ACCELERATED PUBLICATION

Anaerobic Fermentation of Glycerol by *Escherichia coli*: A New Platform for Metabolic Engineering

Yandi Dharmadi, Abhishek Murarka, Ramon Gonzalez

Department of Chemical and Biomolecular Engineering, Rice University, Houston, Texas 77251-1892; telephone: (713)-348-4893; fax: (713) 348-5478; e-mail: Ramon.Gonzalez@rice.edu

Received 14 March 2006; accepted 5 May 2006

Published online 20 May 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.21025

Abstract: The worldwide surplus of glycerol generated as inevitable byproduct of biodiesel fuel and oleochemical production is resulting in the shutdown of traditional glycerol-producing/refining plants and new applications are needed for this now abundant carbon source. In this article we report our finding that *Escherichia coli* can ferment glycerol in a pH-dependent manner. We hypothesize that glycerol fermentation is linked to the availability of CO_2, which under acidic conditions is produced by the oxidation of formate by the enzyme formate hydrogen lyase (FHL). In agreement with this hypothesis, glycerol fermentation was severely impaired by blocking the activity of FHL. We demonstrated that, unlike CO_2, hydrogen (the other product of FHL-mediated formate oxidation) had a negative impact on cell growth and glycerol fermentation. In addition, supplementation of the medium with CO_2 partially restored the ability of an FHL-deficient strain to ferment glycerol. High pH resulted in low CO_2 generation (low activity of FHL) and availability (most CO_2 is converted to bicarbonate), and consequently very inefficient fermentation of glycerol. Most of the fermented glycerol was recovered in the reduced compounds ethanol and succinate (93% of the product mixture), which reflects the highly reduced state of glycerol and confirms the fermentative nature of this process. Since glycerol is a cheap, abundant, and highly reduced carbon source, our findings should enable the development of an *E. coli*-based platform for the anaerobic production of reduced chemicals from glycerol at yields higher than those obtained from common sugars, such as glucose.

Keywords: glycerol fermentation; *Escherichia coli*; metabolic engineering; bacteria

INTRODUCTION

In recent years, there has been a significant increase in the production and use of biofuels, such as biodiesel and bioethanol. These technologies represent sustainable, secure, renewable, and environmentally safe alternatives to fossil fuels. Biodiesel is produced by a transesterification reaction using vegetable oils or animal fats and an alcohol, a process that inevitably generates large amounts of glycerol as a byproduct. The availability of crude glycerol is predicted to increase in the next years because of the tremendous growth in the production of biodiesel worldwide. The current surplus of glycerol is already resulting in the shutdown of traditional glycerol-producing plants. For example, Dow Chemical (Midland, MI) and Procter & Gamble Chemicals (Cincinnati, OH) have recently closed their glycerol-producing/refining plants (McCoy, 2006). The flood of glycerol is also a problem for oleochemical firms, for which glycerol refining represents a longtime revenue source (McCoy, 2005). In addition, it is well known that some companies pay for the appropriate disposal of glycerol and in some cases bankruptcy has been attributed, in part, to the collapse in glycerol prices (Willke and Vorlop, 2004). The development of processes to convert low-priced glycerol into higher value products is therefore an excellent opportunity to add value to the production of biodiesel. In fact, the need for obtaining new chemicals from glycerol has prompted US government agencies, such as the Department of Energy to adopt the promotion of new glycerol platform chemistry and product families as one of their most important goals (Tyson et al., 2004).

Crude glycerol sells at 5–15 cents/lb (Tyson et al., 2004), a price approaching that of sugars typically used in fermentation processes. Not only is glycerol abundant but its higher reduced state, compared to sugars, such as glucose, xylose, etc., promises to significantly increase the product yield of chemicals, such as succinate, ethanol, and propanediols whose production from these sugars is limited by the availability of reducing equivalents. The potential use of glycerol as carbon source in fermentation processes could be hampered, however, by the inability of *Escherichia coli* (the workhorse of modern biotechnology) to ferment glycerol in the absence of external electron acceptors (Booth, 2005). This article describes our recent finding that *E. coli* is in fact capable of anaerobically fermenting glycerol in a pH-dependent manner. We investigated the mechanisms

Correspondence to: Ramon Gonzalez
Contract grant sponsor: NRI, CSREES, USDA
Contract grant number: 2005-35504-16698

mediating this process and showed that the production of CO_2 from formate (catalyzed by the enzyme formate hydrogen lyase (FHL)) is required for glycerol fermentation to proceed. High pH resulted in low CO_2 generation (low activity of FHL) and availability (most CO_2 is converted to bicarbonate), thus resulting in very inefficient fermentation of glycerol. The identity and amount of fermentation products (mainly ethanol and succinic acid) reflect the highly reduced state of glycerol and corroborate the fermentative nature of this process. We showed that supplementation of the medium with a low-priced byproduct of the corn-processing industry, corn steep liquor, facilitates the fermentation of glycerol thus demonstrating that this process is amenable to industrial applications.

MATERIALS AND METHODS

Strains and Genetic Methods

Wild-type strains *E. coli B* (ATCC 11303) and the K12 strain W3110 (ATCC 27325) were obtained from the American Type Culture Collection (Manassas, VA). *E. coli* K12 strain MG1655 (F-lambda-*ilvG*-*rfb*-50 *rph*-1) and several derivatives (see below) were obtained from the *E. coli* Genome Project (University of Wisconsin, Madison). The mutants were constructed using a transposon-mediated mutagenesis method that has been described elsewhere (Kang et al., 2004). Mutant strains include (name of wild-type followed by mutated gene given in parenthesis: i.e., MG1655*gene_name::Tn5KAN*-I-SceI): FB21975 (MG1655*mgsA::Tn5KAN*-I-SceI), FB20908 (MG1655*hycB::Tn5KAN*-I-SceI), and FB21104 (MG1655*trkA::Tn5KAN*-I-SceI). Since a similar fragment was inserted in each mutated gene, for clarity, these strains will be referred to using the following nomenclature: MG1655*gene_name::*. 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 with 50 μg/mL kanamycin, where appropriate.

