

UNDERSTANDING AND IMPROVING RESPIRATORY SUCCINATE PRODUCTION
FROM GLYCEROL BY *ACTINOBACILLUS SUCCINOGENES*

By

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ABSTRACT

UNDERSTANDING AND IMPROVING RESPIRATORY SUCCINATE PRODUCTION FROM GLYCEROL BY *ACTINOBACILLUS SUCCINOGENES*

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Succinic acid tops the U.S. Department of Energy's list of value-added products from biomass, because it has the potential, if produced economically, to become the feedstock for a bulk chemical industry currently based on maleic anhydride, a petrochemical. In addition to the large market potential for succinate and its immediate derivatives, bio-based succinate production has the added environmental benefit of using CO₂, a greenhouse gas, as a substrate. *Actinobacillus succinogenes* 130Z naturally produces among the highest levels of succinate from a variety of inexpensive carbon substrates. Previous reports of *A. succinogenes*'s metabolic capabilities mainly used glucose as a feedstock and provided insight into several key factors controlling succinate production. Conversely, little is known about how *A. succinogenes* metabolizes glycerol, a waste product of biodiesel manufacture and an inexpensive feedstock with potential application in bio-based succinate production.

As suggested by our manual annotation of its genome, *A. succinogenes* cannot ferment glycerol in defined minimal medium but it can metabolize glycerol by aerobic or anaerobic respiration. We investigated *A. succinogenes*'s glycerol metabolism in a variety of respiratory conditions by comparing growth, metabolite production, and *in vitro* activity of terminal oxidoreductases. Under conditions of nitrate-respiration and fully aerobic respiration, acetate was the primary acid produced from glycerol. However, succinate was the primary product of dimethyl sulfoxide-respiring cultures and cultures grown in microaerobic conditions. The highest succinate yield observed was 0.69 mol succinate/mol glycerol (69% of the maximum theoretical

yield) under microaerobic conditions. We also show that *A. succinogenes* can grow and produce succinate on partially refined glycerols obtained directly from biodiesel manufacture.

We used recently developed genetic tools to create knockout mutants of *A. succinogenes*. The gene knockout strategy uses natural transformation to introduce linearized DNA into the cells. The isocitrate dehydrogenase gene (*icd*) from *Escherichia coli* was used as a selection marker, enabling positive selection of recombination events based on the glutamate auxotrophy of *A. succinogenes*. After successful deletion of the target gene, we employed the *Saccharomyces cerevisiae* flippase recombinase to remove the *icd* marker, enabling its re-use. With the aim of increasing succinate yields, the *A. succinogenes pflB* gene (encoding pyruvate formate-lyase, PFL) was targeted for deletion. Strain $\Delta pflB$ produced higher succinate yields than strain 130Z (0.85 mol/mol glycerol) under microaerobic conditions.

In summary, in optimized respiratory conditions, *A. succinogenes* can conserve most of the reducing power available in glycerol for succinate production. The increased understanding of *A. succinogenes*'s glycerol metabolism, combined with new genetic tools, sets the stage for future strain and process development towards a highly productive and economic glycerol-to-succinate conversion process.

DEDICATION

This dissertation is dedicated to my entire family. Particularly to my encouraging and patient wife, Christina, who kept me laughing during this process even at times when exciting results in the lab were difficult to come by. Without her, this accomplishment would not be nearly as fulfilling. I must thank my loving parents, Alan and Marla Schindler, who would have supported any career path I chose as long as it made me happy. I would not have gotten this far without their sacrifices for and dedication to my success. Finally, I thank my terrific in-laws, David and Judy Lam, who have included me as a part of their family from day one and have given me their fullest support.

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SYMBOLS AND ABBREVIATIONS

1,2-PD, 1,2-propanediol

1,3-PD, 1,3-propanediol

1,3-PDDH, 1,3-propanediol dehydrogenase

3HPA, 3-hydroxypropionaldehyde

ADH, alcohol dehydrogenase

AK, acetate kinase

AKR, aldo-keto reductase

AO, acridine orange

DHA, dihydroxyacetone

DHAK, dihydroxyacetone kinase

DHAP, dihydroxyacetone phosphate

DMS, dimethyl sulfide

DMSO, dimethyl sulfoxide

FOA, 5-fluoroorotic acid

FHL, formate hydrogen lyase

Fm, fumarase

ForDH, formate dehydrogenase

FR, fumarate reductase

FRT, Flp recognition target

GD, glycerol dehydratase

GDH, glycerol dehydrogenase

GF, glycerol facilitator

GK, glycerol kinase

Gly, glycerol

Gly3P, glycerol-3-phosphate

HA, hydroxyacetone

ICD, isocitrate dehydrogenase

MCF, microbial fuel cell

MDH, malate dehydrogenase

ME_{enz}, malic enzyme

MFA, metabolic flux analysis

MG, methylglyoxal

MGS, methylglyoxal synthase

NR, neutral red

OAA, oxaloacetate

OAA_{dec}, oxaloacetate decarboxylase

OMP_{dec}, orotidine-5-phosphate decarboxylase

PEP, phosphoenolpyruvate

PyrDH, pyruvate dehydrogenase

PEPCK, PEP carboxykinase

PFL, pyruvate formate-lyase

PK, pyruvate kinase

PMF, proton motive force

S/A ratio, ratio of succinate to acetate

TMAO, trimethylamine oxide

USS, uptake signal sequence

Chapter 1

Opportunities for *Actinobacillus succinogenes* as a biocatalyst for the conversion of glycerol to succinate

1.1 The biodiesel market

Success in the United States' push for energy independence will undoubtedly rely on a combination of technological advancements in alternative energy sources including solar, wind, and renewables. In particular, development of a sustainable domestic biofuel industry could obviate the daily import of expensive foreign petrochemical fuels, strengthen national security, and promote rural agriculture. The environmental benefit of replacing petroleum fuels with biofuels is a potential reduction of pollutants, particularly greenhouse gas emissions that contribute to global warming. Among all biofuels, biodiesel stands out because it can be used as a pure fuel or blended with petroleum in any proportion, and it can be used in existing diesel-consuming engines, making it an attractive substitute for traditional diesel (1).

Biodiesel is not yet cost-competitive with traditional diesel fuels. The cost of feedstocks (e.g., soybean oil) is largely responsible for the high price of biodiesel. It was not until the U.S. government enacted tax credit policies and biodiesel usage mandates that domestic biodiesel production expanded. Government action included (i) a 1998 amendment to the 1992 Energy Policy Act, (ii) the USDA Commodity Credit Corporation's Bioenergy Program, (iii) the American Jobs Creation Act of 2004, (iv) the Energy Policy Act of 2005, (v) and the Environmental Protection Agency mandates for biodiesel blends (1). These programs lowered production costs for biodiesel producers and required the blending of biodiesel with petroleum diesel to increase demand. As a result, biodiesel prices competed with petroleum diesel and the U.S. biodiesel industry rapidly expanded (1, 87). For example, production increased from 25 million gallons of biodiesel in 2004 to 700 million gallons in 2008. However, after tax credits expired, production dropped in 2009 to 545 million gallons (87), evidence that the industry's viability still depends on continued government support. Currently, legislation to extend expired

government tax credit programs for biodiesel consumption and production is stalled in congress. Considering also the recent decreases in crude oil prices and narrow biodiesel production margins, it is estimated that domestic biodiesel producers are currently operating at less than 25% capacity (6).

The biodiesel industry would undoubtedly benefit from technological advances to improve the performance and price of biodiesel. The presence of double bonds in the chemical structure of biodiesel makes biodiesel more reactive with oxygen than traditional diesel fuels. Exposure of biodiesel to air over time can lead to oxidative degradation and loss of fuel quality due to increased acidity, higher concentrations of insoluble impurities, and greater viscosity. Issues of storage stability are being investigated (8). Biodiesel manufacturers would also benefit from technologies that reduce feedstock prices. Efforts to increase oil yields in both traditional (e.g., soybean) and non-traditional (e.g., algae) feedstock crops are promising (11, 18). Besides reducing the cost of feedstocks, another means of cost reduction is to create value from the wastestream of biodiesel production.

