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Fermentation of Glycerol to Succinate by Metabolically Engineered Strains of *Escherichia coli*▽†

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The fermentative metabolism of *Escherichia coli* was reengineered to efficiently convert glycerol to succinate under anaerobic conditions without the use of foreign genes. Formate and ethanol were the dominant fermentation products from glycerol in wild-type *Escherichia coli* ATCC 8739, followed by succinate and acetate. Inactivation of pyruvate formate-lyase (*pflB*) in the wild-type strain eliminated the production of formate and ethanol and reduced the production of acetate. However, this deletion slowed growth and decreased cell yields due to either insufficient energy production or insufficient levels of electron acceptors. Reversing the direction of the gluconeogenic phosphoenolpyruvate carboxykinase reaction offered an approach to solve both problems, conserving energy as an additional ATP and increasing the pool of electron acceptors (fumarate and malate). Recruiting this enzyme through a promoter mutation (*pck) to increase expression also increased the rate of growth, cell yield, and succinate production. Presumably, the high NADH/NAD$^+$ ratio served to establish the direction of carbon flow. Additional mutations were also beneficial. Glycerol dehydrogenase and the phosphotransferase-dependent dihydroxyacetone kinase are regarded as the primary route for glycerol metabolism under anaerobic conditions. However, this is not true for succinate production by engineered strains. Deletion of the *ptsI* gene or any other gene essential for the phosphotranferase system was found to increase succinate yield. Deletion of *pflB* in this background provided a further increase in the succinate yield. Together, these three core mutations (*pck**, *ptsI*, and *pflB*) effectively redirected carbon flow from glycerol to succinate at 80% of the maximum theoretical yield during anaerobic fermentation in mineral salts medium.**

Renewable bioenergy offers the potential to solve many environmental problems associated with petroleum-based fuels and chemicals. Biodiesel is produced by reacting vegetable oil or animal fat with alcohol (methanol or ethanol) and used as a transportation fuel in many countries (33). Glycerol is formed as an abundant waste product with limited commercial uses. As the worldwide production of biodiesel continues to increase, the development of effective uses for glycerol may prove essential for the economics and competitiveness of the biodiesel industry. The value of glycerol waste from biodiesel is similar to that of sugars currently used to produce fuel ethanol. Bioconversion of glycerol to higher-value products that replace petroleum, such as polymers, surfactants, solvents, and chemical intermediates, represents an opportunity to decrease waste and improve the economics of the biodiesel industry (5).

Many previous investigations have focused on the fermentative production of 1,3-propanediol (1,3-PD) from glycerol (2, 26, 35). Microorganisms including *Klebsiella* (14), *Citrobacter* (6), *Enterobacter* (1), *Lactobacillus* (29), and *Clostridium* (10, 28) have the native ability to ferment glycerol into this product. Dupont and Genencor have commercialized a 1,3-PD-based polyester, a condensation product of 1,3-PD and terephthalic acid using glucose as the feedstock. Potential demand for this polymer is estimated to be 1 billion to 2 billion pounds per year over the next 10 years (26). Other investigations of glycerol fermentation have described the production of hydrogen and ethanol (15), polyhydroxyalkanoates (PHAs) (20, 27), glyceric acid (13), and small amounts of succinate (21).

Succinic acid is currently used as a specialty chemical in the agricultural, food, and pharmaceutical industries (24, 34). It has also been identified by the U.S. Department of Energy as one of the top 12 building block chemicals (31) because it can be converted into a wide variety of products, including green solvents, pharmaceutical products, and biodegradable plastics (24, 34). Succinate is primarily produced from petroleum-derived maleic anhydride. Recent increases in the petroleum price have generated considerable interest in the fermentative production of succinate from sugars using either natural succinate-producing rumen bacteria or metabolically engineered *Escherichia coli* strains (24, 36, 38). Succinate can also be produced from glycerol by rumen bacteria, such as *Anaerobiospirillum succiniciproducens* (21). However, these strains require complex nutrients that increase costs of production, purification, and waste treatment.