Culture Medium and Cultivation Conditions

Wild-type and recombinant strains were cultivated using MOPS (4-Morpholinepropanesulfonic acid) minimal medium (Neidhardt et al., 1974) with 1.32 mM Na_2HPO_4 in place of K_2HPO_4. Two versions of this medium were prepared; one containing MOPS for pre-culture, and one with no MOPS for fermentation. Unless otherwise stated, these media were supplemented with 10 g/L of glycerol, 5 g/L of yeast extract, and 10 g/L of tryptone. Some experiments were conducted with minimum medium supplemented with specified concentrations of tryptone or corn steep liquor. Selenite (required for the activity of the enzyme FHL), at a final concentration of 1 μM, was included in all medium formulations. All experiments were conducted at 37°C under anaerobic conditions. Fermentations were conducted in a SixFors multi-fermentation system (Infors HT, Bottmingen, Switzerland) with six 500 mL working volume fermenters and independent control of temperature, pH, and stirrer speed (200 rpm). The system is fully equipped and computer controlled using manufacturer IRIS NT software. Each fermenter is fitted with a condenser to prevent evaporation, which was operated with a 0°C cooling methanol–water supply. Anaerobic conditions were maintained by flushing the headspace with ultrahigh purity argon (Matheson Tri-Gas, Inc., Houston, TX) at 0.01 LPM. An oxygen trap (Alltech Associates, Inc., Deerfield, IL) was used to eliminate traces of oxygen from the gas stream. To maintain sterile conditions, 0.2 μm and 0.45 μm HEPA filters (Millipore, Billerica, CA) were used to fit the inlet and outlet lines, respectively. Strains (stored as glycerol stocks at −80°C) were streaked onto LB plates and incubated overnight at 37°C in an Oxoid anaerobic jar with the CO_2 gas generating kit (Oxoid Ltd, Basingstoke, Hampshire, UK). A single colony was used to inoculate 17.5 mL Hungate tubes completely filled with medium. The tubes were incubated at 37°C until an OD_{550} of ∼0.4 was reached. An appropriate volume of this actively growing pre-culture was centrifuged and the pellet washed and used to inoculate 350 mL of medium in each fermenter, with the target starting optical density of 0.05 at 550 nm.

Analytical Methods

Optical density was measured at 550 nm and used as an estimate of cell concentration (1 OD = 0.34 g DW/L). After centrifugation, the supernatant was stored at −20°C for subsequent analyses. To quantify the concentrations of glycerol, lactate, acetate, formate, succinate, and ethanol, samples were analyzed with ion-exclusion HPLC using a Shimadzu Prominence SIL 20 system (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with an HPX-87H organic acid column (Bio-Rad, Hercules, CA). Operating conditions to optimize peak separation (30 mM H_2SO_4 in mobile phase, column temperature 42°C) were determined using a previously described approach (Dharmadi and Gonzalez, 2005). Hydrogen production was calculated as the difference between molar amounts of (ethanol + acetate) and formate. This relationship assumes that pyruvate dissimilation takes place via the enzyme pyruvate-formate lyase (PFL) and therefore negligible activity of the enzyme pyruvate dehydrogenase (PDH). PDH activity is known to be negligible during the anaerobic fermentation of glucose and negatively correlated with the NADH/NAD ratio (de Graef et al., 1999). Glycerol fermentation should result in a more reduced environment (compared to glucose fermentation, see Fig. 3), and thus very negligible activity of PDH. Therefore, pyruvate dissimilation via PFL is a reasonable assumption. The identities of the fermentation products were confirmed through a 1D 1H (Proton) NMR (nuclear magnetic spectroscopy) experiment. Sixty microliters D_2O and 1 μL of 600 mM NMR internal standard TSP (3-(trimethylsilyl) propionic acid-D4, sodium salt) were added to 540 μL of the sample. The resulting solution was then transferred to a 5 mm-NMR tube and 1D proton NMR spectroscopy was

DOI 10.1002/bit

performed at 25°C in a Varian 500 MHz Inova spectrometer equipped with a Penta probe. The following parameters were used: 8,000 Hz sweep width; 2.8 s acquisition time; 256 acquisitions; 6.3 μs pulse width; 1.2 s pulse repetition delay; and pre-saturation for 2 s. The resulting spectrum was analyzed using the software FELIX 2001 (Accelrys Software, Inc., Burlington, MA). Peaks were identified by their chemical shifts and the J-coupling values, which were obtained in separate experiments where samples were spiked with metabolite standards (2 mM final concentration).

RESULTS AND DISCUSSION

Anaerobic Fermentation of Glycerol Results in a Distinctive Mixture of Products

Figure 1A shows a typical profile for the fermentation of glycerol by *E. coli* strain MG1655 at pH 6, also including cell growth and generation of fermentation products. The supernatant of the fermentation samples were analyzed via HPLC, and the HPLC profiles for samples taken at times 0 h and 84 h are shown in Figure 2. A validation of the HPLC method used in the quantification of glycerol and fermentation products is presented in Table I. A 1D 1H (Proton) NMR experiment was conducted to confirm the identity of the fermentation products identified in the HPLC chromatograms. Ethanol, succinic acid, formic acid (mainly at alkaline pH), and minor amounts of acetic acid were found in the NMR spectrum of the extracellular medium (data not shown).