1.2 The glycerol market

Biodiesel is produced by transesterification of vegetable or animal oils with methanol in the presence of a base catalyst (NaOH or KOH), yielding fatty acid methyl esters (biodiesel) and

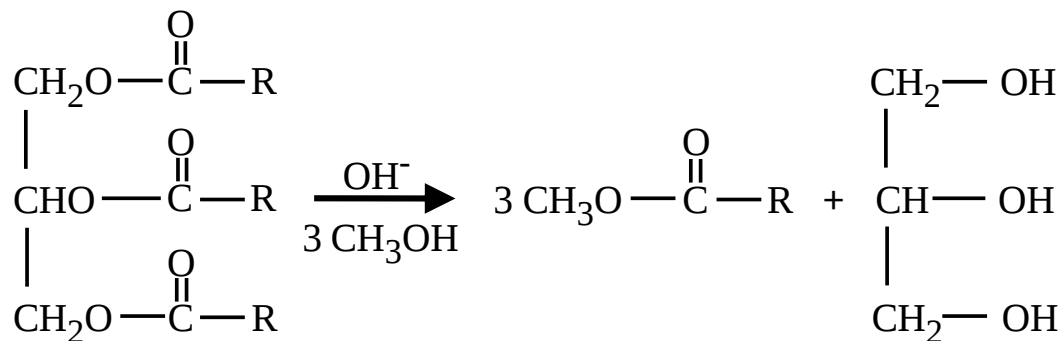


Figure 1.1 Schematic of biodiesel production.

the co-product glycerol (or glycerin). Because crude glycerol is denser than biodiesel the two phases are separated by gravity in a settling vessel or by centrifugation and the glycerol is drawn off from the bottom (87). Most of the unreacted methanol is removed from both phases by distillation and later re-used. The resulting crude glycerol contains variable amounts of glycerol (60-80% wt), unused catalyst, soaps, salts, water, alcohol, protein, and un-reacted fats and oils (76).

Although the transesterification and purification steps of biodiesel production are inexpensive, significant costs arise from the refining or disposal of the by-product crude glycerol. Approximately 10 pounds of crude glycerol are formed for every 100 pounds of biodiesel produced. Rapid expansion of the biodiesel industry has produced a surplus of crude glycerol in the market. This growth resulted in a 10-fold decrease in crude glycerol prices between 2004 and 2006 from \$0.25 to \$0.025 lb⁻¹, forcing some synthetic glycerol-producing facilities to close (88).

Refined glycerol (i.e., 99% free of salt, methanol, and free fatty acids) has applications in the pharmaceutical, personal care, and food and beverage industries. However, these markets are currently satisfied and not projected to grow (34). There is growing interest in catalytically converting pure glycerol into value-added chemicals and fuels, including 1,3-dihydroxyacetone (through oxidation), glycerol tertiary butyl ether (through etherification), propylene glycol (through hydrogenolysis), and acrolein (through dehydration) (12, 21). Steam reforming of glycerol yields varying amounts of H₂, carbon monoxide, carbon dioxide, and methane depending on the temperature and ratio of water and glycerol (84). However, nearly all of these processes are currently hindered by the need for costly upstream purification steps and lack of methods that can directly use crude glycerol (96). Thus, finding value-added uses for crude

glycerol would improve the economics of biodiesel production. In its crude form, glycerol is also being considered as a feed additive for poultry to partially replace conventional feeds (i.e., corn). This can only take place if crude glycerol production can be economically streamlined to maintain consistent composition (safe levels of impurities, especially salts, soaps, and methanol) to ensure the safety of the poultry (55). At its lowest price, crude glycerol can be burned for energy. However, compared to kerosene and gasoline, glycerol is challenging to burn given its low energy density, high viscosity, and high auto-ignition temperature, and this process needs to be optimized (6).

1.3 Glycerol as a potential feedstock for biobased fuel and chemical production: metabolic determinants

In 2010, the price of crude glycerol ranged between \$0.02 and \$0.04 lb⁻¹, near or below the price of traditional fermentation feedstocks (82). In addition to its abundance and low price, the chemical nature of glycerol makes it an attractive feedstock for the production of reduced fuels and chemicals. Indeed, microbial fermentations of glycerol have produced high yields of fuels and other high-value products (12, 88). Table 1.1 lists select examples of glycerol-based fermentations producing reduced fuels and chemicals with preference given to reports of high

Table 1.1 Bacterial products of glycerol metabolism

Product (oxidation state)/Organism	Conditions ^a	Glycerol Form	Medium (g L ⁻¹) ^b	Molar yield (product/glycerol)	Vol. Prod (g L ⁻¹ h ⁻¹)	Other products ^c	Ref.
Hydrogen	Glycerol → ethanol + H ₂ + CO ₂ (theoretical yield of 1.00)						(87)
<i>Enterobacter aerogenes</i> HU-101	an, Ba	Crude	YE (5), T (5)	1.12	<0.01	E, A, PD, F	(27)
<i>Escherichia coli</i> SY03 (pZSKLMgldA)	mO ₂ , Ba	Pure	T (2)	0.96	0.01	E	(87)
Ethanol (6.00)	Glycerol → ethanol + formate (or H ₂ + CO ₂) (theoretical yield of 1.00)						(87)
<i>E. coli</i> SY04 (pZSKLMgldA)	an, Ba	Crude	T (2)	1.02	0.16	F	(87)
<i>E. aerogenes</i> NBRC 12010	MFC, Ba	Crude	Defined	0.92	0.08	H, C, F, L	(61)
<i>E. coli</i> TCS099 e50rep1/pLOI297	mO ₂ , Ba	Pure	Defined	0.90	0.39	A, S	(77)
1,3-PD (5.33)	8 Glycerol → 7 1,3-PD + 3 CO ₂ + 4 H ₂ O (theoretical yield of 0.88)						(75)
<i>Clostridium butyricum</i> VPI 3266	an, FB	Pure	Defined	0.69	1.2	C, B	(60)
<i>Klebsiella pneumoniae</i> ATCC 15380	Aerobic, FB	Crude	YE (5)	0.85	0.6	E, A, L ^d	(25)
<i>E. coli</i> ER2925 (pDY220)	DP, FB	Pure	YE (9)	1.09	2.6	P, A, L, F, S	(72)
Butanol (6.00)	2 Glycerol → butanol + 2 CO ₂ + 2 H ₂ + H ₂ O (theoretical yield of 0.50)						(3)
<i>Clostridium pasteurianum</i> ATCC 6013	an, Ba	Pure	YE (1)	0.45	ND ^e	E, PD, B, A	(71)
<i>Clostridium pasteurianum</i> ATCC 6013	an, Ba	Crude	YE (1)	0.37	ND	E, PD, A, B	(71)
2,3-butanediol (5.50)	2 Glycerol → 2,3-butanediol + 2CO ₂ + 3 H ₂ (theoretical yield of 0.50)						
<i>Klebsiella pneumoniae</i> G31	mO ₂ , FB	Pure	YE (2)	0.45	0.36	PD, A, L, E, S ^f	(59)
3-hydroxypropionaldehyde (4.67)	Glycerol → 3-hydroxypropionaldehyde + H ₂ O (theoretical yield of 1.00)						
<i>Klebsiella pneumoniae</i> NRRL B-199 (ATCC 8724)	Aerobic	Pure	Def.	0.83	ND	ND	(67)

Table 1.1 (cont'd)

