from Fisher Scientific (Pittsburgh, PA) and Sigma–Aldrich Co. (St Louis, MO).

Fermentations were conducted in a multi-fermentation system with six 300-mL working volume vessels (CGS-The Kontes Custom Glass Shop, Vineland, NJ). Each vessel was fitted with a double-tube condenser maintained at 4°C by using a Fisher Scientific Isotemp Refrigerating Circulator 30165 (Thermo Fisher Scientific, Waltham, MA). The vessels were immersed in a water bath, whose temperature was maintained at 37.5°C with a Lauda E-100 series immersion circulator (Brinkmann, Westbury, NY). The pH was controlled at either 6.3 or 7.5 with a Jenco 3671 pH Controller fitted with a Jenco 600p pH probe (Jenco, San Diego, CA). Base (2 M NaOH) for pH control was added by gravity flow using a Pinch Valve (Bio-Chem Inc., Boonton, NJ) connected to the pH controller. The stirrer speed was

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

Strain/plasmid/primer	Genotype/structure/description	References
Strains		
MG1655	F-λ-*ilvG-rfb*-50 *rph*-1	Kang et al. (2004)
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR*514 Δ*araBA-D*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Baba et al. (2006)
Δ*gldA*	BW25113; Δ*gldA-FRT-Km-FRT*	Baba et al. (2006)
Δ*dhaK*	BW25113; Δ*dhaK-FRT-Km-FRT*	Baba et al. (2006)
Δ*glpD*	BW25113; Δ*glpD-FRT-Km-FRT*	Baba et al. (2006)
Δ*glpK*	BW25113; Δ*glpK-FRT-Km-FRT*	Baba et al. (2006)
Δ*glpA*	BW25113; Δ*glpA-FRT-Km-FRT*	Baba et al. (2006)
Δ*frdA*	BW25113; Δ*frdA-FRT-Km-FRT*	Baba et al. (2006)
Δ*pta*	BW25113; Δ*pta-FRT-Km-FRT*	Baba et al. (2006)
Δ*ldhA*	BW25113; Δ*ldhA-FRT-Km-FRT*	Baba et al. (2006)
Δ*adhE*	BW25113; Δ*adhE-FRT-Km-FRT*	Baba et al. (2006)
Δ*pflB*	BW25113; Δ*pflB-FRT-Km-FRT*	Baba et al. (2006)
Δ*aceF*	BW25113; Δ*aceF-FRT-Km-FRT*	Baba et al. (2006)
Δ*fdhF*	BW25113; Δ*fdhF-FRT-Km-FRT*	Baba et al. (2006)
Δ*poxB*	BW25113; Δ*poxB-FRT-Km-FRT*	Baba et al. (2006)
SY03	MG1655, Δ*frdA*::*FRT* Δ*pta-FRT-Km-FRT*	Yazdani and Gonzalez (2008)
SY04	MG1655, Δ*fdhF*::*FRT* Δ*frdA*::*FRT* Δ*pta-FRT-Km-FRT*	Yazdani and Gonzalez (2008)
EH03	MG1655, Δ*frdA*::*FRT* Δ*pta*::*FRT*	This study
EF04	MG1655, Δ*fdhF*::*FRT* Δ*frdA*::*FRT* Δ*pta*::*FRT*	This study
EH05	MG1655, Δ*frdA*::*FRT* Δ*pta*::*FRT* Δ*ldhA-FRT-Km-FRT*	This study
EF06	MG1655, Δ*fdhF*::*FRT* Δ*frdA*::*FRT* Δ*pta*::*FRT* Δ*ldhA-FRT-Km-FRT*	This study
Plasmids		
pCP20	rep$^{ts}_{pSC101}$ Ap^R Cm^R *cI*857 l P$_R$ flp+	Datsenko and Wanner (2000)
pZSKLMGldA	*E. coli dhaKLM* and *gldA* genes under control of P$_{LtetO-1}$ (tetR, oriR SC101*, cat)	Yazdani and Gonzalez (2008)
Primers[a]		
ldhA	cgtaatatcagggaatgaccc gggcaaagaatgtcaaaaacaa	This study

[a]Primers (5′ to 3′: forward sequence following reverse sequence) were used for verification of the *ldhA* mutation transferred from strain Δ*ldhA* to strains EH03 and EF04 via P1 phage transduction.

maintained at 300 rpm by using a Lab-Line Multi-Magnestir Model 1278 (Thermo Fisher Scientific, Waltham, MA). Microaerobic conditions were established by flushing air through the headspace of the fermenters at a flow rate of 0.01 L/min. The dissolved oxygen concentration was measured with a DO-BTA dissolved oxygen sensor (Vernier Software & Technology, Beaverton, OR). Operation under these conditions resulted in decreasing dissolved oxygen concentrations that fell below the detection limits after 2 h of cultivation (i.e., undetectable dissolved oxygen concentrations during almost the entire course of the fermentation).

Prior to use, the cultures (stored as glycerol stocks at -80°C) were streaked onto LB plates and incubated overnight at 37°C. Ten colonies were used to inoculate 250-mL Pyrex bottles (Thermo Fisher Scientific) containing 175 mL of minimal medium supplemented with 10 g/L of glycerol. The bottles were incubated at 37°C and 150 rpm in an NBS C24 Benchtop Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ) until an OD_{550} of ~0.3 was reached. An appropriate volume of this actively growing pre-culture was centrifuged, and the pellet was washed and used to inoculate 350 mL of medium in each fermenter, with a target initial optical density at 550 nm of 0.05. In some experiments, specified in each case, the minimal medium for the inoculum phase was supplemented with 10 g/L tryptone and 5 g/L yeast extract and/or the initial optical density in the fermenters was higher.

Analytical Methods

Optical density was measured at 550 nm and used as an estimate of cell mass (1 $OD_{550} = 0.34$ g dry weight/L) (Dharmadi et al., 2006). After centrifugation, the supernatant was stored at -20°C for high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) analysis. The identity of fermentation products was established by NMR as previously described (Murarka et al., 2008). Glycerol, organic acids, and ethanol were measured via HPLC and hydrogen was quantified as previously described (Dharmadi and Gonzalez, 2005; Dharmadi et al., 2006). The transfer of oxygen in microaerobic cultures was characterized by calculating the volumetric oxygen transfer rate (N_{O_2} in mg O_2/L/h) as $N_{O_2} = k_L a(C^* - C_L)$. k_L is the oxygen transport coefficient (cm/h), a is the gas–liquid interfacial area (cm^2/cm^3), $k_L a$ is the volumetric oxygen transfer coefficient (h^{-1}), C^* is the saturated dissolved oxygen concentration (mg/L), and C_L is the actual dissolved oxygen concentration in the liquid (mg/L) (Shuler and Kargi, 2002). The volumetric oxygen transfer coefficient ($k_L a$) was estimated by the static gassing out method (Shuler and Kargi, 2002). In these calculations, C^* was assumed to be 6.41 mg/L. During microaerobic operation, the dissolved oxygen concentration was essentially zero and therefore the volumetric rate of oxygen uptake equals the volumetric rate of oxygen transfer. The latter can be calculated as $N_{O_2} = k_L a \times C^*$($N_{O_2}$ in mg O_2/L/h). The average specific rate of consumption of oxygen (mg O_2/g Cell/h) was then obtained by dividing N_{O_2} by the time-average concentration of cells.

Enzyme Activities

Cells from microaerobic cultures were harvested by centrifugation (2 min, 10,000*g*), washed twice with 9 g/L NaCl, and stored as cell pellets at -20°C. The pellets were resuspended in 0.1 M potassium phosphate buffer to obtain ~3.4 mg dry cell weight/mL, and permeabilized with chloroform (Murarka et al., 2008; Osman et al., 1987; Tao et al., 2001). Anaerobic and aerobic glycerol-3-phosphate dehydrogenase (G3PDH) activities were assayed as previously reported (Murarka et al., 2008). Glycerol kinase activity was assayed as described by Kistler and Lin (1972), by measuring the change in absorbance at 340 nm and 25°C in a 1 mL reaction mixture containing 0.15 M glycine, 11 mM $MgCl_2$, 0.27 M hydrazine, 1.2 mM NAD^+, 5 mM ATP, 2 mM glycerol, 20 U of α-glycerophosphate dehydrogenase, and 50 μL crude cell extract prepared as described above.