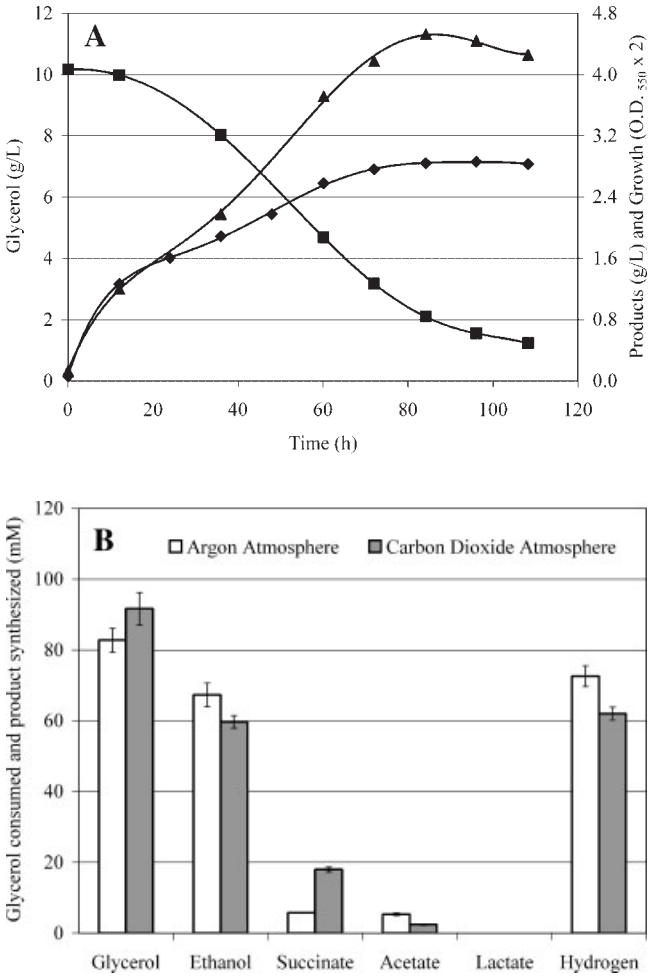

Figure 1. Anaerobic fermentation of glycerol by strain MG1655 at pH 6. **A**: A typical profile for glycerol consumption (squares), cell growth (diamonds), and accumulation of products in the fermentation broth (triangles). **B**: Effect of the composition of the gas atmosphere on the distribution of fermentation products. Values represent the means and bars standard deviations for samples taken once the cultures reached stationary phase.

Glycerol was almost completely consumed within 84 h of active growth (~8 g/L of the 10 g/L initially present in the medium), resulting in a maximum cell concentration of 486.2 mg/L. In the remaining 24 h (in stationary phase) the cells consumed only one-tenth (~0.8 g/L) of the glycerol consumed during active growth, and 1.2 g/L were left unfermented in the medium. As shown in Figure 1B, ethanol and succinic acid accounted for 93% (molar basis) of the products (86% ethanol and 7% succinic acid) present in the fermentation broth. Only minor amounts of acetate were found and no detectable formate or lactate (estimated to be below 0.6 mM and 0.2 mM, respectively, based on the detection limit of the HPLC method: see Table I). These results sharply contrast with the composition of products obtained during the fermentation of glucose by *E. coli*: excluding CO_2 and H_2, lactate is the main product of glucose fermentation (45%) followed by ethanol (29%), acetate (21%), and succinate (6%) (Sawers and Clark, 2004). The almost exclusive synthesis of reduced products during glycerol fermentation (compared to those of glucose fermentation) reflects the highly reduced state of glycerol whose metabolism results in the generation of twice the reducing equivalents produced during glucose fermentation (see Fig. 3). The high carbon recovery in fermentation products (95%) along with the almost exclusive synthesis of reduced compounds ethanol and succinate corroborates the fermentative nature of the process. Otherwise, the presence of an electron acceptor would consume the reducing equivalents generated from glycerol and large amounts of oxidized products (such as acetate) would be produced instead of ethanol. Since formate was completely converted into CO_2 and H_2, these gases would be present in large amounts in the gas phase. The higher levels of ethanol compared to succinate could reflect the higher generation of energy in the conversion of glycerol into ethanol and/or the limited availability of carbon dioxide and phosphoenolpyruvate required to convert glycerol into succinate. These results can be interpreted by examining two key factors that determine the feasibility of a fermentation process: energy generation by substrate-level phosphorylation and overall redox balance. Only two fermentative pathways can provide closure of redox balance during glycerol fermentation: synthesis of succinate or ethanol (Fig. 3). Although the two pathways are equivalent regarding the overall redox balance, the energetic contribution of the ethanologenic pathway is much higher. While 1 net ATP is produced per each molecule of glycerol converted into ethanol, production of energy in

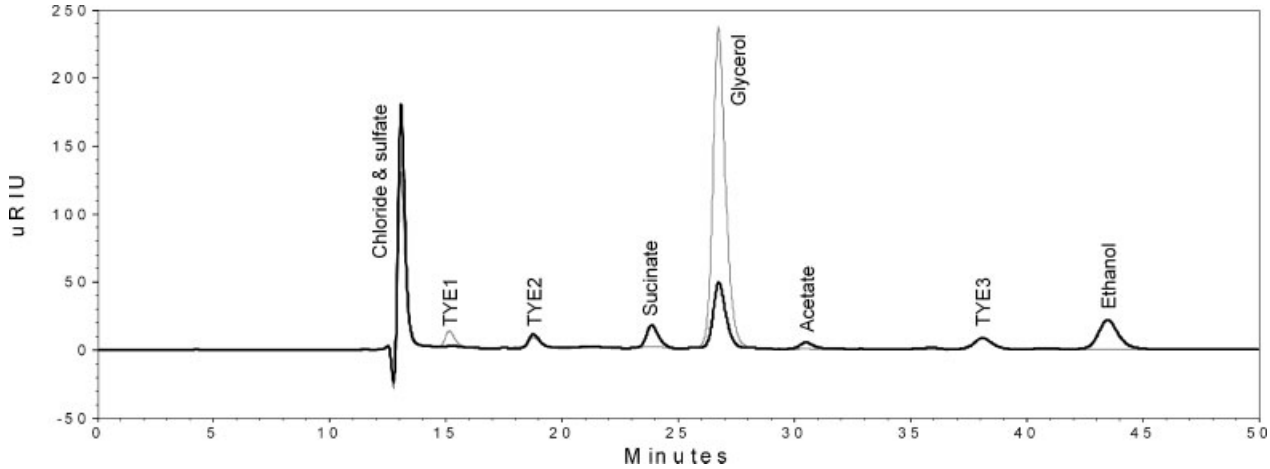

Figure 2. HPLC profiles (refraction index detector) for the supernatant of samples taken at times 0 h (gray line) and 84 h (black line) in the fermentation shown in Figure 1. Peaks are identified by compound name. TYE peaks correspond to tryptone and yeast extract components.

the succinate pathway is limited to the potential generation of a proton motive force by fumarate reductase (Unden and Kleefeld, 2004).