Propionic acid (4.67)	Glycerol → propionic acid + H ₂ O (theoretical yield of 1.00)						
<i>Propionibacterium acidipropionici</i> (ACK-Tet)	An, B	Crude	YE (10)	0.88	0.09	S, A	(92)
<i>P. acidipropionici</i> ATCC 4965	mO ₂ , B	Pure	YE (5)	0.90	0.05	A	(14)
<i>P. acidipropionici</i> CGMCC 1.2230 (evolved for acid tolerance)	An, FB	Pure	YE (10)	0.98	0.13	A, S	(97)
1,2-propanediol (5.33)	8 Glycerol → 7 1,2-PD + 3 CO ₂ + 4 H ₂ O (theoretical yield of 0.88)						
<i>E. coli</i> MG1655 $\Delta ackA \Delta pta \Delta ldhA \Delta dhaK$ (pTHKLcfgldAmgsAyqhD)	An, B	Pure	YE (5), T (10)	0.26		E, F, P, S, A	(13)
<i>E. coli</i> MG1655 $\Delta ackA \Delta pta \Delta ldhA \Delta dhaK$ (pTHKLcfgldAmgsAyqhD)	An, B	Crude	YE (5), T (10)	0.29		E, F, P, S, A	(13)
Succinic acid (4.67)	Glycerol + CO ₂ → succinic acid + H ₂ O (theoretical yield of 1.00)						
<i>Anaerobiospirillum succiniciproducens</i>	an, FB	Pure	YE (10)	1.25	0.15	A	(42)
<i>A. succiniciproducens</i>	an, C	Pure	YE (25)	1.17	2.10	A	(40)
<i>Basfia succiniciproducens</i> DD1	an, Ba	Crude	YE (5), P (5)	0.94	0.90	A, F, L	(66)
<i>B. succiniciproducens</i> DD1	an, C	Crude	YE (0.5)	0.80	0.09	A, L	(67)
<i>E. coli</i> XZ721 $pck^{*g} \Delta ptsI \Delta pflB$	an, Ba	Pure	Defined	0.80	ND	A, L	(95)
<i>E. coli</i> MG1655 $\Delta adhE \Delta pta \Delta poxB \Delta ldhA \Delta ppc$ [pZS- <i>pyc</i>]	mO ₂ , Ba	Pure	Defined	0.54	0.26	A, P	(5)
<i>E. coli</i> KJ073 $\Delta ldhA \Delta adhE \Delta ackA \Delta focA \Delta pflB \Delta mgsA \Delta poxB$	an, Ba	Pure	Defined	0.89	ND	ND	(31)

Table 1.1 (cont'd)

^a an, anaerobic; mO₂, microaerobic; Ba, batch culture; C, continuous culture; DP, dual phase (aerobic then anaerobic); FB, fed batch culture; MCF, microbial fuel cell with 2 mM thionine as electron mediator

^b P, peptone; T, tryptone; YE, yeast extract

^c Listed in order of abundance (mol/mol glycerol consumed): A, acetate; B, butyrate; C, CO₂; E, ethanol; F, formate; H, H₂; L, lactate; P, pyruvate; PD, 1,3-propanediol; S, succinate

^d Trace amounts

^e ND, not determined

^f Lactate, ethanol, and succinate were consumed by the end of the fermentation

^g *pck**, phosphoenolpyruvate carboxykinase expressed constitutively

yield processes. The reduced nature of glycerol also makes it an attractive co-substrate for fermentations to improve product yields. For example, the propionic acid yield produced by *Propionibacterium acidipropionici* was improved from 0.475 and 0.303 g/g with glycerol and glucose alone, respectively, to 0.572 g/g with a glycerol and glucose mixture (44).

Glycerol is a highly reduced substrate, with an oxidation state of -0.67 , as calculated using equation 1:

$$\text{Oxidation state} = -1 \times [(n_{\text{hydrogen}} \times 1) + (n_{\text{oxygen}} \times -2) + (n_{\text{nitrogen}} \times -3)] / n_{\text{carbon}}$$

(Eq. 1)

where n is the number of a particular atom in the molecules. With the elemental composition for a microbial cell given as $\text{CH}_2\text{O}_{0.5}\text{N}_{0.2}$ (70), cellular biomass (oxidation state, -0.4) is less reduced than glycerol, but more reduced than glucose (oxidation state, 0.0), the most commonly used fermentation feedstock. To ferment glucose, bacteria must achieve redox balanced growth by excreting a mixture of products with a combined oxidation state lower than that of glucose (i.e., less than 0.0). For example, this can be achieved by producing a greater proportion of formic acid (oxidation state, 2.0) or pyruvic acid (oxidation state, 0.67) than succinic acid (oxidation state, -0.67) and ethanol (oxidation state, -2.0). Conversely, bacteria growing fermentatively on glycerol must excrete products with a combined oxidation state lower than that of glycerol (i.e., less than -0.67).

Although ethanol is more reduced than glycerol, the route from glycerol to ethanol (oxidation state, -2.0) is redox-neutral because of the concomitant production of CO_2 (oxidation state, 4.0). Succinate is less reduced than glycerol when using the formula $\text{C}_4\text{H}_6\text{O}_4$ (oxidation state, 0.5 ; Table 1.1). However, succinate biosynthesis requires CO_2 fixation and, if CO_2 is

Table 1.2 Oxidation state of typical fermentation substrates and products.

Compound	Formula	Oxidation state
Substrates		
Glucose	$C_6H_{12}O_6$	0.00
Glycerol	$C_3H_8O_3$	-0.67
Products		
Cellular biomass	$CH_2O_{0.5}N_{0.2}^a$	-0.40
1,3-propanediol	$C_3H_8O_2$	-1.33
Acetic acid	$C_2H_4O_2$	0.00
Carbon dioxide	CO_2	+4.00
Ethanol	C_2H_6O	-2.00
Formic acid	CH_2O_2	+2.00
Fumaric acid	$C_4H_4O_4$	+1.00
Lactic acid	$C_3H_6O_3$	0.00
Pyruvic acid	$C_3H_4O_3$	+0.67
Succinic acid (3C) ^a	$C_3H_6O_2$	-0.67
Succinic acid (4C)	$C_4H_6O_4$	+0.50

^a(70).

supplied exogenously, the part of succinate coming from glycerol is $C_3H_6O_2$ (oxidation state, -0.67 ; Table 1.1). Taken together, succinate production from glycerol plus CO_2 is also redox-neutral, and it cannot, alone, support growth by glycerol fermentation.

1.3.1 Fermentative glycerol metabolism: 1,3-propanediol production

One redox-consuming pathway that does allow for glycerol fermentation is the production of 1,3-propanediol (1,3-PD, oxidation state $= -1.33$). 1,3-PD production is a metabolic determinant of all glycerol-fermenting microorganisms from the genera *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*, *Lactobacillus*, and *Bacillus* (86). In these organisms, glycerol enters the cell via a glycerol facilitator and is processed in two parallel enzymatic pathways. It is either oxidized to 1,3-PD and excreted, or reduced to dihydroxyacetone phosphate (DHAP) for assimilation through glycolysis (Figure 1.2A) (4). In these natural glycerol-fermenting organisms, 1,3-PD is often the main fermentation product.

Species of the genera *Propionibacterium* (24) and *Anaerobiospirillum* (40) have been described as fermenting glycerol without producing 1,3-PD. However, their growth was always demonstrated in the presence of complex nutrient supplementation (i.e., yeast extract or tryptone) that likely contributed to achieving redox balance. Furthermore, the pathways involved in glycerol fermentation were not investigated. Supplementation with high levels of tryptone and yeast extract is costly and prevents the use of these organisms as biocatalysts for the production of fuels and chemicals from glycerol.

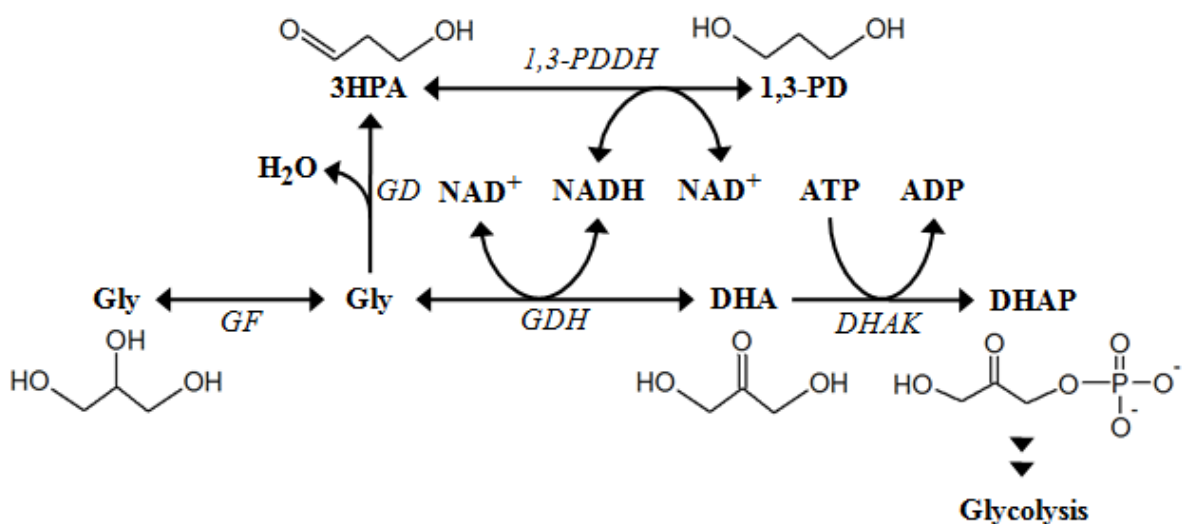


Figure 1.2 Glycerol fermentative pathways producing 1,3-PD. Metabolites: 1,3-PD, 1,3-propanediol; 3HPA, 3-hydroxypropionaldehyde; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; Gly, glycerol. Enzyme reactions: *1,3-PDDH*, 1,3-propanediol dehydrogenase; *DHAK*, dihydroxyacetone kinase; *GD*, glycerol dehydratase; *GDH*, glycerol dehydrogenase; *GF*, glycerol facilitator; ►►, multiple enzymatic steps.