E. coli has been previously engineered for the commercial production of 1,3-PD from sugars by Dupont and Genecor (26). It is an excellent organism for biotechnology applications but was long thought incapable of anaerobic growth on glycerol (23). Recent studies demonstrated that *E. coli* can ferment glycerol anaerobically (8, 11, 25, 33), and a new model was proposed for glycerol fermentation (11). In this model, glycerol is metabolized through the glycerol dehydrogenase (encoded by *gldA*) and dihydroxyacetone kinase (encoded by *dhaKLM*) pathway with the production of ethanol and acetate as primary fermentation products (11). Small amounts of succinate and

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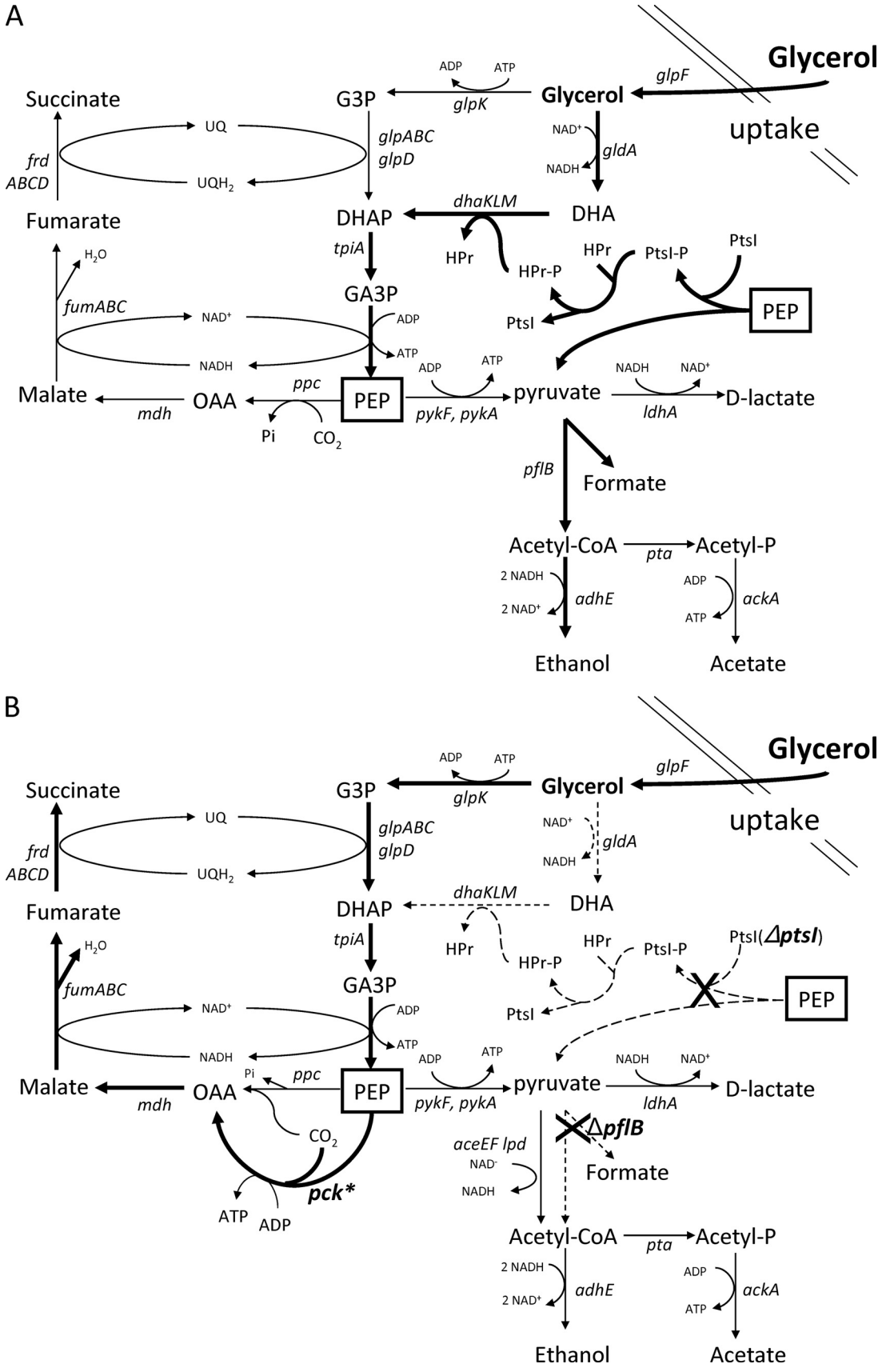
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Glycerol
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PEP
Pi
CO2
pykF, pykA
pyruvate
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HPr
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OAA
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ppc
PEP
pykF, pykA
pyruvate
NADH
NAD+
ldhA
D-lactate
CO2
ΔpflB
aceEF lpd
NAD-
NADH
Formate
pck*
ATP
ADP
Acetyl-CoA
pta
Acetyl-P
2 NADH
2 NAD+
adhE
ackA
Ethanol
Acetate