Although the analysis of fermentative pathways presented above reveals that conversion of glycerol into ethanol or succinate permits achieving redox poise (see Fig. 3), it is widely believed that *E. coli* cannot ferment glycerol because of the highly reduced state of this carbon source. Essentially, this means that an electron acceptor needs to be present to dispose the reducing equivalents generated from glycerol. Dissimilation of glycerol in wild-type *E. coli* strains is thought to involve its conversion into glycerol-3-phosphate (G3P) by the enzyme glycerol kinase. G3P can be further metabolized to glycolytic intermediate dihydroxyacetone-phosphate (DHAP) by either of two respiratory, membrane-bound enzymes encoded by *glpD* and *glpABC* operons (Booth, 2005). GlpD is coupled to glycerol metabolism in the presence of oxygen while GlpABC transfer the electrons to fumarate or nitrate. Experimental evidence for the requirement of electron acceptors is completely based on *in vitro* studies for the two G3P dehydrogenases, GlpABC and GlpD (Schryvers and Weiner, 1981; Schryvers et al., 1978). In the absence of a G3P dehydrogenase activity, it is expected that accumulation of high levels of G3P would reach a point where it becomes inhibitory for cell growth (Booth, 2005). However, there are several alternatives for the conversion of glycerol into glycolytic intermediate DHAP, which could include or not the synthesis of G3P. For example, glycerol can be converted into dihydroxyacetone (DHA) by glycerol dehydrogenase (GldA) and the resulting DHA then phosphorylated by a DHA kinase (Booth, 2005; Jin et al., 1983; Tang et al., 1982a,b). In this scenario, G3P would not be part of the glycerol dissimilation pathway thus invalidating the above hypothesis. Identifying the pathways that mediate the

Table I. HPLC calibration curves for succinate, lactate, glycerol, formate, acetate, and ethanol.

	Equation	R^2	Retention time (min)
Succinate	$y = 93569x - 27235$	1.000	23.87
Lactate	$y = 66920x - 10857$	1.000	25.63
Glycerol	$y = 84421x - 14513$	1.000	26.75
Formate	$y = 17311x - 10455$	1.000	28.22
Acetate	$y = 31480x - 37291$	0.999	30.51
Ethanol	$y = 20966x - 20907$	1.000	43.62

Data were generated with 25, 50, 75, and 100 mM of each analyte in triplicates (total number of points = 12 for each analyte). Injection volume was 10 μL in all runs. In the linear equation, x denotes the analyte concentration in mM and y denotes the peak area count.

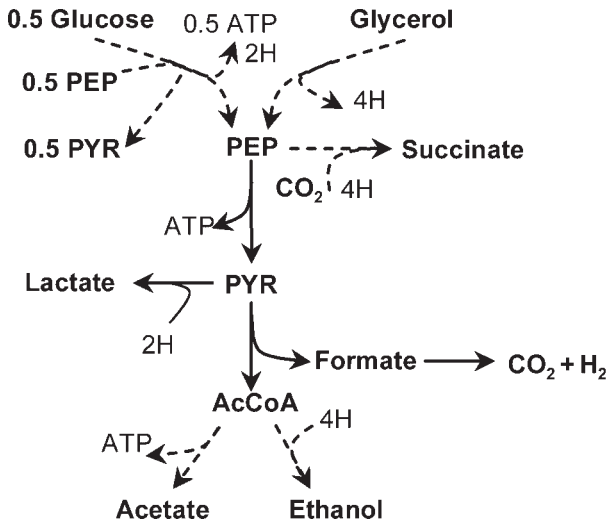

Figure 3. Schematic representation of the anaerobic fermentation of glucose and glycerol in *E. coli*. Converting glycerol into phosphoenolpyruvate or pyruvate results in the generation of twice the amount of redox equivalents generated during the conversion of glucose. Note that conversion of glycerol into ethanol or succinate are redox-balanced processes (ethanol being more energetically favorable). Conversion of pyruvate into acetyl-CoA is assumed to proceed through enzyme pyruvate-formate lyase (PFL). Broken lines represent linear pathways composed of several reactions. Abbreviations: H, reducing equivalents (2H = 1NADH/NADPH/FADH2); AcCoA, acetyl coenzyme A; PEP, phosphoenolpyruvate; PYR, pyruvate.

DOI 10.1002/bit

anaerobic fermentation of glycerol is the objective of current research efforts in our group.

Feasibility of Glycerol Fermentation in *E. coli* Is Determined by the pH of the Culture Medium

Figure 4 illustrates the feasibility of glycerol fermentation by *E. coli* strain MG1655 at neutral, acidic, and alkaline pH. Clearly, the metabolism of glycerol proceeds optimally under acidic conditions (~9 g/L of glycerol fermented at pH 6) but is impaired at alkaline conditions (~1 g/L of glycerol fermented at pH 8). Cell growth followed the same pattern: biomass produced at alkaline pH was almost exclusively synthesized from growth supplements rather than from glycerol. That is, experiments in the same media lacking glycerol resulted in an average OD_{550} of 0.5 over the range of pH reported here (data not shown).

The striking similarity between the effect of pH on glycerol fermentation and carbon dioxide-bicarbonate (CO_2-HCO_3^-) equilibrium (see Fig. 4) motivated us to investigate whether the feasibility of glycerol fermentation could be linked to CO_2 availability. *E. coli* requires a supply of bicarbonate/CO_2 as a metabolic substrate during normal growth for the biosynthesis of small molecules, and also in fatty acids biosynthesis and central metabolism (e.g., oxaloacetate). For example, this organism grows anaerobically without a lag period if a suitable concentration of CO_2 is provided, and subsequent exponential growth rate can be controlled solely by the concentration of this species (Lacoursiere et al., 1986; Repaske and Clayton, 1978). Further, it has been shown that *E. coli* responds to CO_2 concentration, not to bicarbonate ion concentration (Repaske and Clayton, 1978). This behavior is due to the fact that

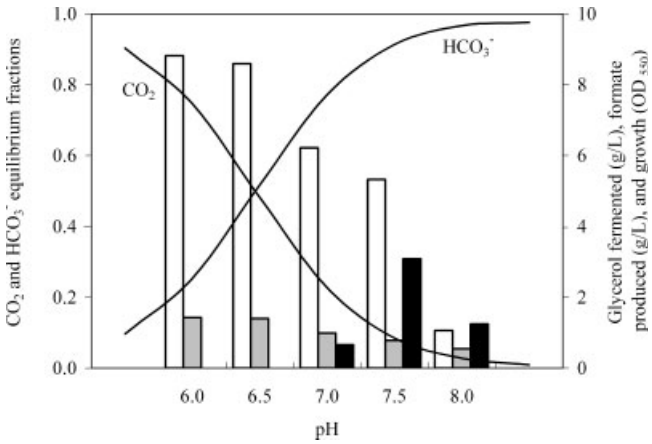

Figure 4. Effect of pH on fermented glycerol (open bars), cell growth (gray bars), formate accumulation (solid bars), and CO_2-HCO_3^- equilibrium (lines). Values represent the means and bars standard deviations for samples taken once cultures of strain MG1655 reached stationary phase. Equilibrium fractions of CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-} were calculated considering the following events along with their respective equilibrium constants: CO_2 (aq) $<>$ CO_2 (g), CO_2 (aq) + H_2O $<>$ H_2CO_3 (aq), H_2CO_3 (aq) $<>$ H^+ (aq) + HCO_3^- (aq), HCO_3^- (aq) $<>$ H^+ (aq) + CO_3^{2-} (aq), H_2O $<>$ H^+ (aq) + OH^- (aq).