1.3.2 Pseudo-fermentative glycerol metabolism in *Escherichia coli*

Only recently has *Escherichia coli*, the workhorse for molecular biology, proven to be a possible biocatalyst for conversion of glycerol to reduced products. It had long been understood that, while wild-type *E. coli* can grow on glycerol aerobically, it cannot ferment glycerol (7). The group of Dr. Ramon Gonzalez at Rice University (14, 21, 55) exhaustively investigated the environmental and genetic determinants of glycerol metabolism in *E. coli*. These investigations led to the following observations:

Glycerol fermentation by *E. coli* is possible and requires specific conditions. Early studies of glycerol metabolism in *E. coli* did not observe the pseudo-fermentative nature of anaerobic glycerol metabolism in *E. coli* due to the particular growth conditions tested (21). Early studies used high levels of potassium and phosphate, later shown to be detrimental to glycerol metabolism. These studies also used low initial glycerol concentrations (20-30 mM) instead of 100 mM in the most recent tests. The K_m of GldA, the *E. coli* glycerol dehydrogenase is 4-40 mM (78). It may explain why, even in recent studies, complete glycerol consumption was not observed (21).

Glycerol consumption is dependent on the F_0F_1 -ATPase system and a proton motive force.

Maintenance of a proton motive force (PMF) for F_0F_1 -ATPase activity is essential for ATP production and metabolite transport during glycerol metabolism in *E. coli* (21). The mechanism of glycerol fermentation was different in acidic and alkaline conditions (21). In acidic conditions (pH 6.3), fermentation was dependent on the activity of formate hydrogen lyase (FHL), as a $\Delta fdhF$ mutant lost the ability to ferment glycerol (*fdhF* encodes the formate dehydrogenase

subunit of FHL). Growth and fermentation were restored by gene complementation. In alkaline conditions (pH 7.5), the $\Delta fdhF$ mutant fermented glycerol as well as the wild type strain. Regardless of the culture pH, the F_0F_1 -ATPase system was required for glycerol metabolism.

H₂ accumulation and recycling impairs glycerol metabolism. When H₂ is not flushed out of fermenters, glycerol consumption and cell growth are reduced at least two-fold (55). H₂ accumulation has little inhibitory effect on a fumarate reductase mutant ($\Delta frdA$), suggesting that H₂ is recycled (via hydrogenase and the quinone pool) and used as an electron donor for fumarate reduction to succinate, resulting in a redox imbalance (55).

***E. coli* produces low levels of 1,2-propanediol (1,2-PD) from glycerol as a redox sink.** 1,2-PD is produced from DHAP through methylglyoxal and hydroxyacetone (Figure 1.3). This pathway consumes one reducing equivalent (i.e., NADH). In a tryptone-supplemented medium, the 1,2-PD produced (0.5 mM from 85 mM glycerol) was equivalent to the NADH produced during biomass production (21). Over-expression of the 1,2-PD pathway genes (*mgsA* and *gldA*) supported growth in the absence of tryptone. However, the growth yield (maximum OD_{550 nm} of 0.17) and glycerol consumption (1.6 g/L) were low (21), suggesting that the 1,2-PD pathway alone is insufficient for glycerol fermentation, even after over-expression. It is possible that NADPH production is limiting 1,2-PD production and growth on glycerol (Figure 1.3).

Growth on glycerol is limited by biosynthesis of biomass protein. Supplementation of the growth medium with 0.3 g L⁻¹ tryptone or amino acids at concentrations close to those provided by tryptone results in similar growth yields and glycerol consumption. This observation suggests

that biomass production from glycerol is limited by the redox demands of protein biosynthesis. This conclusion is supported by the fact that in cultures supplemented with 2 g L^{-1} tryptone, only 20% of the proteinogenic amino acids are derived from glycerol. In these cultures, in contrast, nearly 100% of the carbon atoms in ethanol, acetate, and formate originate from glycerol (55). By using mainly tryptone, and not glycerol, to satisfy amino acid demands for growth, only a small flux through the 1,2-PD pathway is necessary to oxidize the excess NADH produced during biomass production from glycerol.

The *gldA-dhaKLM* pathway is required for anaerobic glycerol metabolism in the absence of electron acceptors. Under anoxic, non-respiratory conditions, *E. coli* uses a type II glycerol dehydrogenase (encoded by *gldA*) and dihydroxyacetone (DHA) kinase (encoded by *dhaKLM*) to convert glycerol to DHAP (Figure 1.3). Glycerol metabolism is completely eliminated by disrupting these genes, but can be rescued by gene complementation.

Based on these observations, Shams Yazdani and Gonzalez (87) engineered *E. coli* strains for highly efficient production of ethanol from glycerol with the co-production of hydrogen and formate. First, succinate and acetate productions were reduced to negligible levels after disruption of the *frdA* and *pta* genes, respectively. This strain, SY03, co-produced H_2 and CO_2 with ethanol at near equimolar concentrations when grown in slightly acidic conditions (pH 6.3). Strain SY04 was engineered for the co-production of formate with ethanol. Formate oxidation was eliminated by inactivating the formate dehydrogenase subunit of FHL (encoded by *fdhF*). Strain SY04 produced 0.92 mol ethanol per mol glycerol with a volumetric productivity of $0.3 \text{ mmol L}^{-1} \text{ h}^{-1}$. Overexpression of the genes encoding glycerol dehydrogenase and dihydroxyacetone kinase increased productivity to $3.6 \text{ mmol L}^{-1} \text{ h}^{-1}$. A similar increase in