The activity of alcohol dehydrogenase was measured as described by Kessler et al. (1991). Cells pellets, harvested as described above, were resuspended in 0.2 mL of 0.1 M MOPS–KOH and permeabilized by vortex mixing with chloroform. The activity was assayed by measuring the change in absorbance at 340 nm and 30°C in a 1 mL reaction mixture containing 0.4 mM NADH, 10 mM acetadehyde and 50 μL crude cell extract.

The activity of glycerol dehydrogenase towards the oxidation of glycerol was measured as previously described (Gonzalez et al., 2008) using 100 mM potassium carbonate buffer (pH 9.5). Dihydroxyacetone kinase activity was assayed using the method reported by Kornberg and Reeves (1972) with minor modifications. Cells from microaerobic cultures (OD_{550} of ~0.7) were harvested by centrifugation (2 min, 10,000*g*), washed with decryptification buffer (0.1 M-sodium-potassium phosphate, pH 7.2, and 5 mM $MgCl_2$) and stored as cell pellets at -20°C. The pellets were resuspended in decryptification buffer to obtain ~1 mg dry cell weight/mL. A portion of the ice-cold cell suspension was placed in a test tube, vigorously mixed for 1 min, and 0.2 volume of toluene–ethanol (1:9, v/v) added. The assay was conducted in a 1 mL reaction mixture containing 1 mM phosphoenolpyruvate (trisodium salt), 0.1 mM NADH, 2 U lactate dehydrogenase, and 50 μg dry weight of toluene–ethanol treated cells. The assay mixture was incubated at 30°C for 15 min. Dihydroxyacetone was added to a concentration of 1 mM and the decrease in absorbance was followed at 340 nm.

Linearity of the reactions (protein concentration and time) was established for all preparations. All spectrophotometric measurements were conducted in a BioMate 5 spectrophotometer (Thermo Scientific, Waltham, MA). The nonenzymatic rates were subtracted from the observed

initial reaction rates. Enzyme activities are reported as μmoles of substrate/min/mg of cell protein and represent averages for at least three cell preparations. A protein content of 50% (w/w) was assumed in these calculations.

Calculation of Fermentation Parameters

Data for cell growth, glycerol consumption, and product synthesis were used to calculate volumetric rates/productivities (mmoles/L/h). Specific rates (mmoles/g cell/h) were calculated by dividing the volumetric rates by the time-average concentration of cells. Growth and product yields (mole or gram of product per mole or gram of substrate consumed) were calculated as the amount of cell mass or product synthesized per amount of glycerol consumed once the cultures reached the stationary phase, unless otherwise stated. The "oxygen yield," defined as the amount of oxygen consumed in the metabolism of a given carbon source (molar basis), was calculated by dividing the volumetric rate of oxygen consumption by the volumetric rate of substrate consumption during the active growth phase. In the above calculations, we used an average molecular weight for an *E. coli* cell of 24.7, which corresponds to an average cell of a molecular formula $CH_{1.9}O_{0.5}N_{0.2}$ (Nielsen et al., 2003).

Results and Discussion

Glycerol Utilization Under Microaerobic Conditions and Its Metabolic Products

Given the high degree of reduction of carbon in glycerol, the synthesis of cell mass from this substrate is accompanied by the generation of reducing equivalents: $\kappa_{glycerol} = 4.7$ while $\kappa_{biomass} = 4.3$. Therefore, attaining redox balance in the absence of external electron acceptors requires the operation of a pathway(s) capable of converting glycerol to a more reduced fermentation product(s): that is, a pathway(s) leading to the consumption of reducing equivalents. The synthesis of 1,3-propanediol (1.3-PDO) and 1,2-PDO from glycerol are examples of such pathways (Gonzalez et al., 2008; Yazdani and Gonzalez, 2007): $\kappa_{glycerol} = 4.7$ while $\kappa_{1,2\text{-PDO}/1,3\text{-PDO}} = 5.3$.

We have recently reported that *E. coli* anaerobically ferments glycerol in a 1,2-PDO-dependent manner (Dharmadi et al., 2006; Gonzalez et al., 2008; Murarka et al., 2008). Given the low activity of the 1,2-PDO pathway, this metabolic process required supplementation of the medium with rich nutrients such as tryptone and yeast extract. Such supplementation with costly nutrients could prevent the use of *E. coli* as a biocatalyst for the production of fuels and chemicals from glycerol. Although the use of respiratory conditions enables redox balance and eliminates the requirement of medium supplementation, this metabolic mode would lead to low product yields as most of the carbon is incorporated in cellular mass and converted to carbon dioxide. The use of microaerobic conditions, on the other hand, has the potential to eliminate the requirement of rich nutrients while preserving the capacity to synthesize large amounts of products. In fact, the metabolism of glycerol under microaerobic conditions (i.e., limited amounts of oxygen) enabled redox balance while preserving the ability to convert glycerol to reduced products, as discussed below.

A typical fermentation profile for the microaerobic utilization of 20 g/L of glycerol by strain BW25113 is shown in Figure 2A. Similar results were obtained with the use of other strains, including MG1655, W3110, MC4110, and *E. coli B* (data not shown). When compared to the fermentative utilization of glycerol, the microaerobic process is much faster, consuming about 10 times the amount of glycerol utilized by the anaerobic culture (Fig. 2A). More importantly, the microaerobic utilization of glycerol does not require supplementation of the medium with rich nutrients and still results in a high recovery of carbon in

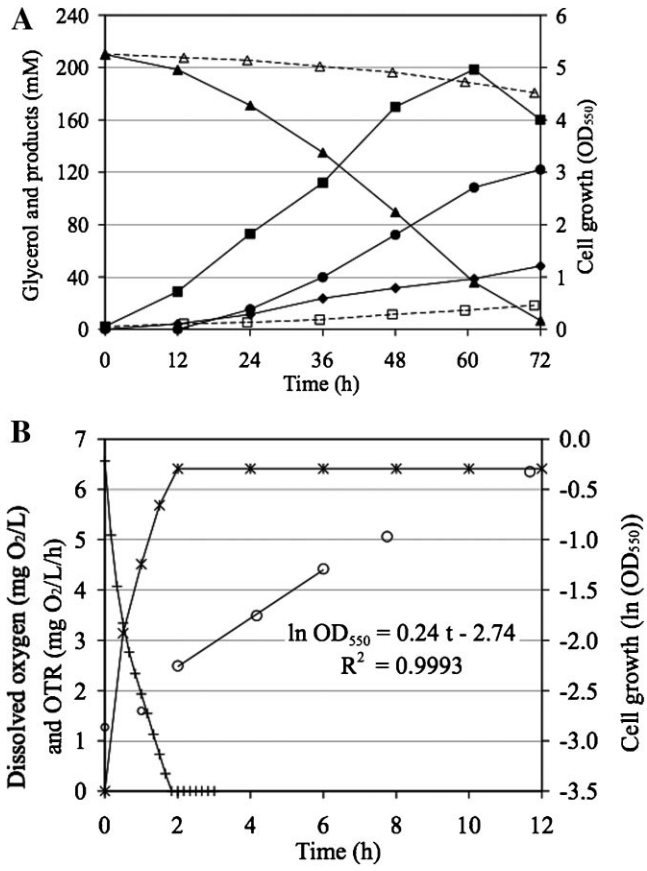

Figure 2. Glycerol utilization by *E. coli* BW25113 on minimal medium under microaerobic conditions. **A**: Concentration of cells (■), glycerol (▲), ethanol (●), and organic acids (◆). Concentration of cells (□) and glycerol (△) for the fermentative utilization of glycerol (same mineral salts medium but supplemented with 2 g/L tryptone) are also shown (open symbols and broken lines). **B**: Dissolved oxygen concentration (+), volumetric rate of oxygen transfer, OTR (✻), and log-linear plot of cell density (○) around the exponential growth phase. Fitting of the exponential-phase data to a straight-line model once microaerobic conditions are established is shown (least-squares method).

Table II. Calculation of the fermentation balance for the growth of *E. coli* BW25113 on glycerol under microaerobic conditions at pH 6.3 and 37°C.