negatively charged HCO_3^- is highly soluble in an aqueous solution but poorly soluble in lipids; CO_2, however, is highly soluble in both. Therefore, while CO_2 can freely diffuse in and out of the cell, HCO_3^- is a membrane-impermeable molecule. Although bicarbonate transport proteins have been identified in mammalian cells (Sterling and Casey, 2002) and cyanobacteria (Shibata et al., 2002), no such transporters have been reported in *E. coli*. While the principal reactions consuming bicarbonate/CO_2 in *E. coli* do so in the form of bicarbonate (whose only source is the hydration of CO_2), the reactions producing bicarbonate/CO_2 do so in the form of CO_2. Conversion of HCO_3^- to CO_2 may then facilitate its transport into the cell, while conversion of CO_2 to HCO_3^- may be important for trapping CO_2 in the cell. In fact, it was recently shown that the *can* gene of *E. coli* encodes a carbonic anhydrase (interconverts CO_2 and bicarbonate), which is essential for growth under CO_2-limiting conditions (Merlin et al., 2003). Above pH 6.3 the equilibrium between the two species shifts toward HCO_3^- (Fig. 4), thus posing problems in maintaining required intracellular CO_2 and HCO_3^- concentrations.

The effect of pH on the equilibrium fractions of CO_2 and HCO_3^- should result in very low amounts of CO_2 present in the medium at pH 8, as HCO_3^- is the main species. Low CO_2 availability, in turn, possesses a problem for the cells since the species transported across the cell membrane is CO_2, not HCO_3. Interestingly, the optimum pH for glycerol fermentation is very similar to the pK_a of the equilibrium reaction between CO_2 and HCO_3^- ($pK_a = 6.3$).

Conversion of Formate into CO_2 by Formate Hydrogen Lyase (FHL) Is Required for Glycerol Fermentation at Acidic pH

Since the decrease in glycerol fermentation correlates well with the accumulation of formate in the extracellular media (Fig. 4), we hypothesize that the availability of CO_2 produced by the oxidation of formate at acidic pH could be the reason why *E. coli* is able to ferment glycerol. Under fermentative conditions the disproportionation of formate into CO_2 and H_2, catalyzed by the enzymatic complex FHL, is the main source of CO_2. The generation of CO_2 by dehydrogenases (e.g., PDH, 2-oxoglutarate dehydrogenase, isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, etc.) is constrained by the need to recycle the NADH also produced by these enzymes. This situation is especially relevant during the fermentation of a highly reduced compound like glycerol, which generates a larger amount of reducing equivalents than other carbon sources (see Fig. 3). Transcription of FHL (composed of formate dehydrogenase isoenzyme, FDH-H, and hydrogenase-3 isoenzyme, Hyd-3) occurs only during fermentative growth and is dependent on acidic pH in the medium and on formate availability (Sawers et al., 2004). As shown in Figure 4, glycerol fermentation under acidic conditions is accompanied by formate oxidation (i.e., no formate accumulation), while at alkaline pH accumulation of formate at levels equivalents to the sum of ethanol and acetate

is observed (i.e., formate was not converted into CO_2 and H_2). If the oxidation of formic acid by the enzyme FHL is providing the CO_2 required for growth at acidic pH, it would be expected that blocking FHL will impair glycerol fermentation at pH 6. Deletion of *hycB* (encoding a subunit of Hyd-3) has been shown to result in no production of CO_2 and H_2 from formate under acidic conditions (Sawers et al., 2004; Trchounian et al., 2000). We tested strain MG1655*hycB::* and found that glycerol fermentation at pH 6 had been severely impaired (Fig. 5) and significant amounts of formate accumulated in the fermentation broth (data not shown). These results clearly demonstrate that glycerol fermentation at acidic pH requires conversion of formate into H_2 and CO_2, a process that in turn appears to fulfill cellular requirements for CO_2 (see previous section for discussion on the cellular requirements of CO_2). Due to the highly reduced state of glycerol it is very unlikely that the cells would require hydrogen, the other product of formate oxidation, for glycerol fermentation. In fact, when the produced hydrogen was not allowed to escape the fermenter (i.e., no flushing with argon) glycerol fermentation and cell growth were also impaired (Fig. 5). Molecular hydrogen, coupled to ferredoxin reduction, can be used as electron donor in the reduction of a variety of substances. For example, a hydrogen-enriched gas phase is known to increase the synthesis of succinic acid during glucose fermentation by stimulating the conversion of fumarate into succinate (Vemuri et al., 2002). The excess of reducing equivalents generated by the presence of hydrogen during glycerol fermentation creates a redox imbalance that cannot be compensated by any other pathway because the conversion of glycerol into ethanol or succinic acid are redox balanced processes (see Fig. 3). Although CO_2 also accumulated in the headspace, this gas has a positive effect on cell growth and glycerol fermentation as shown in Figures 1B and 5.

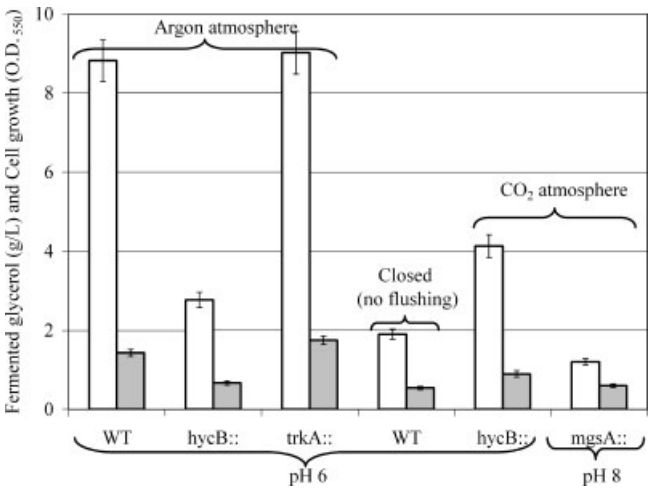

Figure 5. Role of FHL-catalyzed oxidation of formate and MGS-mediated MG production on glycerol fermentation (open bars) and cell growth (gray bars). Mutants are indicated by the name of disrupted gene. Experiments were conducted at pH and under gas atmosphere indicated in each case. "Closed (no flushing)" represents an experiment conducted in a closed fermenter, which was flushed with argon at time 0 only. Values represent the means and bars standard deviations for samples taken once the cultures reached stationary phase. WT, wild-type strain MG1655.