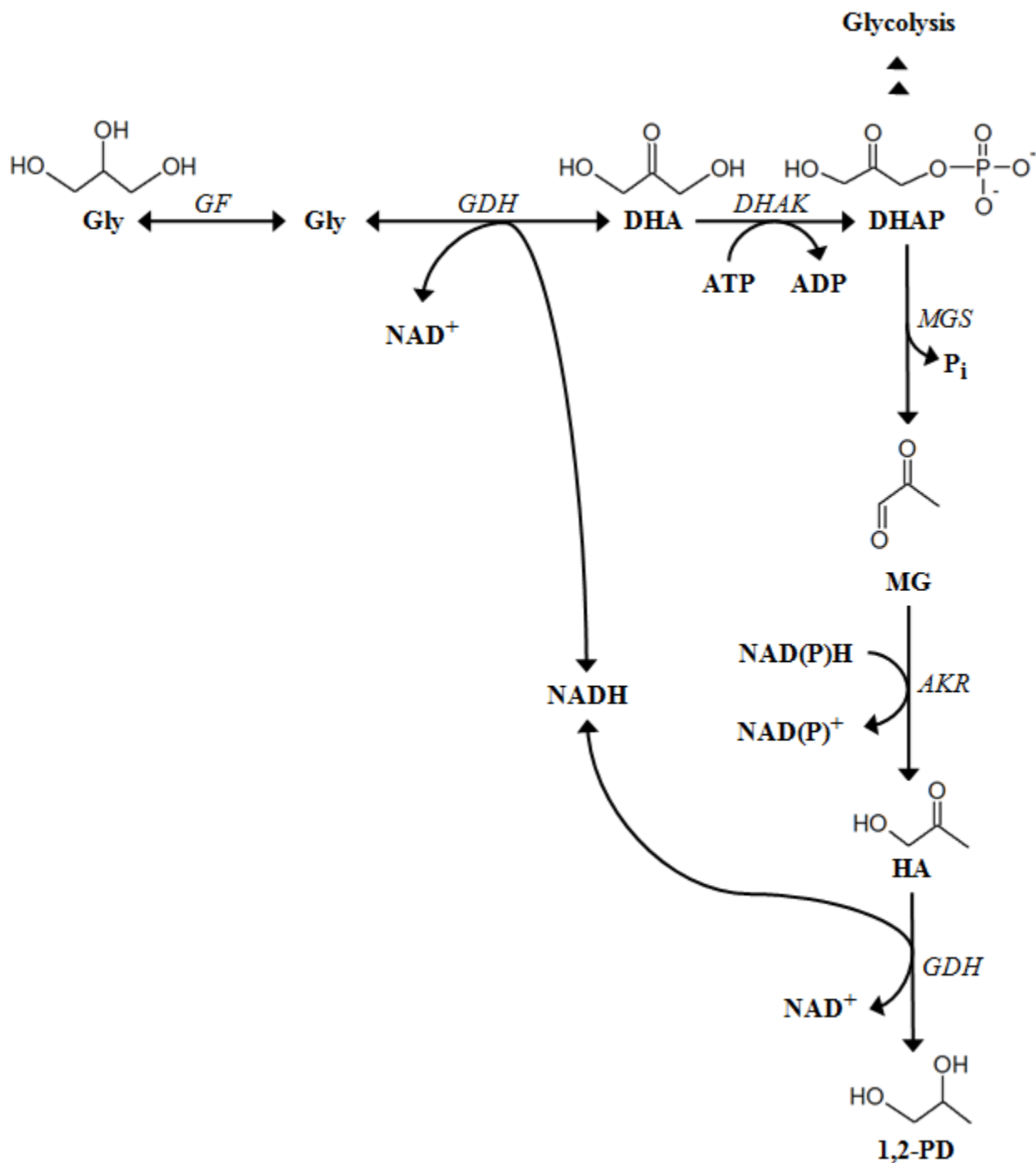


Figure 1.3 Pseudo-fermentative glycerol metabolic pathways in *E. coli*. Metabolites: 1,2-PD, 1,2-propanediol; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; Gly, glycerol; HA, hydroxyacetone; MG, methylglyoxal. Enzyme reactions: AKR, aldo-keto reductase; DHAK, dihydroxyacetone kinase; GDH, glycerol dehydrogenase; GF, glycerol facilitator; MGS, methylglyoxal synthase; ►►, multiple enzymatic steps.

productivity was observed for strain SY03 overexpressing the same genes. While these investigations went far in demonstrating *E. coli*'s ability to produce high yields of reduced products from glycerol (1.02 mol ethanol per mol crude glycerol with strain *E. coli* SY04[pZSKLMgldA]; Table 1.1) (87), they fall short of proving the process to be a true fermentation, since tryptone supplementation is required.

1.3.3 Respiratory glycerol metabolism in bacteria

Glycerol can be used as a carbon source by many bacteria growing by aerobic or anaerobic respiration. *E. coli* is the model system of respiratory glycerol metabolism in bacteria (21). In *E. coli*, glycerol first enters the cell via a glycerol facilitator (GlpF) belonging to the aquaglyceroporin family. Once inside the cell, glycerol is phosphorylated by glycerol kinase (GlpK) to glycerol-3-phosphate (Gly3P). Gly3P is oxidized to DHAP by either the aerobic (GlpD) or anaerobic (GlpABC) Gly3P dehydrogenase (Figure 1.4) (7). The latter enzyme complex links G3P oxidation to the reduction of fumarate (50, 52) or nitrate (51). DHAP, a glycolytic intermediate, can subsequently be metabolized into pyruvate and further converted to fermentation products.

Respiratory conditions enable redox-balanced growth from glycerol and eliminate the requirement for medium supplementation. However, if the goal is to produce fermentation products, careful design is necessary to minimize respiratory metabolism. Otherwise, respiration as the dominant metabolic mode leads to low product yields, especially of reduced products, as most of the carbon is incorporated into biomass or released as CO₂. For example, acetate and CO₂ are the only end-products of glycerol metabolism in nitrate-respiring *E. coli* (76). In dimethylsulfoxide-respiring cultures grown on glycerol, *E. coli* produced mainly acetate (0.4 mol

per mol glycerol), followed by CO₂, ethanol, and succinate (76). Alternatively, microaerobic conditions have the potential to preserve *E. coli*'s ability to synthesize large amounts of reduced products. In fact, the microaerobic metabolism of glycerol enabled redox balance while preserving the ability of *E. coli* to convert large amounts of glycerol to ethanol (0.52 to 0.66 mol/mol glycerol for wild-type strains and 0.84 mol/mol glycerol for engineered strain EH05) in the absence of rich nutrient supplementation (15).

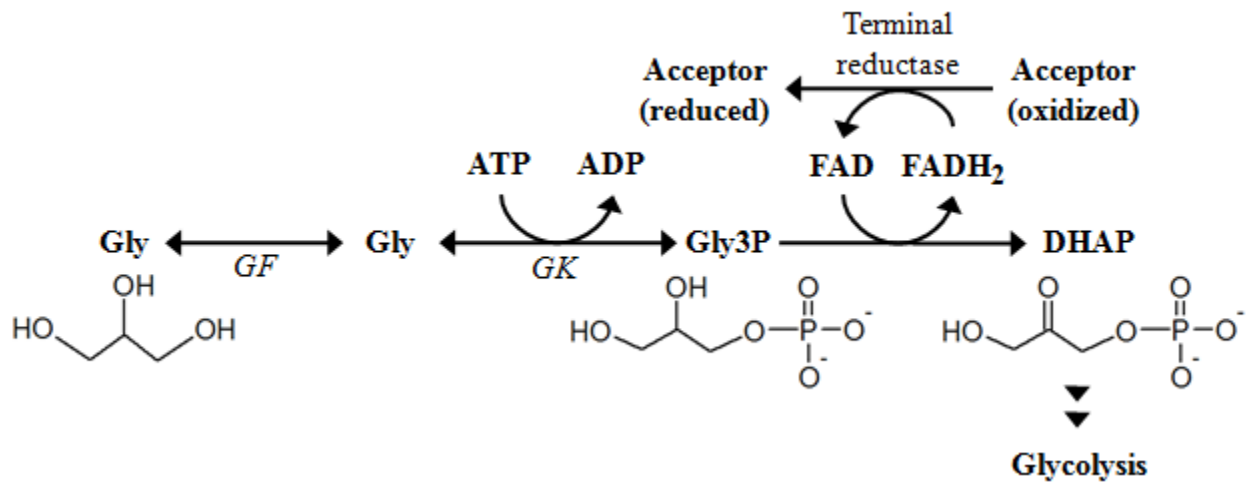


Figure 1.4 Respiratory pathway of glycerol metabolism. Metabolites: DHAP, dihydroxyacetone phosphate; Gly, glycerol; Gly3P, Glycerol-3-phosphate. Enzyme reactions: *G3PDH*, glycerol-3-phosphate dehydrogenase; *GF*, glycerol facilitator; *GK*, glycerol kinase; ►►, multiple enzymatic steps.