TABLE 1. Sources and characteristics of *E. coli* strains used in this study

Strain	Relevant characteristic(s)[a]	Source or reference
ATCC 8739	Wild type	Lab collection
XZ17	Δ*pflB*	This study
XZ462[b]	*pck** Δ*glpA*	This study
XZ464	*pck** Δ*gldA*	This study
XZ465[b]	*pck** Δ*dhaKL*	This study
XZ466[b]	*pck** Δ*dhaM*	This study
XZ467[b]	*pck** Δ*ptsA*	This study
XZ468[b]	*pck** Δ*ptsH*	This study
XZ632	*pck**	This study
XZ647	*pck** Δ*ptsI*	This study
XZ721	*pck** Δ*ptsI* Δ*pflB*	This study

[a] The abbreviation *pck** denotes a mutated form of *pck* (G to A at −64 relative to the ATG start) that results in an 8-fold increase in expression (36, 38).

[b] The one-step gene deletion method (7) was used to delete the *glpA*, *dhaKL*, *dhaM*, *ptsA*, and *ptsH* genes.

1,2-propanediol were also produced. Native genes encoding glycerol dehydrogenase and dihydroxyacetone kinase were expressed from a plasmid to increase the rates of glycerol metabolism and ethanol production (32). Succinate production has also been increased by expressing *Clostridium freundii* dihydroxyacetone kinase (encoded by *dhaKL*) (11). However, neither of these enhanced pathways would appear suitable for efficient succinate production due to the absence of net ATP production and the requirement for phosphoenolpyruvate as a phosphoryl donor for dihydroxyacetone, limiting the carboxylation of this intermediate (Fig. 1).

Previous studies in our laboratory (16, 17, 36, 38) have engineered *E. coli* ATCC 8739 for the efficient production of succinate from glucose by recruiting genes from alternative pathways (36, 38). In this paper, we report the use of a similar approach to engineer strains for succinate production from glycerol in mineral salts medium.

MATERIALS AND METHODS

Strains, media, and growth conditions. Strains used in this study are listed in Table 1. During strain construction, cultures were grown aerobically at 30, 37, or 39°C in Luria broth (10 g liter^{-1} Difco tryptone, 5 g liter^{-1} Difco yeast extract, and 5 g liter^{-1} NaCl) containing 2% (wt/vol) glucose or 5% (wt/vol) arabinose. Ampicillin (50 mg liter^{-1}), kanamycin (50 mg liter^{-1}), or chloramphenicol (40 mg liter^{-1}) was added as needed during construction.

Genetic methods. Chromosomal genes were deleted either seamlessly without leaving segments of foreign DNA as described previously (17, 37) or through one-step gene deletion methods (7). Red recombinase technology (Gene Bridges GmbH, Dresden, Germany) was used to facilitate chromosomal integration. Plasmids and primers used during construction are listed in Supplement S1 in the supplemental material.