Substrate consumed and products formed	Moles of product or substrate per mole of glycerol[a]	Oxidation state difference[b]	Redox balance[c]	Carbon recovery (C atoms)[d]
Glycerol	1	−2.0	−2.00	3.00
Ethanol	0.66	−4.0	−2.64	1.32
Acetic acid	0.18	+0.0	+0.00	0.37
Succinic acid	0.05	+2.0	+0.11	0.16
Formate	0.01	+2.0	+0.03	0.01
CO_2[e]	0.83	+4.0	+3.32	0.83
Hydrogen[e]	0.83	+2.0	+1.66	—
Cell mass[f]	0.10	−0.3	−0.03	0.10
Oxygen[g]	0.08	−4.0	−0.31	—
Sum[h]	—	—	+2.14	2.80

The data correspond to a 72-h sample from the culture shown in Figure 2A.

[a]Values are net moles produced/consumed per mole of glycerol utilized.

[b]The oxidation state difference was calculated assuming oxidation states of −3, −2, and +1 for nitrogen, oxygen and hydrogen, respectively (Sawers and Clark, 2004).

[c]Data in this column were obtained by multiplying the "moles of product per mole of glycerol" times the "oxidation state."

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

[e]Moles of CO_2 or hydrogen generated in the dissimilation of pyruvate were calculated assuming that moles of "CO_2 *plus* formate" *equals* moles of "ethanol *plus* acetate".

[f]A molecular formula of $CH_{1.9}O_{0.5}N_{0.2}$ was assumed for an average *E. coli* cell (Nielsen et al., 2003).

[g]Moles of oxygen per mole of glycerol were calculated by dividing the volumetric rate of oxygen consumption by the volumetric rate of glycerol utilization during the active growth phase.

[h]The "sum" does not account for glycerol.

products, primarily highly reduced compounds such as ethanol (Table II).

Microaerobic conditions were characterized by measuring the dissolved oxygen concentration and estimating the volumetric oxygen transfer coefficient ($k_La = 2.5\ h^{-1}$). This, in turn, allowed the calculation of the volumetric and specific rates of oxygen transfer and oxygen consumption (see Materials and Methods Section). The concentration of dissolved oxygen fell under the detection limit within 2 h after inoculation (Fig. 2B), defining two distinctive phases: an aerobic phase characterized by a continuously decreasing dissolved oxygen concentration, followed by a microaerobic phase whose onset is determined by a zero dissolved oxygen concentration. Once microaerobic conditions were established, cells grew exponentially at a maximum specific growth rate of 0.24 h^{-1} (Fig. 2B).

We used 1D proton NMR to identify the products of glycerol metabolism under microaerobic conditions. Figure 3 shows the spectrum of a 60-h fermentation sample from the culture in Figure 2A. The only products found in the extracellular medium were ethanol and succinic, acetic and formic acids. Although we have reported the synthesis of 1,2-propanediol during the fermentative metabolism of glycerol (Gonzalez et al., 2008; Murarka et al., 2008), this product was not found in the fermentation broth of microaerobic cultures. Figure 2A shows the time profiles for the identified metabolic products. The composition of the product mixture resembles that found during the fermentative metabolism of glycerol (Dharmadi et al., 2006; Gonzalez et al., 2008; Murarka et al., 2008). A fermentation balance showed close to 90% recovery of carbon in products, primarily the reduced compound ethanol, while about 10% of the carbon was consumed in the synthesis of cellular biomass (Table II). The overall redox balance was excellent as well, with less than 7% discrepancy between the amounts of reducing equivalents generated and consumed (Table II). The product formic acid accumulated in large proportions only when the cells were cultivated at basic pH (data not

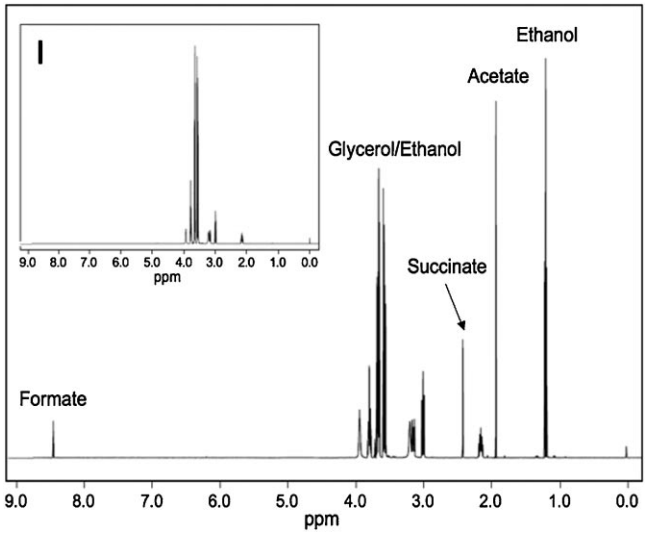

Figure 3. 1D 1H-NMR spectrum of the culture medium in a late fermentation sample (60 h in the fermentation shown in Fig. 2A). The inset "I" corresponds to the NMR spectrum of the time zero sample.

shown), owing to the lower activity of the enzyme formate hydrogen-lyase (FHL) at alkaline conditions: FHL converts formate to CO_2 and H_2 (Sawers and Clark, 2004).

Comparative Analysis of Glycerol, Glucose, and Xylose Utilization Under Microaerobic Conditions

The high carbon recovery in reduced products during the microaerobic utilization of glycerol is a direct consequence of the high degree of reduction of this carbon source. To illustrate the unique characteristics of glycerol in this regard, we compared its metabolism to that of glucose and xylose under the microaerobic conditions described above. These two sugars were selected due to the fact that they represent the most abundant carbon sources available for the production of chemicals and fuels via microbial fermentations. It is noteworthy that the degree of reduction per carbon, κ, of glycerol is significantly higher than that of glucose or xylose: $\kappa_{glycerol} = 4.67$ while $\kappa_{glucose/xylose} = 4$.

The product mixtures generated by the microaerobic utilization of glycerol, glucose, and xylose were very different (Fig. 4). The most abundant product of glycerol metabolism was ethanol (>60% w/w of the final product mixture) while no lactate was found in the medium. However, acetate and lactate were the main products of glucose and xylose metabolism, together accounting for more than 50% (w/w) of the product mixture. The yield of ethanol, the most reduced compound among these metabolic products, was two- to threefold higher when glycerol was used as carbon source (Fig. 4). The ethanol-to-acetate ratio was also much higher on glycerol, 2.8, compared to 0.65 on glucose or xylose. This 4.3-fold increase in ethanol-to-acetate ratio clearly reflects the higher reduced state of carbon in glycerol.

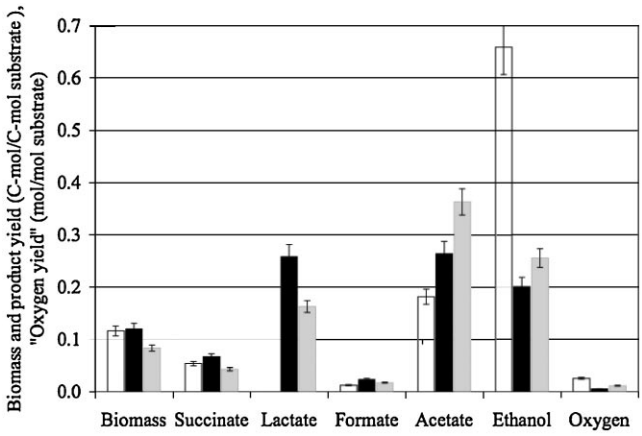

Figure 4. Product, biomass and oxygen yield during the microaerobic utilization of glycerol (white bars), glucose (black bars) and xylose (gray bars) by *E. coli* BW25113. The "oxygen yield" represents the amount of oxygen consumed in the metabolism of each substrate. Values represent the means and bars standard deviations from three samples taken in stationary phase cultures.