An alternative approach to respiratory glycerol metabolism is the use of microbial fuel cells (MFCs) (43). MFCs can be assembled in several different ways, but one common arrangement is a two-electrode, two-compartment system where bacteria growing in the MFC's anode compartment oxidize substrates and transfer electrons to the anode (positive terminal). Electrons are transferred to the anode either by artificial electron mediators (e.g., neutral red), or by direct contact between the bacteria and the electrode. Electrons are transferred to the cathode through the outside electrical current. Protons generated in the anode compartment are transferred to the cathode compartment through a proton-permeable membrane separating the two compartments. Electrons and protons are used in the cathode compartment to reduce cheap substrate, often ferricyanide. The end product is a fuel cell in which electricity production consumes the extra electrons detrimental to the bacterial transformation of interest. This approach has been used successfully to permit bacterial growth on glycerol. For example, *E. coli* produced (in decreasing order of magnitude) acetate, ethanol, lactate, and H₂ from glycerol in the anode compartment in combination with ferricyanide reduction in the cathode compartment (18). In a three-electrode, three-compartment MCF *Propionibacterium freudenreichii* oxidized glycerol to acetate and CO₂ (17). Using this particular MFC setup, the electron mediator (i.e., ferricyanide) was continuously reoxidized at the working electrode. By maintaining the potential of the working electrode (+ 280 mV) constant against the reference electrode with a potentiostat, a constant electrical current was produced between the working and counter electrodes during bacterial growth on glycerol. For the production of reduced products within a MFC, *Enterobacter aerogenes* NBRC 12010, using thionine as an electron mediator, oxidized crude glycerol to produce H₂ and ethanol (0.92 mol/mol glycerol each) (61). *Shewanella oneidensis*, genetically engineered to convert glycerol to ethanol, produced ethanol (at 0.85 mol/mol

glycerol) and CO₂, with acetate as a co-product. Rather than using soluble electron mediators, *S. oneidensis* directly contacts and donates electrons to an electrode surface (20).

Less than half of the transformations described above and listed in Table 1.1 used crude glycerol as the feedstock. However, with the increased availability and low price of crude glycerol, the number of fermentations using crude glycerol is expected to increase. The crude glycerol used in past studies ranged in purity from 18% to 90% and contained various concentrations of methanol (0.01% to 15%), salts (5%–6%), free fatty acids, and soaps. In nearly all reports, crude glycerol feedstocks resulted in higher product yields than pure glycerol. For instance, propionic acid yield was higher from crude glycerol than pure glycerol (0.88 mol/mol vs. 67 mol/mol) (90). In contrast, using crude glycerol does not necessarily increase volumetric productivity. Some studies reported higher productivities with pure glycerol (25, 90), while others reported higher productivities with crude glycerol (64). Lower productivities with crude glycerol were attributed to possible inhibitory levels of salts or toxic compounds in the feedstock (90). Tolerance of bacteria to different concentrations of crude glycerol also varied among the different reports. For example, *Clostridium pasteurianum* grew similarly in pure and crude glycerols at 0.5 and 1% (w/v), but growth and solvent production were appreciably delayed when using 2.5% crude glycerol (growth slowed to 14–24 days compared to 10 days with pure glycerol) (71). However, many reports used 1–2% (w/v) crude glycerol without observing inhibitory effects (61, 87, 90), and one report used 5% glycerol in a fedbatch culture, eventually adding a total of 8% crude glycerol (25).

1.4 Biobased succinate production

To maintain their economic sustainability, future biodiesel refineries would benefit from

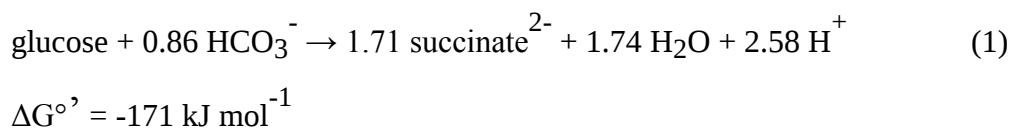
being able to produce a variety of high-value products from crude glycerol and from adjusting their production to changing market demands (54). Succinic acid (or succinate), a four carbon, saturated dicarboxylic acid, is one of the most valuable products that can be made from glycerol. Succinate's largest markets are as a surfactant, detergent extender, and foaming agent. Next, succinate also acts as an ion chelator to prevent metal corrosion. In the food market, succinate's applications are as an acidulant, a flavoring agent, and an anti-microbial agent. Lastly, succinate is used in the production of pharmaceuticals and other health-related agents (89). As of 2010, the market for biobased succinate is 30,000 tons per year with a market value of approximately \$225 million (66).

The primary interest in biobased succinate is not to satisfy this specialty chemical market, but rather to tap succinate's potential as a platform chemical for commodity chemical production. Except for uses in human consumption, for which it is produced fermentatively, succinate is largely produced petrochemically from butane through maleic anhydride. Maleic anhydride is also the chemical feedstock for bulk chemical production of many other carbon compounds-including 1,4-butanediol (a precursor to "stronger-than-steel" and biodegradable plastics), γ -butyrolactone, tetrahydrofuran, ethylene diamine disuccinate (a biodegradable chelator), diethyl succinate (a "green" solvent), and adipic acid (a nylon precursor) (89). If succinate can be produced by fermentation at a cost competitive with that of maleic anhydride, biobased succinate could replace maleic anhydride as the feedstock for the production of all these commodity chemicals. Based on the market demand of several succinate derivatives (i.e., 1,4-butanediol, γ -butyrolactone, polysuccinate esters, and polyamides) the worldwide demand for succinic acid could reach 30 million tons per year (44) with a future potential market value of \$3 to \$10 billion (66, 73). By 2015, it is estimated that 180,000 tons of succinate will be

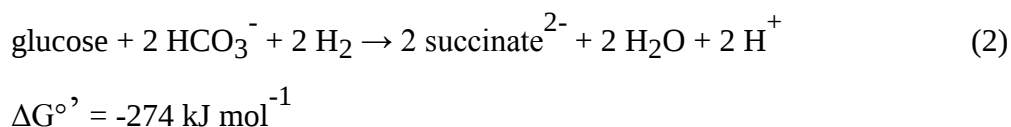
produced per year (66). Consequently, succinate tops the list of the DOE's Top Value Added Chemicals from Biomass (83).

Besides utilizing renewable resources, biobased succinate production also requires fixation of CO₂, a greenhouse gas, further strengthening its environmental impact. In theory, CO₂ produced in another process, (e.g., in a cellulosic ethanol fermentation) could be fed into a succinate fermentation, reducing the refinery's overall carbon footprint. To this end, a suitable strain must be engineered to produce succinate with high titer and productivity from inexpensive fermentations. Due to the high cost of product purification, developing a process with a high succinate yield would further increase the fermentation's cost effectiveness.

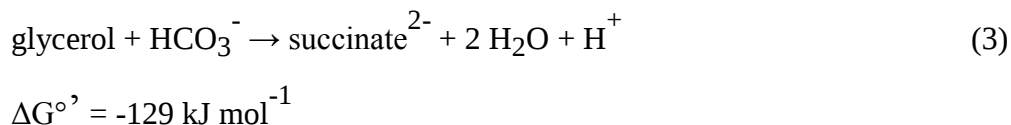
While the sole biological production of succinate from sugars is possible, it has yet to be observed. Bacteria that produce succinate as a major fermentation product also produce other organic acids in significant quantities. The theoretical yields of succinate (based on available electrons) from glucose (equations 1 and 2) or glycerol (equation 3) demonstrate the advantage of using glycerol as a feedstock. Theoretically, in the presence of additional CO₂, ~1.71 mol succinate could be produced per mol glucose (i.e., 24 electrons in glucose divided by 14 electrons in succinate):



In the presence of CO₂ and additional reducing power (e.g., in the form of H₂), 2 mol succinate can be produced per mol glucose:



However, due to the more reduced nature of glycerol, when CO₂ is provided the theoretical succinate yield is 1 mol per mol glycerol:



These theoretical yields are constrained by the production of biomass and alternative products, as well as the available metabolic pathways.

1.5 Bacteria with potential use for fermentative succinate production

The many uses of succinate and the advantages of a succinate fermentation process described above have driven the search for organisms that produce succinate naturally and the engineering of these organisms to further enhance succinate production.

A homosuccinate process would significantly reduce the high cost of succinate purification, one current limiting factor of making biobased succinate economically viable. Succinate production occurs naturally during mixed acid fermentations, where succinate is produced along with other organic acids (e.g., formate and acetate) and ethanol. In most organisms, succinate is produced by the reductive branch of the tricarboxylic acid cycle, which requires reductant for the malate dehydrogenase and fumarate reductase reactions. Altogether, a homosuccinate fermentation can only take place when the competing fermentation pathways are eliminated and the high reductant demand of succinate production is satisfied. Few bacteria naturally produce succinate to levels high enough to be considered for industrial use. The metabolism and production capabilities of these bacteria (both natural and engineered strains) have been the focus of several reviews (2, 48, 69).