Glycerol fermentation. Strains were grown without antibiotics at 37°C (150 rpm) in NBS (New Brunswick Scientific) mineral salts medium supplemented with 5% (wt/vol) glycerol and 100 mM potassium bicarbonate as previously described (38). Inocula were prepared by transferring fresh colonies into a 250-ml flask (100 ml NBS mineral salts medium, 2% glycerol). After 16 h (37°C, 120 rpm), this culture was diluted into a pH-controlled fermentation vessel containing 300 ml NBS medium (5% glycerol, 100 mM potassium bicarbonate) to provide an inoculum of 0.033 g cell dry weight (CDW) liter^{-1}. Fermentations were automatically maintained at pH 7.0 by adding base containing additional CO_2 (2.4 M potassium carbonate in 1.2 M potassium hydroxide).

Analysis. Cell mass was estimated by measuring the optical density at 550 nm (OD_{550}). Organic acids and the glycerol concentration were measured by high-performance liquid chromatography (37).

RESULTS

Inactivating competing pathway. Wild-type *E. coli* ATCC 8739 grew very slowly during glycerol fermentation. After 6 days, 153 mM glycerol was metabolized with a cell yield of 0.55 g liter^{-1} CDW (Table 2). Formate and ethanol were the major products, with smaller amounts of succinate (38 mM). Although no lactate was detected, a small amount of acetate was produced. To minimize production of ethanol and formate, the *pflB* gene was deleted (Fig. 1), with unexpected results. Although the production of formate and ethanol was eliminated in the resulting mutant (strain XZ17), glycerol metabolism and cell yield were lowered (45% and 69%, respectively). Succinate production and succinate yield (5 mM succinate after 6 days; 0.11 mol succinate per mol glycerol) were reduced by the *pflB* deletion. Lactate was produced as the dominant fermentation product (Table 2). Since lactate does not provide redox balance with glycerol, small amounts of dissolved oxygen and air leakage are presumed to have also contributed to NADH oxidation.

Recruiting phosphoenolpyruvate carboxykinase. No net ATP would be produced during the anaerobic metabolism of glycerol to succinate using the native phosphoenolpyruvate (PEP) carboxylase for carboxylation (Fig. 1).

In contrast, the high energy of PEP can be conserved by replacing this enzyme with phosphoenolpyruvate carboxykinase (36, 38) to produce 1 mole of ATP per mole of succinate (Fig. 1B). A chromosomal promoter mutation was used to upregulate *pck* expression by approximately 8-fold (36, 38). In the resulting mutant strain, XZ632, cell yield and glycerol metabolism were increased by 27% compared to those of the wild-type parent (ATCC 8739) (Table 2). Production of formate and ethanol were also lower, with a corresponding increase in the yield of succinate

FIG. 1. Glycerol uptake and fermentation by *E. coli.* (A) Native *E. coli* pathways. Bold black arrows represent dominant fermentation reactions prior to engineering; thin black arrows represent minor fermentation reactions. GlpK and GlpD are thought to function primarily during aerobic metabolism. Pathways are based on current reviews in EcoSal (3, 4, 22), data available in Ecocyc (19), and primary literature (11, 12, 18, 25, 30). (B) Engineered pathway for the fermentative metabolism of glycerol to succinate. Bold black arrows represent the engineered reactions for glycerol fermentation to succinate as the dominant product; thin black arrows represent minor fermentation reactions in the engineered strain. Dashed arrows represent reactions that are not functional due to deletions in *ptsI* and *pflB*. Deleted genes are marked with a black X. In native *E. coli* strains, phosphoenolpyruvate carboxykinase functions during gluconeogenesis to produce phosphoenolpyruvate. Mutational activation of the *pck* gene (denoted *pck**) allows this enzyme to function in the reverse direction and to serve as the dominant carboxylation step, conserving energy as ATP. With this engineered pathway, competing needs for PEP have been eliminated and net ATP production has been increased. PEP is boxed to indicate a common pool. Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetone 3-phosphate; PEP, phosphoenolpyruvate; G3P, glycerol 3-phosphate; GA3P, glyceraldehydes 3-phosphate.