Note that, excluding formate, ethanol ($\kappa = 6$) and acetate ($\kappa = 3.5$) are the most reduced and oxidized compounds, respectively, among the products being analyzed. Although glucose was consumed much faster than xylose and glycerol (18, 30, and 60 h for glucose, xylose, and glycerol consumption, respectively), the biomass yield was very similar on glucose and glycerol and about 25% lower on xylose (Fig. 4). Another parameter shown in Figure 4 is the "oxygen yield", which represents the amount of oxygen consumed during the utilization of each carbon source (moles oxygen/moles of substrate). This parameter was much higher on glycerol: 1.4- and 2.4-fold higher than xylose and glucose, respectively. If expressed per C-mol basis, the differences in oxygen yield are even more significant: 2.3- and 4.8-fold higher than xylose and glucose, respectively. Since the synthesis of different metabolic products is largely determined by energy and redox constraints, an analysis of the generation and consumption of reducing equivalents and ATP during the utilization of glycerol, glucose, and xylose serves as another important point of comparison and is discussed below.

E. coli generates 1 net ATP per each molecule of pyruvate synthesized from glycerol or glucose via the Embden-Meyerhof-Parnas pathway (Fig. 5). Xylose, however, is a less energetically efficient carbon source that produces ~ 0.4 net ATP per pyruvate (Fig. 5). This lower energy generation in the glycolytic pathway would explain the higher levels of acetate produced during the metabolism of xylose (Fig. 4), as the synthesis of a molecule of acetate from acetyl-CoA generates one molecule of ATP. Since the synthesis of acetate

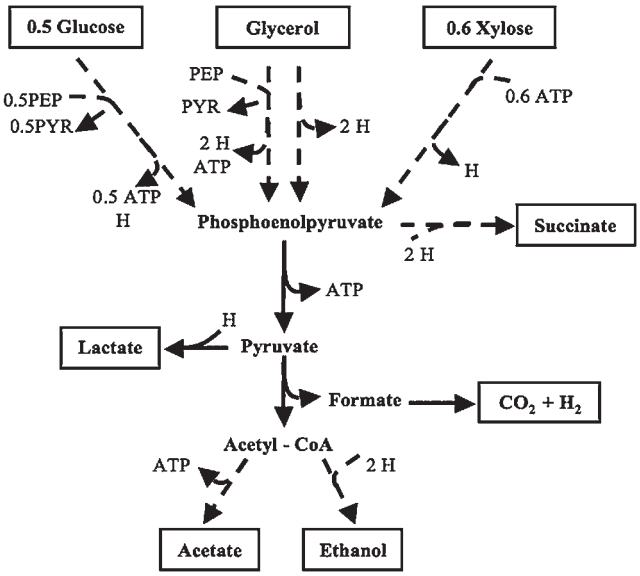

Figure 5. Generation and consumption of ATP and reducing equivalents during the conversion of glycerol, glucose, and xylose to glycolytic intermediates and metabolic products. Extracellular metabolites are boxed. Broken lines illustrate multiple steps. H, $NADH/FADH_2/QH_2$; PEP, phosphoenolpyruvate; PYR, pyruvate.

from xylose results in the net generation of reducing equivalents (Fig. 5), the utilization of this carbon source requires a larger amount of oxygen than the utilization of glucose (Fig. 4). Higher lactate production on glucose, compared to xylose (Fig. 4), can also be related to higher energy generation: lactate is produced from pyruvate, and therefore its synthesis from glucose and xylose would generate 1 and 0.4 moles of ATP, respectively (Fig. 5). Although favorable in terms of ATP generation, synthesis of acetate and lactate from glycerol are greatly limited by redox constraints, as discussed below.

A redox balance analysis can also help explain the observed differences in the microaerobic utilization of glycerol, glucose, and xylose. As discussed in previous sections, the incorporation of glycerol in cellular biomass results in the generation of reducing equivalents. Under conditions of limited availability of electron acceptors (e.g., microaerobic conditions), the utilization of glycerol would be favored by the production of metabolites whose synthesis does not involve the net generation of reducing equivalents. This appears to be the reason why the primary product of glycerol utilization is ethanol (Fig. 4), as its synthesis from glycerol takes place through a redox-balanced pathway (Fig. 5). While conversion of glycerol to succinate is also a redox-balanced pathway, its contribution to ATP generation, if any, is very limited (Fig. 5). Synthesis of cell mass from glucose or xylose ($\kappa_{glucose/xylose} = 4.0$), on the other hand, is a redox-consuming process and thus favors the synthesis of acetate, which is a redox-generating pathway (Fig. 5). This would explain the higher levels of acetate produced by glucose- and xylose-grown cultures and their lower ''oxygen yield,'' when compared to glycerol cultures (Fig. 4). Synthesis of lactate from glycerol, although energetically advantageous (1 mole of ATP per mole of lactate), is a redox-generating pathway (Fig. 5) and therefore not favored during the microaerobic metabolism of this carbon source. Indeed, no lactate was found in the glycerin-grown cultures. Production of lactate from sugars, on the other hand, is redox-balanced and therefore a favored metabolic pathway (Fig. 4). The higher levels of lactate produced by cells grown on glucose, compared to xylose-grown cells, would reflect the aforementioned advantages of glucose over xylose in terms of energy generation via the glycolytic pathway: lactate synthesis from glucose and xylose generates 1 and 0.4 mole of ATP, respectively.

Conversion of Glycerol to Glycolytic Intermediates

Two alternative routes have been reported to mediate the conversion of glycerol to dihydroxyacetone phosphate (DHAP) in *E. coli* (Fig. 1): (i) the GlpK-GlpD/GlpABC route under respiratory conditions (Booth, 2005) and (ii) the GldA-DHAK route under fermentative conditions (Gonzalez et al., 2008; Murarka et al., 2008). As a first approach to assess the contribution of these pathways, we measured the activities of the corresponding enzymes and the results are shown in Table III. Values are reported for two phases: the initial aerobic phase in which dissolved oxygen concentrations were above detection limits (50% of saturation, which corresponds to a 1-h culture), and the microaerobic phase in which dissolved oxygen concentrations were undetectable (a 24-h culture) (Fig. 2B shows the profile of dissolved oxygen concentration). Significant activity was detected for all enzymes assayed, thus indicating that both routes are active during the dissimilation of glycerol under microaerobic conditions. The activity of glycerol kinase and aerobic glycerol-3-P dehydrogenase was higher than that of the other enzymes during the initial, aerobic phase. Glycerol dehydrogenase and dihydroxyacetone kinase activities were very minimal during this phase. The transition to microaerobic conditions, characterized by undetectable amounts of dissolved oxygen, triggered a 2.5-fold increase in the anaerobic glycerol-3-phosphate dehydrogenase activity and a 10-fold increase in both glycerol dehydrogenase and dihydroxyacetone kinase activities (Table III). This transition was also accompanied by a 60% decrease in glycerol kinase activity. These results indicate that the respiratory and fermentative pathways appear to have ''complementary'' functions, with the GlpK-GlpD pathway having a predominant role during the initial phase of the culture and the GldA-DhaKLM pathway being more active at late stages. This behavior would be in agreement with the proposed role of each of these pathways in the respiratory (GlpK-GlpD) and fermentative (GldA-DhaKLM) utilization of glycerol (Gonzalez et al., 2008; Murarka et al., 2008; Yazdani and Gonzalez, 2007 and references therein).

We also evaluated strains containing single deletions in *gldA*, *dhaK*, *glpK*, *glpD*, and *glpA* (see Fig. 1 for roles of these genes). In previous studies we characterized these mutants and demonstrated that the genetic manipulations indeed eliminated the target activities (Gonzalez et al., 2008; Murarka et al., 2008). Inactivation of glycerol dehydrogen-

Table III. Activities of enzymes involved in the conversion of glycerol to glycolytic intermediate dihydroxyacetone phosphate.*

	Harvesting time[a]	
Activity measured	1-h culture	24-h culture
Glycerol kinase	0.167 ± 0.002	0.097 ± 0.049
Aerobic glycerol-3-phosphate dehydrogenase	0.028 ± 0.006	0.029 ± 0.002
Anaerobic glycerol-3-phosphate dehydrogenase	0.017 ± 0.005	0.029 ± 0.007
Glycerol dehydrogenase	0.004 ± 0.0003	0.033 ± 0.001
Dihydroxyacetone kinase	0.001 ± 0.0002	0.009 ± 0.001

*All activities were measured as described in Materials and Methods Section and values (μmoles/min/mg protein) are reported as average ± standard deviation for triplicate assays.