1.5.1 *Escherichia coli*

Besides exploiting natural succinate producers, tremendous effort has been applied to engineer *E. coli* into a homosuccinate producer. Wild-type *E. coli* produces succinate as only a minor fermentation product, at about 0.11 mol/mol glucose (63). While early *E. coli* engineered strains showed significant increases in succinate production, these strains still could not compete with the natural succinate producers in terms of titer, and volumetric productivity (48).

Only recently has Dr. Lonnie Ingram's group at the University of Florida made significant advances in succinate production by *E. coli* (29, 31, 91, 92). This group used a combination of gene knockouts and metabolic evolution to engineer *E. coli* strains with dramatically increased succinate production from glucose. First, the genes largely responsible for lactate, ethanol, and acetate production (*ldhA*, *adhE*, and *ackA*, respectively) were knocked out. In this strain, the ATP produced during succinate production was required for growth. This strain, KJ012, grew poorly anaerobically in glucose minimal medium. It was subjected to over 2,000 generations of selective pressure to improve growth rate and succinate yield. This evolution process selected for mutants that had increased energy efficiency (i.e., they produced more ATP per glucose), increased glycolytic flux, and increased succinate yield to satisfy the redox balance. This evolution process, followed by additional gene knockouts (Δ *focA-pflB*), resulted in strain KJ060. Strain KJ060 was able to produce high succinate titers (733 mM) and yields (1.4-1.6 mol succinate per mol glucose, depending on the initial cell density) (29).

Although the main acetate-producing enzyme was inactivated (acetate kinase), the evolution approach activated other acetate-producing pathways to increase ATP yields. The deletion of three additional genes: phosphotransacetylase (*pta*), an *ackA* homologue (*tdcD*), and a *pflB* homologue (*tdcE*), further reduced acetate-linked ATP production in strain KJ134, a KJ060

derivative. Strain KJ134 also contained knockouts of *aspC* (aspartate aminotransferase) and *sfcA* (NADP-linked malic enzyme, an oxaloacetate [OAA] decarboxylating enzyme), which further contributed to increased succinate yields by increasing the flux to OAA. Strain KJ134 produces succinate at more than 90% of the theoretical yield of 1.71 mol/mol glucose (30, 31).

Zhang *et al.* (91) investigated the metabolic changes responsible for the increased energy efficiency and succinate production by the evolved strains. First, PEPCK (encoded by *pckA*), which in *E. coli*, normally functions in the gluconeogenic direction, replaced PEP carboxylase as the most active PEP-carboxylating enzyme. While PEP carboxylation by PEP carboxylase dissipates energy as P_i , PEP carboxylation by PEPCK conserves energy in the form of ATP (1 ATP per OAA). In strain KJ060, A point mutation in the *pckA* promoter region increased *pckA* expression, and PEPCK activity increased 28-fold over that of wild type *E. coli* and 79% over that of *A. succinogenes*, which is known to have high PEPCK activity (81). Second, a frameshift mutation inactivated the PEP-dependent phosphotransferase glucose uptake system. This mutation was compensated by an increase in GalP permease and glucokinase activities. These changes in the glucose uptake mechanism conserved PEP for succinate production. It is interesting to note that this evolved *E. coli* pathway closely resembles the metabolic pathways of natural succinate-producing bacteria found in the rumen (i.e., *Actinobacillus succinogenes* and *Mannheimia succiniciproducens*) (see below). These two major changes were confirmed by re-engineering wild-type *E. coli*. After over-expressing *pckA* and knocking out *ptsI*, succinate became the predominant product of glucose fermentation. Further inactivation of *pflB* in strain XZ721 increased succinate yield to 1.25 mol/mol glucose (92).

E. coli has also been engineered to efficiently produce succinate from glycerol using a number of different approaches. Blankschien *et al.* engineered *E. coli* MG1655 $\Delta adhE \Delta pta$

$\Delta poxB \Delta ldhA \Delta ppc$ [pZS-*pyc*] to produce succinate (0.54 mol/mol glycerol) with little by-product formation (acetate and pyruvate) under microaerobic conditions (5). The authors tested several PEP- and pyruvate-carboxylating enzymes for improved succinate production and chose to overexpress the *E. coli* pyruvate carboxylase in their final engineered strain. Although succinate production was improved significantly compared to the wild-type strain, the low carbon recovery of products (70%, including biomass) suggests that significant amounts of carbon are released as CO₂ or as other unidentified products.

Using another approach, Jantama *et al.* combined metabolic engineering and metabolic evolution to produce strain KJ073 ($\Delta ldhA \Delta adhE \Delta ackA \Delta focA \Delta pflB \Delta mgsA \Delta poxB$) that produces 0.89 mol succinate per mol glycerol (29). Zhang *et al.* (91) discovered that this strain's most active carboxylating enzyme is PEPCK (the significance of this result is discussed in section 1.5). The details of this fermentation are limited to the succinate yield. Lastly, Zhang *et al.* engineered a highly efficient succinate producing strain, *E. coli* XZ721, with three simple engineering steps: (1) overexpression of *pckA*, (2), inactivation of the phosphotransferase system ($\Delta ptsI$), and (3) inactivation of pyruvate formate lyase ($\Delta pflB$) (93). Strain XZ721 produces 0.80 mol succinate per mol glycerol while also producing acetate and lactate. While the authors described the growth of strain XZ721 as a fermentation, they also presumed that minimal air leakage was necessary to achieve complete NADH oxidation, suggesting that the process was microaerobic (93).

1.5.2 Natural succinate producers

The energy-conserving strategies employed by natural succinate producers make them attractive biocatalysts for succinate production. Isolation of natural succinate overproducing

microorganisms has been most successful after screening enrichment cultures of rumen microbiota, whose succinate production plays an important role in the energy production for the host. In the rumen, succinate produced by saccharolytic organisms is quickly decarboxylated to propionate. This decarboxylation step serves as an energy source for other rumen microorganisms and the resulting propionate is converted in the liver of the ruminant to glucose via gluconeogenesis to maintain blood glucose levels (62, 88). Over the past 15 years, several rumen succinate-producing bacteria have received significant attention for industrial succinate production.

1.5.2.1 *Anaerobiospirillum succiniciproducens* and *Basfia succinogenes*

With the high theoretical yield of a glycerol to succinate fermentation and the availability of inexpensive glycerol, succinate production processes have been studied with a number of organisms. *Anaerobiospirillum succiniciproducens*, isolated from the throat of a beagle dog (13), produced succinate in high yields (1.17 mol/mol glycerol) and with high productivity ($2.1 \text{ g L}^{-1} \text{ h}^{-1}$) during continuous culture (38). However, the medium used contained 25 g L^{-1} yeast extract and is not amenable to scale-up for industrial production. *Basfia succiniciproducens*, isolated from bovine rumen by BASF, can convert crude glycerol to succinate at 80% of the maximum theoretical yield with minimal yeast extract supplementation (0.5 g L^{-1}) (65). As expected, the succinate yield increases with added supplementation with yeast extract and peptone (64).

1.5.2.2 *Mannheimia succiniciproducens* and *Actinobacillus succinogenes*

The two most studied natural succinate producers are *Actinobacillus succinogenes* and *Mannheimia succiniciproducens*. Both organisms were isolated from bovine rumen, A.

succinogenes from a University of Wisconsin cow (isolated at MBI international, Lansing, MI) and *M. succiniciproducens* from a Korean cow (39). *M. succiniciproducens* is *A. succinogenes*'s closest relative in the *Pasteurellaceae* family and the two species share many genomic and metabolic similarities (26, 45). For instance, *A. succinogenes* shares 78% of its open reading frames with *M. succiniciproducens*. Both organisms are auxotrophic for cysteine, methionine, nicotinic acid, pantothenate, pyridoxine, and thiamine, with the same genetic bases for each auxotrophy. While the two organisms share many similar genes involved in central metabolism and succinate production, they differ in a few aspects. First, the *A. succinogenes* genome encodes only one PEP-carboxylating enzyme, PEPCK (45), while *M. succiniciproducens*' genome encodes both PEPCK and PEP carboxylase (26). Second, while both succinate producers are auxotrophic for cysteine and methionine, only *A. succinogenes* is auxotrophic for glutamate (49, 68). *A. succinogenes*'s glutamate auxotrophy is due to an incomplete oxidative tricarboxylic acid cycle branch that lacks the enzymes citrate synthase and isocitrate dehydrogenase. The absence of these enzymes makes *A. succinogenes* unable to synthesize α -ketoglutarate, the precursor to glutamate (49). Third, *M. succiniciproducens*'s genome contains the gene *ldhA*, the fermentative lactate dehydrogenase, and *M. succiniciproducens* produces lactate during fermentative growth (41). *A. succinogenes* lacks an *ldhA* homolog and, in our hands, does not produce lactate. For extensive reviews of metabolic features of both organisms see (48) and (45).