It has been recently shown that oxidation of formate and reduction of protons by FHL results in the formation of a proton gradient across the bacterial membrane, which in turn could be involved in the regulation of proton potassium exchange (using the TrkA system) (Hakobyan et al., 2005). We demonstrated that the feasibility of glycerol fermentation at acidic pH is not linked to this process since glycerol fermentation in a TrkA-deficient strain (MG1655*trkA::*) proceeded in a wild-type manner (Fig. 5). If the reason why a strain lacking an active FHL complex is unable to ferment glycerol is the absence of CO_2 production, externally provided CO_2 should facilitate glycerol fermentation by this strain. MG1655*hycB::* partially recovered its ability to ferment glycerol upon supplementation of the medium with CO_2 (Fig. 5). Although the action of decarboxylases involved in the catabolism of amino acids (e.g., arginine and lysine decarboxylases) could represent an additional source of CO_2, they are induced at higher concentration of amino acids and lower pH than those used in this study (Reitzer, 2005). In agreement with this hypothesis we observed that mutants defective in these systems fermented glycerol in a wild-type fashion (data not shown). Taken together, these results support our belief that the feasibility of glycerol fermentation at acidic pH is determined by the availability of CO_2, which in these conditions is produced mainly via the oxidation of formate by FHL.

Inability to Ferment Glycerol at Basic pH: CO_2 Availability and Methylglyoxal Toxicity

The inability of *E. coli* to ferment glycerol at alkaline pH is particularly surprising since this organism grows optimally in the pH range from 6 to 8 on many carbon sources (Ingraham and Marr, 1996). However, it is expected that at alkaline pH the activity of FHL would be very limited thus resulting in low CO_2 generation. In three separate experiments in the presence of externally supplied CO_2 (2% CO_2 in Argon gas phase, an atmosphere of pure CO_2, and 1 g/L of $NaHCO_3$) the same pH-dependence identified earlier was observed for wild-type MG1655 (data not shown). These results can be explained by the effect of pH on the equilibrium fraction of CO_2 and HCO_3^- (Fig. 4): at pH 8, hardly any CO_2 can be found in the medium because HCO_3^- is the main species.

Another factor that could contribute to the impairment of glycerol fermentation under alkaline conditions could be the accumulation of electrophiles, which can be highly toxic at alkaline pH. For example, there are several reports of methylglyoxal (MG) accumulation and its negative effect during the anaerobic metabolism of glycerol in recombinant *E. coli* strains (Booth, 2005). The ability to withstand the toxic effects of MG is influenced by the permeability of the membrane, the capacity for DNA repair, and the levels of detoxification enzymes (Ferguson et al., 1995). MG is produced in *E. coli* from DHAP via the enzyme MG synthase (MGS), encoded by the *mgsA* gene (Totemeyer et al., 1998).

DOI 10.1002/bit

It is well known that the mechanism the cells use to cope with MG toxicity involves decreasing the intracellular pH. In fact, it has been shown that good protection against MG can be achieved if the cytoplasmic pH lies below 7.5 (Booth, 2005). Although not detected in the extracellular medium, MG could be toxic at concentrations below those detectable with the analytical techniques used in our study. To test whether MG toxicity could be impairing glycerol fermentation at alkaline conditions, we evaluated a MGS-deficient strain (strain MG1655*mgsA::*) at pH 8. This strain still was not able to ferment glycerol at alkaline pH (Fig. 5).

Taken together, the above results demonstrate that glycerol fermentation proceeds optimally at acid pH, conditions that appear to facilitate the availability of carbon dioxide required for cell growth. On the other hand, alkaline pH impaired cell growth and glycerol fermentation, apparently due to low production and availability of carbon dioxide rather than of accumulation of MG. Previous studies of glycerol metabolism were conducted in conditions that we have shown in this study to impair glycerol fermentation, namely at alkaline pH and in closed vessels (the later results in the accumulation of hydrogen) (see for example Bouvet et al., 1995; Tanaka et al., 1967). It is important to emphasize that the culture media used in those studies were in fact supplemented with growth factors (mainly tryptone/casein hydrolysates). As we will show in the next section, glycerol fermentation is also feasible in a medium supplemented with low levels of tryptone (Table II). Current research in our group focuses on elucidating why glycerol fermentation requires the supplementation of the medium with growth factors.

Implications of the Feasibility of Glycerol Fermentation for Metabolic Engineering

The production of chemicals and fuels via microbial fermentation has been largely based on the use of sugars (e.g., glucose, lactose, xylose, etc.) as carbon source. This trend, however, will change in the near future due to the large surplus of glycerol generated as byproduct in the production of biodiesel fuel (McCoy, 2005, 2006). The development of processes to convert low-priced glycerol into higher value products is expected to add value to the production of biodiesel and help the development of biorefineries.

Glycerol is not only cheap and abundant but its greater degree of reduction, when compared to the aforementioned sugars, offers the opportunity to obtain reduced chemicals (e.g., succinate, ethanol, xylitol, propionate, hydrogen, etc.) at yields higher than those obtained using sugars. Converting glycerol to phosphoenolpyruvate or pyruvate generates twice the amount of reducing equivalents produced from glucose or xylose (Fig. 3). This permits, for example, the co-production of ethanol and formic acid (or ethanol and hydrogen), which would result in doubling the *overall* product yield compared with glucose fermentation to ethanol, a process in which half of the sugar is lost as CO_2 due to the oxidation state of glucose. The equations below illustrate these advantages by comparing the maximum production of ethanol from glucose and glycerol, considering redox-balanced routes:

$$^1/_2C_6H_{12}O_6 \rightarrow CH_3{}^-CH_2OH + CO_2$$

Ethanol from glucose

$$C_3H_8O_3 \rightarrow CH_3{}^-CH_2OH + CH_2O_2/(CO_2{+}H_2)$$

Ethanol and formate/H_2 from glycerol

Our results indicate that wild-type *E. coli* is already a good biocatalyst for the conversion of glycerol into ethanol and hydrogen: Figure 1B shows that these are, by far, the two main fermentation products. Alternatively, co-production of ethanol and formic acid can also be achieved if glycerol is used as carbon source. It is noteworthy to mention that energy generation should not be a limiting factor, as conversion of glycerol into ethanol and formate/H_2 would result in the same net ATP yield of currently available *E. coli* strains that produce ethanol from glucose (Gonzalez et al., 2002).