[a]Two BW25113 cultures were grown under the conditions described in Materials and Methods Section. One culture was harvested after 1 h, when the dissolved oxygen concentration was 50% of the saturation level. The second culture was harvested after 24 h, when the dissolved oxygen concentration in the liquid was below detection limits.

ase (Δ*gldA*) or dihydroxyacetone kinase (Δ*dhaK*) resulted in a reduction in cell growth and glycerol utilization to approximately 70% and 50% of that observed in wild-type BW25113, respectively (Fig. 6A). These results clearly indicate that the GldA-DHAK pathway contributes to the microaerobic utilization of glycerol, although it is not strictly required for this metabolic process. Disruption of the genes encoding glycerol kinase (Δ*glpK*) or the aerobic glycerol-3-P dehydrogenase (Δ*glpD*), on the other hand, prevented cell growth at the inoculum preparation phase (data not shown). The lack of growth under these conditions appeared to indicate that the GlpK-GlpD route was an absolute requirement for the microaerobic utilization of glycerol. This was a surprising result, given the significant activity of the alternative GldA-DHAK pathway in BW25113 (Table III) and the observation that knockouts of *gldA* and *dhaK* negatively affected glycerol utilization (Fig. 6A). Since the initial phases of our cultures were significantly aerobic (inocula are prepared in closed bottles with air in the headspace: see Materials and Methods Section for details), we hypothesize that the activity of the GlpK-GlpD pathway is required under these initially aerobic conditions. To test this hypothesis, inocula for strains Δ*glpK* and Δ*glpD* were prepared using MOPS minimal medium supplemented with 5 g/L yeast extract and 10 g/L tryptone, which allowed the growth of both strains. Actively growing cultures were washed with MOPS minimal medium in order to avoid carry over of rich nutrients to the fermenters and the effect of inoculum size was evaluated: high and low initial OD_{550} of 0.05 and 0.5, respectively. The cultures inoculated with larger inocula grew with no detectable lag phase, although cell growth and glycerol consumption were significantly impaired: 40–80% of that observed for wild-type BW25113 (Fig. 6A: Δ*glpK*** and Δ*glpD***). Cultures with small inocula grew very slowly for about 24 h, at which point they had reached an OD_{550} of about 0.12 (data not shown). These cultures were also significantly impaired in cell growth and glycerol fermentation (Fig. 6A: Δ*glpK** and Δ*glpD**). Taken together, these results indicate that the GlpK-GlpD pathway contributes to the efficiency of microaerobic growth on glycerol but it is not essential for this metabolic process. In fact, overexpression of the GldA-DhaKLM pathway in strain Δ*glpK* or Δ*glpD* resulted in cell growth and glycerol fermentation at levels equivalents to those observed for wild-type BW25113 (Fig. 6A). Finally, disruption of the anaerobic glycerol-3-P dehydrogenase had little effect on cell growth and glycerol fermentation (Fig. 6A).

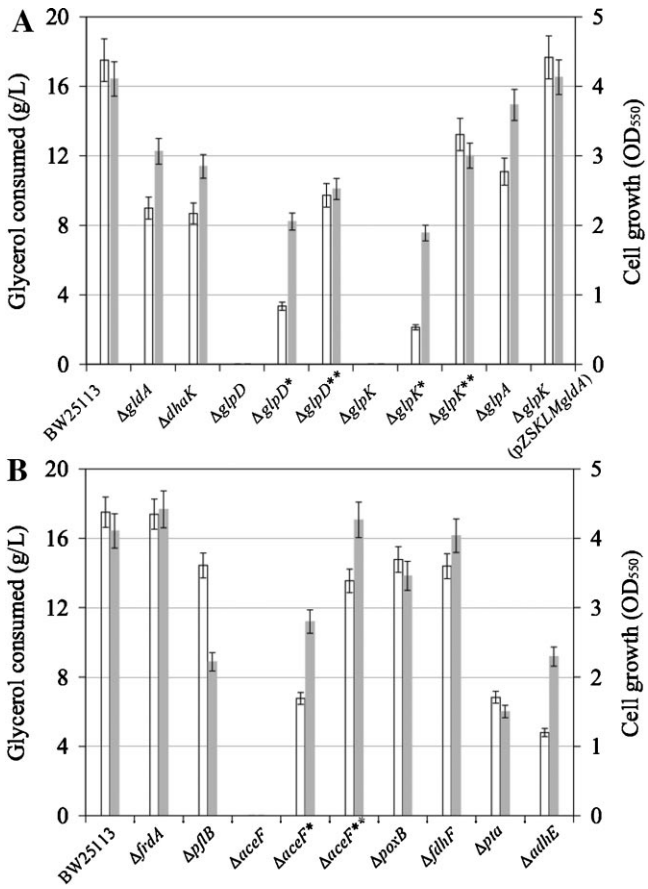

Figure 6. Effect of single knockout mutations on glycerol utilization (white bars) and cell growth (gray bars). **A**: Mutated genes are involved in the respiratory and fermentative conversion of glycerol to glycolytic intermediate dihydroxyacetone phosphate (DHAP). **B**: Mutated genes are involved in the synthesis of metabolic products originating from phosphoenolpyruvate, pyruvate, and acetyl-CoA. Unless otherwise specified, values represent the means and bars standard deviations for three samples taken at the earlier of: (i) the time at which a culture of the specified strain entered stationary phase or (ii) the time at which the BW25113 culture entered stationary phase. One asterisk (*) indicates that the inoculum was prepared in minimal medium supplemented with 5 g/L yeast extract and 10 g/L tryptone. Two asterisks (**) indicate that the inoculum was prepared in minimal medium supplemented with 5 g/L yeast extract and 10 g/L tryptone and the initial OD_{550} in the fermenter was ~0.5.

Figure 6A also indicates that knockouts of the aerobic glycerol-3-P dehydrogenase (Δ*glpD*) and the fumarate reductase (Δ*frdA*) had little effect on cell growth and glycerol fermentation. Since the initial phases of our cultures were significantly aerobic (inocula are prepared in closed bottles with air in the headspace: see Materials and Methods Section for details), we hypothesize that the activity of the GlpK-GlpD pathway is required under these initially aerobic conditions. To test this hypothesis, inocula for strains Δ*glpK* and Δ*glpD* were prepared using MOPS minimal medium supplemented with 5 g/L yeast extract and 10 g/L tryptone, which allowed the growth of both strains. Actively growing cultures were washed with MOPS minimal medium in order to avoid carry over of rich nutrients to the fermenters and the effect of inoculum size was evaluated: high and low initial OD_{550} of 0.05 and 0.5, respectively. The cultures inoculated with larger inocula grew with no detectable lag phase, although cell growth and glycerol consumption were significantly impaired: 40–80% of that observed for wild-type BW25113 (Fig. 6A: Δ*glpK*** and Δ*glpD***). Cultures with small inocula grew very slowly for about 24 h, at which point they had reached an OD_{550} of about 0.12 (data not shown). These cultures were also significantly impaired in cell growth and glycerol fermentation (Fig. 6A: Δ*glpK** and Δ*glpD**). Taken together, these results indicate that the GlpK-GlpD pathway contributes to the efficiency of microaerobic growth on glycerol but it is not essential for this metabolic process. In fact, overexpression of the GldA-DhaKLM pathway in strain Δ*glpK* or Δ*glpD* resulted in cell growth and glycerol fermentation at levels equivalents to those observed for wild-type BW25113 (Fig. 6A). Finally, disruption of the anaerobic glycerol-3-P dehydrogenase had little effect on cell growth and glycerol fermentation (Fig. 6A).

The above results clearly demonstrate that both GldA-DhaKLM and GlpK-GlpD pathways contribute to the conversion of glycerol to DHAP. Common glycolytic enzymes catalyze the remaining steps in the conversion of DHAP to pyruvate, thus completing the Embden-Meyerhof-Parnas (EMP) pathway (Romeo and Snoep, 2005). In addition, gluconeogenic metabolism of DHAP is required as the source of 4-, 5-, and 6-C precursor metabolites such as fructose-6-phosphate, glucose-6-phosphate and pentose phosphate pathway intermediates. In this context, the gluconeogenic role of enzymes such as fructose bisphosphate aldolase, fructose 1,6-bisphosphatase, and phosphoglucose isomerase for the growth of *E. coli* on C-3 carbon sources such as glycerol has been well established (Romeo and Snoep, 2005). In agreement with those reports, we found that these enzymes were required for the microaerobic utilization of glycerol (data not shown).