TABLE 2. Succinate production from glycerol by engineered *E. coli* strains in NBS mineral salts medium

Strain	Genetic modification[a]	Time (days)	Cell mass (g liter^{-1})	Glycerol used (mM)	Fermentation product (mM)[b]					
					Suc	Suc yield	For	EtOH	Lac	Ace
ATCC 8739	Wild type	6	0.55	153	38 ± 6	0.25 ± 0.03	110	81		16
XZ17	Δ*pflB*	6	0.3	48	5 ± 1	0.11 ± 0.02			20	5
XZ632	*pck**	6	0.7	149	64 ± 13	0.44 ± 0.05	83	46		20
XZ647	*pck** Δ*ptsI*	6	0.4	89	63 ± 4	0.71 ± 0	25	5		13
XZ721	*pck** Δ*pflB* Δ*ptsI*	6	0.5	128	102 ± 25	0.80 ± 0.01			6	12
XZ632	*pck**	6	0.7	149	64 ± 13	0.44 ± 0.05	83	46		20
XZ467	*pck** Δ*ptsA*	6	0.79	168	72 ± 16	0.43 ± 0.03	85	56		16
XZ462	*pck** Δ*glpA*	6	0.5	77	28 ± 4	0.36 ± 0.05	50	22		17
XZ464	*pck** Δ*gldA*	6	0.47	150	112 ± 29	0.75 ± 0.03	37	11		15
XZ465	*pck** Δ*dhaKL*	6	0.43	125	95 ± 25	0.77 ± 0.02	35	8		16
XZ466	*pck** Δ*dhaM*	6	0.5	152	108 ± 33	0.71 ± 0.05	30	9		14
XZ468	*pck** Δ*ptsH*	6	0.5	125	85 ± 22	0.68 ± 0.07	32	14		10

[a] *pck** denotes a mutated form of *pck* (G to A at −64 relative to the ATG start). This mutation increases expression by 8-fold (36, 38).
[b] Fermentations were carried out in NBS medium with 5% glycerol and 100 mM potassium bicarbonate (37°C, pH 7.0, 150 rpm). Succinate yield was calculated as moles succinate produced per mole glycerol metabolized. Data represent an average of 4 experiments (±SD). Abbreviations: Suc, succinate; For, formate; EtOH, ethanol; Lac, lactate; Ace, acetate.

(from 0.25 to 0.44 mol per mol glycerol; $P < 0.05$), a 75% increase in carbon partitioning into product.

Inactivating the glycerol dehydrogenase-dihydroxyacetone kinase (encoded by *gldA* and *dhaKLM*) pathway. Glycerol enters the cell by facilitated diffusion using the GlpF permease. Two pathways have been proposed for the initial metabolism of glycerol in *E. coli* (9, 11). During aerobic (oxidative) metabolism (Fig. 1), glycerol is phosphorylated using ATP as the phosphoryl donor (GlpK) before being oxidized to phosphorylated dihydroxyacetone by GlpD. Quinones serve as electron acceptors. In the absence of oxygen, glycerol is oxidized to dihydroxyacetone by GldA using NAD^+ as the electron acceptor (11). Dihydroxyacetone is subsequently phosphorylated by an intracellular phosphorelay system (PtsI, Hpr, and DhaKLM) using phosphoenolpyruvate as the phosphoryl donor (Fig. 1). Glycerol may also be metabolized through GlpK and GlpABC as a minor pathway during anaerobic conditions (Fig. 1).

Based on this model, a mutation in *ptsI* (strain XZ632) would be expected to impede the fermentative production of succinate. However, the opposite was found (Table 2; Fig. 1). Deletion of *ptsI* significantly increased the succinate yield (from 0.44 to 0.71 mol succinate per mol glycerol; $P < 0.05$). The shift in fermentation products resulting from a *ptsI* deletion confirms that glycerol dehydrogenase and dihydroxyacetone kinase (encoded by *gldA* and *dhaKLM*) represent the primary pathway in native strains, as proposed elsewhere (11). However, this native anaerobic pathway does not appear desirable for succinate production and limits the usage of phosphoenolpyruvate for oxaloacetic acid production (Fig. 1).