Another example of the advantages of using glycerol as carbon source is the conversion of glycerol into succinic acid, which also represents a redox-balanced process (Fig. 3). In this case, the maximum theoretical yield would be twice of that calculated for the anaerobic production of succinic acid from glucose using wild-type *E. coli* strains (see equations below, which also assume redox-balanced routes).

$$C_3H_8O_3 + CO_2 \rightarrow C_4H_6O_4 + H_2O$$

Succinic acid from glycerol

$$C_6H_{12}O_6 + CO_2 \rightarrow C_4H_6O_4 + C_2H_3O_2 + CH_2O_2$$

Succinic acid from glucose

Even if *E. coli* strains are optimized to produce succinic acid from glucose by converting byproduct formate into CO_2 and hydrogen and then channeling these two products into the succinic acid producing pathway (see equation below), the maximum theoretical yield would still be 30% lower than that obtained with glycerol.

$$3C_6H_{12}O_6 + 2CO_2 \rightarrow 4C_4H_6O_4 + 2C_2H_3O_2$$

Succinic acid from glucose

We have already demonstrated that simple modifications of the cultivation conditions can result in dramatic changes in

Table II. Fermentation of glycerol by *E. coli* strains W3110 (K12) and *E. coli* B in the presence of tryptone or CSL (corn steep liquor).

Strain	Supplementation (g/L, supplement)	Fermented glycerol (g/L)	Cell growth (OD_{550})
W3110	10, tryptone	8.42 ± 0.46	1.25 ± 0.06
W3110	5, tryptone	7.11 ± 0.48	1.19 ± 0.08
W3110	2, tryptone	7.03 ± 0.56	0.89 ± 0.05
E. coli B	5, corn steep liquor	6.78 ± 0.41	1.10 ± 0.07

All experiments were conducted at pH 6, an initial concentration of glycerol of 10 g/L, and argon in the headspace. Values represent the means ± SD for samples taken once the cultures reached stationary phase.

the fermentation products. For example, while succinic acid represents only 7% of the products when an atmosphere of argon or nitrogen are used, conducting the fermentations in the presence of CO_2 resulted in the production of succinic acid at levels representing 22%, more than threefold increase in its production (Fig. 1B).

Since *E. coli* has been engineered for the conversion of different sugars into a large number of specialty and commodity chemicals (Gonzalez, 2005; Nielsen, 2001; Stafford and Stephanopoulos, 2001), our finding that this organism can in fact ferment glycerol enables the establishment of a novel platform for the production of these and other chemicals from glycerol. In previous sections we have shown the feasibility of glycerol fermentation in strain MG1655 using a medium containing high concentrations of yeast extract and tryptone. The same results were obtained with two other *E. coli* strains, namely *E. coli B* and the K12 strain W3110 (data not shown). The use of a less-rich medium, however, would make this process more amenable to industrial applications. We now show that supplementation with low levels of tryptone or corn steep liquor (CSL, a byproduct of corn wet-milling widely used as nutrient supplement in fermentations) can equally support this process (Table II). It is noteworthy to mention that CSL, at the levels used here, has been shown to be an economic supplement for the production of ethanol from sugars (Lawford and Rousseau, 1996). Although not yet tested in the fermentation of glycerol, an even cheaper ($\sim$1.7 Euro cent/kg) fermentation supplement of similar characteristics to CSL, but obtained from brown juice, an agricultural byproduct from the green crop drying industry, has been already developed (Thomsen, 2005).

CONCLUSIONS

In this work we have shown that *E. coli* anaerobically ferments glycerol in a pH-dependent manner. We demonstrated that the production of CO_2 from formate (catalyzed by the enzyme FHL) is required for glycerol fermentation to proceed. Unlike CO_2, hydrogen (the other product of FHL-mediated formate oxidation) had a negative impact on cell growth and glycerol fermentation. High pH resulted in low CO_2 generation (low activity of FHL) and availability (most CO_2 is converted to bicarbonate), thus resulting in very inefficient fermentation of glycerol. The identity and amount of fermentation products, mainly ethanol and succinic acid, confirmed the potential of glycerol as a carbon source for the fermentative production of reduced chemicals. Corn steep liquor, a low-priced byproduct from corn-processing industry, is shown to facilitate glycerol fermentation. Considering the worldwide surplus of glycerol and the need to find new uses for this now abundant and cheap carbon source, our findings should enable the development of an *E. coli*-based platform for the anaerobic production of reduced chemicals from glycerol.

This work was supported by the National Research Initiative of the U.S. Department of Agriculture Cooperative State Research, Education and Extension Service, grant number 2005-35504-16698. We thank F.R. Blattner for providing research materials and S. Moran for assistance with NMR experiments.

References

Booth IR. 2005. Glycerol and methylglyoxal metabolism. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE, editors. *Escherichia coli* and *Salmonella*: Cellular and molecular biology (web edition) Washington DC: ASM Press. Available at www.ecosal.org.

Bouvet OM, Lenormand P, Ageron E, Grimont PA. 1995. Taxonomic diversity of anaerobic glycerol dissimilation in the *Enterobacteriaceae*. Res Microbiol 146:279–290.

de Graef MR, Alexeeva S, Snoep JL, Teixeira de Mattos MJ. 1999. The steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolic adaptation in *Escherichia coli*. J Bacteriol 181:2351–2357.

Dharmadi Y, Gonzalez R. 2005. A better global resolution function and a novel iterative stochastic search method for optimization of HPLC separation. J Chromatogr A 1070:89–101.

Ferguson GP, McLaggan D, Booth IR. 1995. Potassium channel activation by glutathione-S-conjugates in *Escherichia coli*: Protection against methylglyoxal is mediated by cytoplasmic acidification. Mol Microbiol 17:1025–1033.