Dissimilation of Phosphoenolpyruvate, Pyruvate, and Acetyl-CoA

The metabolism of phosphoenolpyruvate (PEP), pyruvate and acetyl-CoA resulted in the generation of metabolic products succinate, acetate, ethanol, and formate (Fig. 3). In

this section we investigate the potential role of the enzymes mediating the synthesis of these products from PEP, pyruvate and acetyl-CoA. Succinate is synthesized from PEP in a sequence of reactions that ends with the conversion of fumarate to succinate, a reaction catalyzed by the enzyme fumarate reductase (FRD) (Sawers and Clark, 2004). Although mutant Δ*frdA* produced negligible amounts of succinate (Table IV), it grew and utilized glycerol at levels similar to those observed in strain BW25113 (Fig. 6B). All other metabolic products generated during the microaerobic utilization of glycerol originate from pyruvate, which is metabolized by four different enzymes: lactate dehydrogenase (LDH), pyruvate formate-lyase (PFL), pyruvate dehydrogenase (PDH), and pyruvate oxidase (POX). LDH, encoded by *ldhA*, catalyzes the reduction of pyruvate to lactate. Since lactate was not found in the fermentation broth of wild-type BW25113 (Fig. 3, Table IV), we did not investigate the role of this pathway.

Two enzymes can mediate the synthesis of acetyl-CoA from pyruvate: PFL, encoded by *pflB*, and PDH, encoded by the *aceEF-lpdA* operon. PFL, an oxygen sensitive enzyme, is primarily active during anaerobic fermentation, although its activity has also been reported during microaerobic metabolism of sugars (Sawers and Clark, 2004). PDH, on the other hand, is active primarily in the presence of oxygen or other electron acceptors, although low levels of PDH activity have been reported under fermentative conditions (Sawers and Clark, 2004; Gonzalez, unpublished work). The redox status of the cell determines which of the two enzymes, PDH or PFL, is active under a particular condition. Since dissolved oxygen is detectable in the fermentation broth only for the initial 2 h of the cultures (see Fig. 2B), it is then expected that PDH would be more active during the initial stages of the fermentation while PFL would be the primary enzyme for the rest of the cultivation. Several lines of evidence support this hypothesis. First, as in the case of strains Δ*glpK* and Δ*glpD* (see above), the inoculum of a PDH mutant (strain Δ*aceF*) required the use of complex supplementation (5 g/L yeast extract and 10 g/L tryptone). Once inoculated in the fermenters at an initial OD_{550} of ~0.05, strain Δ*aceF* exhibited a very slow growth during early stages of cultivation: very little growth for about 15 h (2 doublings, an OD_{550} of about 0.2). This behavior resulted in a significant reduction in glycerol utilization and cell growth: only 53% and 68%, respectively, of that observed for BW25113 (Fig. 6B: Δ*aceF**). The use of a larger inoculum for Δ*aceF* resulted in a performance very similar to wild-type BW25113 (Fig. 6B: Δ*aceF***). It is noteworthy that the composition of the fermentation products for Δ*aceF* was similar to that of BW25113 (Table IV). On the other hand, a PFL-deficient strain (Δ*pflB*) exhibited a wild-type behavior up to about 32 h of fermentation, at which point the culture entered into an ''early'' stationary phase. This resulted in a reduction of cell growth and glycerol fermentation to levels

Table IV. Carbon recovery in cell mass and fermentation products during the microaerobic utilization of glycerol in minimal medium.

	Carbon recovery[b]							
Strain and culture conditions[a]	Ethanol	Succinate	Acetate	Formate	Lactate	Biomass	Products	Overall
Wild-type strains								
BW25113	66	5	18	3	0	11	91	101
MG1655	52	13	16	3	0	10	85	95
Strains with deletions in genes involved on glycerol utilization pathways								
Δ*gldA*	29	5	42	3	0	15	76	91
Δ*dhaK*	27	4	44	0	0	15	76	91
Δ*glpK***	76	5	12	6	0	10	93	103
Δ*glpD***	77	3	12	6	0	12	92	104
Δ*glpA*	56	3	19	6	0	13	78	92
Strains with deletions in genes involved on the synthesis of metabolic products								
Δ*frdA*	67	1	19	3	0	11	87	99
Δ*aceF***	65	6	11	6	0	14	82	96
Δ*pflB*	3	1	8	0	67	7	79	86
Δ*poxB*	62	4	21	3	0	11	87	97
Δ*fdhF*	42	4	28	39	3	13	78	90
Δ*pta*	7	3	5	0	48	10	63	73
Δ*adhE*	0	11	62	0	0	21	73	94
Strains engineered for the production of ethanol and co-products								
EH03	79	0	2	12	6	11	87	98
EF04	80	0	2	75	4	9	85	94
EH05, 60 g/L glyc.	84	1	4	9	0	5	89	94

[a]Unless otherwise stated, cells were cultivated in the medium and conditions described in Materials and Methods Section. Data represent the average of three samples taken at the earlier of: (i) the time at which a culture of the specific strain entered stationary phase or (ii) the time at which the BW25113 culture entered stationary phase. The coefficient of variation (standard deviation/mean × 100) was below 9% in all cases. Two asterisks (**) indicate that the inoculum was prepared in minimal medium supplemented with 5 g/L yeast extract and 10 g/L tryptone and the initial OD_{550} in the fermenter was ~0.5.

[b]Carbon recovery is expressed as the percent mole of carbon in product, including biomass, per mole of carbon in glycerol consumed. The column ''Products'' shows the total recovery of carbon in products, 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. The column ''overall'' shows the overall carbon recovery, including products and biomass.

roughly 50% of those observed for wild-type BW25113 (Fig. 6B). More importantly, a significant redistribution of fermentation products took place in response to the *pflB* mutation: strain Δ*pflB* exhibited an almost homolactic behavior, producing about 11.5 g/L of lactate, compared to undetectable amounts in the wild-type (Table IV). Taken together, these results show that although both PFL and PDH are involved in the conversion of pyruvate to AcCoA under microaerobic conditions, PFL is responsible for a larger fraction of the flux through this step. Furthermore, these enzymes appear to have complementary roles during early (PDH) and late (PFL) stages of cell growth, perhaps due to the transition from an "aerobic-like" to an "anaerobic-like" environment as the fermentation proceeds.

A fourth enzyme involved in the metabolism of pyruvate, POX (encoded by *poxB*), generates acetate and CO_2 and is important for the growth efficiency of wild-type *E. coli* under aerobic conditions (Abdel-Hamid et al., 2001; Li et al., 2007). The same studies established that POX was responsible for a significant amount of pyruvate metabolism. The Δ*poxB* strain exhibited only a small change in cell growth and glycerol fermentation and no significant redistribution of carbon among fermentation products (Fig. 6B, Table IV).

Dissimilation of pyruvate via PFL results in the generation of both acetyl-CoA and formate. The latter can be either secreted to the extracellular medium or converted to carbon dioxide and hydrogen by formate hydrogen-lyase (FHL) (Sawers et al., 2004) (Fig. 1B). Since we recently reported that FHL is required for the fermentative metabolism of glycerol at acidic pH (Gonzalez et al., 2008), we investigate here whether this enzyme is also involved in the microaerobic utilization of glycerol. Strain Δ*fdhF* grew and utilized glycerol in a wild-type manner (Fig. 6B). However, the distribution of carbon among metabolic products was significantly affected (Table IV). Δ*fdhF* produced about 0.4 g/L of lactate, compared to undetectable levels in wild-type BW25113. Furthermore, the ratio of ethanol/acetate decreased to about 40% of that observed in the wild-type due to both a decrease in ethanol production and an increase in acetate production (Table IV). These results indicate that inactivation of FHL enhances pathways that lead to the net generation of reducing equivalents from glycerol such as the synthesis of acetate or lactate. Since an internal redox state characterized by a low $NADH/NAD^+$ ratio could promote the activity of these pathways, we hypothesize that such a redox state is created in Δ*fdhF* by the lack of hydrogen generation and recycling. The metabolic recycling of the hydrogen produced by FHL is known to increase the $NADH/NAD^+$ ratio, thus promoting the synthesis of reduced products (Clark, 1989; Sawers and Boxer, 1986; Sawers et al., 1985, 2004; Murarka et al., 2008).