These two glycerol metabolism pathways were further investigated by deleting single genes in XZ632 containing the *pck** up-mutation (Table 2). No growth was observed in mineral salts medium after deletion of *glpK*, suggesting that the *gldA-dhaKLM* pathway alone could not support anaerobic growth with succinate production. A *glpA* deletion was accompanied by lower succinate production and cell yield (Table 2). With this deletion (XZ462), glycerol could be metabolized by the *gldA-dhaKLM* pathway, GlpD, or both. In this *pck** background, deletion of any single gene concerned with the *gldA-dhaKLM* pathway, including *ptsH* (encoding Hpr of the phosphorelay system) and *ptsI*, resulted in a similar increase in succinate yield. Deletion of *ptsA*, a cryptic gene in the *ptsI-glpA-ptsA* operon, had little effect on glycerol metabolism and may not be functional.

Core mutations (*pck, Δ*ptsI*, and Δ*pflB*).** The *pflB* gene was deleted in strain XZ647 (*pck** Δ*ptsI*) to eliminate formate and ethanol accumulation. In this strain, yield was further increased from 0.71 to 0.80 mol succinate per mol glycerol with small amounts of lactate and acetate as side products (Table 2). These three core mutations were sufficient for the fermentation to redirect the fermentative metabolism of glycerol to succinate at 80% of the maximum theoretical yield in mineral salts medium. Growth and metabolism were slow, however, requiring 6 days to produce 102 mM succinate.

DISCUSSION

Previous studies have demonstrated that glycerol can be effectively metabolized to succinate by the rumen bacterium *Anaerobiospirillum succiniproducens* using complex medium containing yeast extract and peptone (21). Succinate yields based on glycerol alone exceeded the maximum theoretical yield, with product titers of 200 mM succinate (5 days). Gonzalez and colleagues (8, 11, 25, 32) recently discovered that *E. coli* can ferment glycerol as a sole carbon source under anaerobic conditions, eliminating the need for complex nutrients. Ethanol and formate were the most abundant products, together with lower levels of succinate and acetate. The succinate yield was increased to 0.4 mol per mol glycerol by expressing the *Clostridium freundii* dihydroxyacetone kinase (*dhaKL*) and providing elevated levels of CO_2 (11).

The anaerobic pathway for glycerol dissimilation described by Gonzalez and colleagues (11, 25, 32) works well for ethanol production but may not be optimal for succinate production using the fermentative phosphoenolpyruvate carboxylase (Fig. 1). No net ATP would be produced. Oxaloacetate production for redox balance would be limited by the competing requirement for phosphoenolpyruvate as the phosphoryl donor of dihydroxyacetone phosphate. We have shown that succinate

production can be substantially increased by conserving energy during the carboxylation of phosphoenolpyruvate using the gluconeogenic phosphoenolpyruvate carboxykinase (36, 38), disrupting the primary pathway for anaerobic glycerol metabolism (deletion of any step), and recruiting the minor (native) anaerobic pathways for glycerol metabolism to dihydroxyacetone phosphate (Fig. 1B). With this assembled pathway for succinate, the ATP yield was increased from 0 to 1 per glycerol and the conflict with phosphoenolpyruvate usage was eliminated. With the further deletion of *pflB*, XZ721 containing three mutations (*pck**, Δ*ptsI*, and Δ*pflB*) achieved a succinate yield of 0.8 mol per mol glycerol. This represents 80% of the maximum theoretical yield for glycerol. Related strains have been shown to produce 1.4 mol succinate per mol glucose (38), also 80% of the maximum theoretical yield (1.7 mol succinate/mol glucose).

Growth and metabolism with glycerol remained slow, however, hindering most biotechnology applications for succinate. Slow anaerobic growth with glycerol has been previously attributed to a redox imbalance resulting from the use of intermediates for biosynthesis (11). Additional potential causes include the limited availability of energy for gluconeogesis (required for cell envelope biosynthesis).

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