Gonzalez R. 2005. Metabolic engineering of bacteria for food ingredients. In: Shetty K, Pometto A, Paliyath G, editors. Food Biotechnology. Florida: CRC Press. p 111–130.

Gonzalez R, Tao H, Shanmugam KT, York SW, Ingram LO. 2002. Global gene expression differences associated with changes in glycolytic flux and growth rate in *Escherichia coli* during the fermentation of glucose and xylose. Biotechnol Prog 18:6–20.

Hakobyan M, Sargsyan H, Bagramyan K. 2005. Proton translocation coupled to formate oxidation in anaerobically grown fermenting *Escherichia coli*. Biophys Chem 115:55–61.

Ingraham JL, Marr AG. 1996. Effect of temperature, pressure, pH, and osmotic stress on growth. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE, editors. *Escherichia coli* and *Salmonella*: Cellular and molecular biology. Washington DC: ASM Press. p 1570–1578.

Jin RZ, Tang JC, Lin EC. 1983. Experimental evolution of a novel pathway for glycerol dissimilation in *Escherichia coli*. J Mol Evol 19:429–436.

Kang Y, Durfee T, Glasner JD, Qiu Y, Frisch D, Winterberg KM, Blattner FR. 2004. Systematic mutagenesis of the *Escherichia coli* genome. J Bacteriol 186:4921–4930.

Lacoursiere A, Thompson BG, Kole MN, Ward D, Gerson DF. 1986. Effects of carbon-dioxide concentration on anaerobic fermentations of *Escherichia coli*. Appl Microbiol Biot 23:404–406.

Lawford HG, Rousseau JD. 1996. Studies on nutrient requirements and cost-effective supplements for ethanol production by recombinant *E. coli*. Appl Biochem Biotechnol 57-58:307–326.

McCoy M. 2005. An unlikely impact. Chem Eng News 83:24–26.

McCoy M. 2006. Glycerin Surplus. Chem Eng News 84:7–8.

Merlin C, Masters M, McAteer S, Coulson A. 2003. Why is carbonic anhydrase essential to *Escherichia coli*? J Bacteriol 185:6415–6424.

Neidhardt FC, Bloch PL, Smith DF. 1974. Culture medium for enterobacteria. J Bacteriol 119:736–747.

Nielsen J. 2001. Metabolic engineering. Appl Microbiol Biotech 55:263–283.

Reitzer L. 2005. Catabolism of amino acids and related compounds. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE, editors. *Escherichia coli* and *Salmonella*: Cellular and molecular biology (web edition) Washington DC: ASM Press. Available at www.ecosal.org.

DOI 10.1002/bit

Repaske R, Clayton MA. 1978. Control of *Escherichia coli* growth by CO_2. J Bacteriol 135:1162–1164.

Sawers G, Clark DP. 2004. Fermentative pyruvate and acetyl-coenzyme A metabolism. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE, editors. *Escherichia coli* and *Salmonella*: Cellular and molecular biology (web edition) Washington DC: ASM Press. Available at www.ecosal.org.

Sawers RG, Blokesch M, Böck A. 2004. Anaerobic formate and hydrogen metabolism. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE, editors. *Escherichia coli* and*Salmonella*: Cellular and molecular biology (web edition) Washington DC: ASM Press. Available at www.ecosal.org.

Schryvers A, Weiner JH. 1981. The anaerobic sn-glycerol-3-phosphate dehydrogenase of *Escherichia coli*. Purification and characterization. J Biol Chem 256:9959–9965.

Schryvers A, Lohmeier E, Weiner JH. 1978. Chemical and functional properties of the native and reconstituted forms of the membrane-bound, aerobic glycerol-3-phosphate dehydrogenase of *Escherichia coli*. J Biol Chem 253:783–788.

Shibata M, Katoh H, Sonoda M, Ohkawa H, Shimoyama M, Fukuzawa H, Kaplan A, Ogawa T. 2002. Genes essential to sodium-dependent bicarbonate transport in cyanobacteria. J Biol Chem 277:18658–18664.

Stafford DE, Stephanopoulos G. 2001. Metabolic engineering as an integrating platform for strain development. Curr Op Microbiol 4: 336–340.

Sterling D, Casey JR. 2002. Bicarbonate transport proteins. Biochem Cell Biol 80:483–497.

Tanaka S, Lerner SA, Lin EC. 1967. Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. J Bacteriol 93: 642–648.

Tang JC, Forage RG, Lin EC. 1982a. Immunochemical properties of NAD+-linked glycerol dehydrogenases from *Escherichia coli* and *Klebsiella pneumoniae*. J Bacteriol 152:1169–1174.

Tang JC, St Martin EJ, Lin EC. 1982b. Derepression of an NAD-linked dehydrogenase that serves an *Escherichia coli* mutant for growth on glycerol. J Bacteriol 152:1001–1007.

Thomsen MH. 2005. Complex media from processing of agricultural crops for microbial fermentation. Appl Microbiol Biotechnol 68:598–606.

Totemeyer S, Booth NA, Nichols WW, Dunbar B, Booth IR. 1998. From famine to feast: The role of methylglyoxal production in *Escherichia coli*. Mol Microbiol 27:553–562.

Trchounian AA, Bagramyan KA, Vassilian AV, Poladian AA. 2000. Relationship between formate hydrogen lyase and proton-potassium pump under heterolactic fermentation in *Escherichia coli*: Functional multienzyme associations in the cell membrane. Membr Cell Biol 13: 511–526.

Tyson KS, Bozell J, Wallace R, Petersen E, Moens L. 2004. Biomass Oil Analysis: Research Needs and Recommendations. Technical Report, National Renewable Energy Laboratory, U.S. Department of Energy. Available at eereweb.ee.doe.gov/biomass/pdfs/34796.pdf

Unden G, Kleefeld A. 2004. C4-Dicarboxylate degradation in aerobic and anaerobic growth. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE, editors. *Escherichia coli* and *Salmonella*: Cellular and molecular biology (web edition) Washington DC: ASM Press. Available at www.ecosal.org.

Vemuri GN, Eiteman MA, Altman E. 2002. Effects of growth mode and pyruvate carboxylase on succinic acid production by metabolically engineered strains of *Escherichia coli*. Appl Environ Microbiol 68:1715–1727.

Willke T, Vorlop KD. 2004. Industrial bioconversion of renewable resources as an alternative to conventional chemistry. Appl Microbiol Biotechnol 66:131–142.