Acetate can be generated from either pyruvate or acetyl-CoA through one of two pathways: (1) the phosphate acetyltransferase (PTA)-acetate kinase (ACK) pathway and (2) the POX pathway (Sawers and Clark, 2004). As shown in Figure 6B, the POX pathway appears to play a minimal role in the metabolism of glycerol under microaerobic conditions. Inactivation of the PTA-ACK pathway, however, had a significant impact on glycerol utilization and cell growth (Fig. 6B, strain Δ*pta*). Synthesis of acetate in strain Δ*pta* was reduced to ~1/4 of the levels produced by the wild-type, demonstrating the importance of the ACK-PTA pathway (Table IV). Even more significant were the changes observed in the distribution of metabolic products: strain Δ*pta* exhibited an almost homolactic behavior, producing about 3 g/L of lactate (48% yield respect to the glycerol consumed) compared to undetectable amounts in the wild-type (Table IV). On w/w basis, this amount of lactate was more than 10-times the amount of any other product synthesized by strain Δ*pta*. These results contrast those reported for the fermentative utilization of glycerol, in which case the inactivation of the PTA-ACK pathway had almost no effect on glycerol fermentation (Murarka et al., 2008). The availability of oxygen in microaerobic cultures relaxes redox constraints and therefore makes oxidative pathways, such as the synthesis of acetate, more active. The same pathway, however, is barely active under fermentative conditions, and hence its disruption is expected to have very different outcomes in each of these two metabolic modes.

Acetyl-CoA can also be converted to ethanol in a two-reaction pathway that involves acetaldehyde as intermediate. A single enzyme catalyzes this conversion: alcohol/acetaldehyde dehydrogenase, ADH, encoded by *adhE* (Sawers and Clark, 2004). Ethanol was found to be the main metabolic product of glycerol utilization under microaerobic conditions (Figs. 2A and 3). This reflects the highly reduced state of carbon in glycerol and suggests an important role for this pathway. To assess the relevance of the ethanologenic pathway, we evaluated glycerol utilization in strain Δ*adhE* (ADH activity in Δ*adhE* was undetectable compared to 0.19 ± 0.02 μmoles/min/mg protein in BW25113). The absence of ADH activity resulted in no ethanol production (Table IV) and significantly impaired cell growth and glycerol utilization (Fig. 6B). These results indicate that, despite the availability of oxygen, the synthesis of ethanol plays a significant role in the microaerobic utilization of glycerol.

Proposed Pathways for the Microaerobic Utilization of Glycerol

Figure 7 shows the pathways involved in the microaerobic utilization of glycerol by *E. coli*, as concluded from the genetic and biochemical evidence collected in this study. The thicknesses of the arrows illustrate the relative contribution of each pathway/reaction to the carbon flux. The metabolic profiling of gene-deletion mutants along with enzyme activity measurements indicate that both respiratory (GlpK-GlpD) and fermentative (GldA-DHAK) pathways play a significant role in the conversion of glycerol to glycolytic intermediate dihydroxyacetone (Fig. 6A, Table III). These pathways significantly contribute to the efficiency of

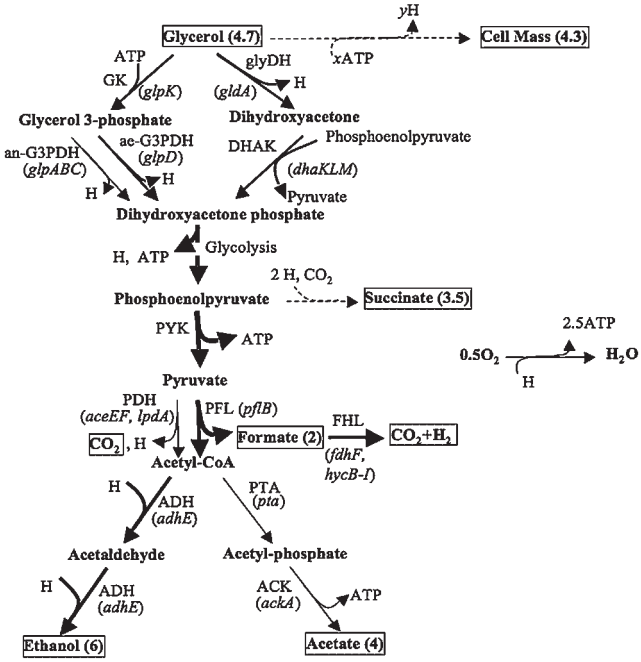

Figure 7. Proposed pathways for the generation of glycolytic intermediates and the synthesis of metabolic products during the microaerobic utilization of glycerol. The thickness of the lines represents the relative contribution to the carbon flux. Extracellular metabolites are boxed. Broken lines illustrate multiple steps. ACK, acetate kinase; ADH, acetaldehyde/alcohol dehydrogenase; ae-G3PDH, aerobic glycerol-3-phosphate dehydrogenase; an-G3PDH, anaerobic glycerol-3-phosphate dehydrogenase; DHAK, dihydroxyacetone kinase; FHL, formate hydrogen-lyase; GK, glycerol kinase; glyDH, glycerol dehydrogenase; H, NADH/$FADH_2$/QH_2; PDH, pyruvate dehydrogenase; PFL, pyruvate formate-lyase; PTA, phosphoacetyl transferase; PYK, pyruvate kinase; QH_2, reduced quinones.

growth under microaerobic conditions and, if expressed at sufficient levels, either one can support growth and glycerol utilization. Similarly, both pyruvate dehydrogenase (PDH) and pyruvate formate-lyase (PFL) contributed to the conversion of pyruvate to acetyl-CoA (Fig. 6B), and appear to have complementary roles during early (PDH) and late (PFL) stages of the cultivation. Nonetheless, several lines of evidence support the conclusion that most of the carbon flux proceeds through PFL. First, a Δ*pflB* mutant converted most of the glycerol to lactate, generating very little ethanol or acetate (Table IV) and clearly indicating a bottleneck at the pyruvate node. Second, a PDH-deficient mutant (Δ*aceF*) did not show significant changes in the distribution of fermentation products when compared to wild-type BW25113, including almost identical ethanol and acetate yields (Table IV). Third, engineered strain SY04 [pZSKLMgldA] produced almost equimolar amounts of formate and ethanol as the main metabolic products (see next section and Fig. 8C), which is possible only if pyruvate is dissimilated via PFL. Among the pathways leading to the synthesis of metabolic products, only those involved in the synthesis of ethanol (alcohol/acetaldehyde dehydrogenase enzyme) and acetate (phosphoacetyltrasferase-acetate kinase pathway) appear to play a significant role in the microaerobic utilization of glycerol (Fig. 6B, Table IV).

Engineered Strains for the Conversion of Glycerol to Ethanol Under Microaerobic Conditions

We recently reported the engineering of *E. coli* for the efficient conversion of glycerol to ethanol under anaerobic conditions (Yazdani and Gonzalez, 2008). Our strategy capitalized on the high degree of reduction of carbon in glycerol, thus enabling the production of not only ethanol but also its co-products, hydrogen and formate. Two strains were created for the co-production of ethanol and hydrogen and ethanol and formate: SY03 [pZSKLMgldA] and SY04 [pZSKLMgldA], respectively. High ethanol yields were achieved in both strains by minimizing the synthesis of by-products succinate and acetate through mutations that inactivated fumarate reductase (Δ*frdA*) and phosphate acetyltransferase (Δ*pta*), respectively. Strain SY04, which produces ethanol and formate, also contains a mutation that blocked formate-hydrogen lyase (Δ*fdhF*), thus preventing the conversion of formate to CO_2 and H_2. High rates of glycerol utilization and product synthesis were achieved by simultaneous overexpression of glycerol dehydrogenase (*gldA*) and dihydroxyacetone kinase (*dhaKLM*), which are the enzymes responsible for the conversion of glycerol to the glycolytic intermediate dihydroxyacetone phosphate under fermentative conditions. Although these strains produced ethanol and co-products hydrogen and formate at high yields and rates, they required supplementation of the medium with rich nutrients such as tryptone (Yazdani and Gonzalez, 2008). Since we have demonstrated the ability of *E. coli* to generate reduced products under microaerobic conditions (primarily ethanol) using a minimal medium without tryptone supplementation, we evaluated the performance of strains SY03 [pZSKLMgldA] and SY04 [pZSKLMgldA] under these conditions (Fig. 8, panels B and C). Both strains produced ethanol and co-products efficiently, reaching yields and productivities of about 85% of the theoretical maxima and 3.3 mmoles/L/h, respectively. This performance is very similar to that reported with the use of anaerobic conditions and a medium supplemented with 2 g/L tryptone (Yazdani and Gonzalez, 2008). However, the use of high concentrations of glycerol led to the synthesis of significant amounts of lactate by the engineered strains (data not shown). To eliminate lactate production, we disrupted the gene *ldhA* in the SY03 and SY04 backgrounds: *ldhA* codes for the fermentative lactate dehydrogenase (LDH). The resulting strains were named EH05 (SY03Δ*ldhA*) and EF06 (SY04Δ*ldhA*). When grown under microaerobic conditions and using high concentrations of glycerol, strains EH05 [pZSKLMgldA] and EF06 [pZSKLMgldA] efficiently converted glycerol to ethanol and co-products hydrogen and formate, respectively. Figure 8D shows a representative example of ethanol production in a medium containing 60 g/L glycerol using strain EH05 [pZSKLMgldA]. The

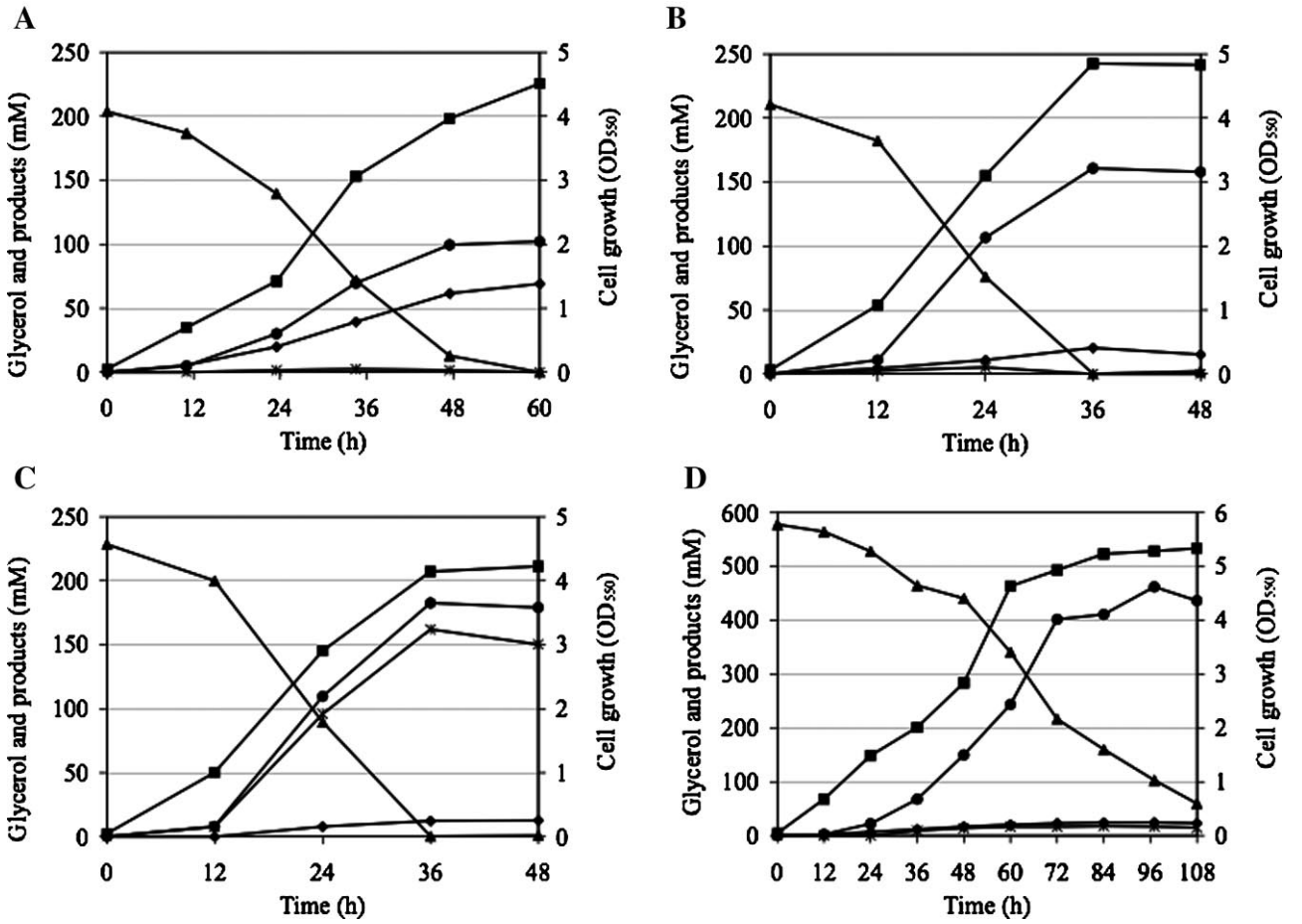

Figure 8. Synthesis of ethanol and co-products formate and hydrogen by strains MG1655 (**A**), SY03 [pZSKLMgldA] (**B**), SY04 [pZSKLMgldA] (**C**), and EH05 [pZSKLMgldA] (**D**). Data are shown for concentration of cells (■), glycerol (▲), ethanol (●), formate (✱), and other products (◆). The coefficient of variation (standard deviation/mean × 100) was below 8% in all cases.

performance of these engineered strains was superior to that reported for the conversion of glycerol to ethanol and hydrogen or ethanol and formate by other microorganisms (Ito et al., 2005; Jarvis et al., 1997; Sakai and Yagishita, 2007) and similar to that achieved with *E. coli* strains engineered to convert cellulosic sugars to ethanol (Underwood et al., 2002). In addition to producing ethanol, strain EF06 [pZSKLMgldA] produces formate, which implies an overall yield and rate of product synthesis almost twice of that reported for the production of ethanol from sugars.

Conclusions

Glycerol is an inevitable byproduct of biodiesel production that has become an attractive carbon source for fermentation processes because of its availability, low price, and high degree of reduction. Although the anaerobic fermentation of glycerol by *E. coli* could be an ideal platform for this purpose, expensive nutrients such as tryptone or yeast extract are required in this process. An alternative approach, exploited in the work reported here, is the supply of limited amounts of an electron acceptor, such as oxygen, which would enable redox balance by consuming the excess reducing equivalents generated by the incorporation of glycerol into cell mass. Based on our results, it is concluded that the use of microaerobic conditions is an effective means of eliminating the need for rich nutrients while still taking advantage of the high degree of reduction of glycerol. Microaerobic utilization of glycerol resulted in a product mixture containing a larger fraction of highly reduced products than those synthesized during the metabolism of sugars such as glucose and xylose. This works has demonstrated that both fermentative and respiratory pathways play a significant role in the microaerobic utilization of glycerol. Finally, it was shown that the microaerobic metabolism of glycerol can be harnessed for the production of ethanol and co-products hydrogen and formate at high

yields and productivities in a medium containing mineral salts without rich supplements.

This work was supported by grants from the U.S. National Science Foundation (CBET-0645188), the National Research Initiative of the U.S. Department of Agriculture Cooperative State Research, Education and Extension Service (2005-35504-16698), the Energy and Environmental Systems Institute at Rice University, and Glycos Biotechnologies, Inc. We thank H. Mori for providing research materials and S.S. Yazdani for introducing an *ldhA* mutation in the SY03 and SY04 backgrounds.

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