Succinate production by *M. succiniciproducens* has been the focus of intense research over the past ten years. The complete genome sequence has been published (26) and used to make in silico predictions to optimize metabolic pathways (35) and environmental conditions (36) for succinate production. *M. succiniciproducens* was genetically engineered to increase the succinate yield from 0.69 mol/mol glucose for the wild-type strain to 0.97 mol/mol glucose for

strain LPK7 ($\Delta ldhA$, $\Delta pflB$, $\Delta pta-ackA$) (41). Genetic tools available for the organism include a shuttle vector (28) for heterologous expression of foreign genes and a temperature-sensitive plasmid for markerless gene knockouts construction (33). Lastly, strain LPK7 was subjected to continuous culture (57) and scale-up optimization (56).

The metabolism of *A. succinogenes* has been studied by a variety of analyses including (i) fermentation balances of batch cultures in different environmental conditions (49, 81), (ii) in vitro enzyme assays (81), (iii) development of a defined growth medium (49), (iv) ^{13}C -based metabolic flux analysis of glucose-grown cultures (46, 47), and most recently (v) manual annotation of its genome sequence (45). Highlighted below are several key traits that make *A. succinogenes* an attractive biocatalyst for biobased succinate production:

***A. succinogenes* produces the highest succinate titer and among the highest succinate yield of known succinate producers.** The wild-type strain, 130Z, produces up to 80 g L^{-1} succinate under optimized conditions. A mutant derivative of strain 130Z., strain FZ53, obtained by chemical mutagenesis, and possibly mutated in pyruvate formate-lyase (PFL), produces 110 g L^{-1} succinate under optimized conditions (22). These succinate titers are equal to or higher than those obtained with the best engineered *E. coli* strains. Acetate and formate are other major fermentation products of strain 130Z, and ethanol is produced in small amounts. *A. succinogenes* is also osmotolerant, surviving up to $160 \text{ g glucose L}^{-1}$ (22, 79).

Table 1.3 Glucose fermentations of facultative anaerobic succinate-producing bacteria

Product/Organism	Condition ^a ; Medium ^b	Succinate			Other products ^c	Ref.
		Molar yield (succinate/ glucose)	Titer (mM)	Vol. Prod (g L ⁻¹ h ⁻¹)		
<i>Escherichia coli</i>						
C; wild type	B; Def.	0.19	49	0.12	F, A, L, P	(29)
KJ017 (KJ012 ^d) 1 st transfer	B; Def.	0.13	6	0.07	A, L	(29)
KJ017 (KJ012) 40 th transfer	B; Def.	0.73	204	0.25	A, F, L	(29)
KJ060 (KJ017, <i>focA</i> , <i>pflB</i>) 86 th transfer, starting OD ₅₅₀ 0.01	B; Def.	1.41	733	0.90	A, M, L	(29)
KJ060 (KJ017, <i>focA</i> , <i>pflB</i>) 86 th transfer, starting OD ₅₅₀ 0.60	B; Def.	1.61	622	0.77	A, M, L, P	(29)
KJ134 (KJ060, <i>mgsA</i> , <i>poxB</i> , <i>tdcDE</i> , <i>citF</i> , <i>aspA</i> , <i>sfcA</i> , <i>pta</i>)	B; Def.	1.53	397	0.75	A, P, M	(31)
XZ721 (<i>pckA</i> [*] , <i>ptsI</i> , <i>pflB</i>)	B; Def.	1.25	327	ND ^e	A, L	(92)
<i>Mannheimia succiniciproducens</i>						
MBEL55E; wild type	B; P, YE	0.69	89	ND	F, A, L	(41)
LPK7; <i>ldhA</i> ::Km ^R , <i>pflB</i> ::Cm ^R <i>pta-ackA</i> ::Sp ^R	FB; YE	1.16	444	2.97	P, M, A, L	(41)
<i>Actinobacillus succinogenes</i>						
130Z; wild type	B; Csl, YE	1.03	568	0.79	A, F, P, Pr	(22)
130Z, wild type	B, Def.	0.70	ND	ND	A, F, E	(49)
FZ53	B, Csl, YE	1.24	896	1.36	A, P, Pr, F	(22)

^aB, batch culture; FB, fed batch culture

^bCsl, corn steep liquor; Def., defined medium; P, polypeptone; YE, yeast extract

^cListed in order of abundance (mol/mol glycerol consumed): A, acetate; F, formate; L, lactate; M, malate; P, pyruvate; Pr, propionate

^dThe genotype of KJ012 is strain C, *ldhA*, *ackA*, *adhE* (29)

^eND, not determined

***A. succinogenes* ferments many different carbohydrates.** *A. succinogenes* grows on and produces succinate from a wide variety of carbon sources. These include the main hemicellulosic sugars (i.e., D-glucose, D-xylose, L-arabinose, and D-mannose) and sugar alcohols (i.e., D-arabitol, D-mannitol, and D-sorbitol) (23, 81). A number of recent studies demonstrate succinate production by *A. succinogenes* from lignocellulosic hydrolysates (9, 10). In total, *A. succinogenes*'s genome sequence suggests that it can transport and degrade about twenty carbon sources (45).

The enzymes involved in succinate production from PEP in *A. succinogenes* are highly expressed. PEPCK, malate dehydrogenase, fumarase, and fumarate reductase are highly expressed, as illustrated by high in vitro activities (81). In contrast to *E. coli*, PEPCK, rather than PEP carboxylase, is *A. succinogenes*'s only CO₂-fixing enzyme in succinate production and the *A. succinogenes* genome does not encode a PEP carboxylase (45). Instead of dissipating energy as inorganic phosphate, *A. succinogenes* produces ATP in this reaction.

***A. succinogenes* is amenable to genetic engineering.** Genetic tools are available, including a shuttle vector for expression of foreign genes (34). This vector, pLGZ920, is a derivative of pGRS-19 (84) containing the pGZRS-1 replicon, allowing plasmid replication in *E. coli* and *Pasteurellaceae* species (*Actinobacillus pleuropneumoniae*, *Pasteurella haemolytica*, *Aggregatibacter actinomycetemcomitans*, and *A. succinogenes*), as well as an ampicillin resistance gene. In pLGZ920, the highly active, constitutive *A. succinogenes* *pckA* promoter allows expression of foreign genes in *A. succinogenes*. pLGZ920 can be transformed into *A.*

succinogenes by electroporation (34). The replication of pLGZ920 in *E. coli* is stabilized by the presence of the ColE1 origin of replication.

***A. succinogenes* is not pathogenic.** As a member of the *Pasteurellaceae* family, *A. succinogenes* is related to many known pathogens. Even though there are no reports of disease caused by *A. succinogenes*, its genome was mined to confirm the lack of virulence factors. *A. succinogenes*'s 2.3-Mb genome was sequenced by the US Department of Energy's Joint Genome Institute, computationally annotated by Oak Ridge National Laboratory, and manually annotated by the Vieille lab (45). *A. succinogenes* is missing several virulence factors common to known pathogens in the *Pasteurellaceae* family, including toxin production and sialic acid incorporation into lipopolysaccharide, suggesting that *A. succinogenes* is not a pathogen.

1.6 *A. succinogenes*'s fermentative metabolism

A. succinogenes's fermentative metabolism from PEP can be divided into two pathways: the C3 pathway (leading to formate, acetate, and ethanol production) and the C4 pathway (leading to succinate production) (Figure 1.5). In the C3 pathway, PEP is converted to pyruvate by pyruvate kinase. PFL then converts pyruvate into acetyl-CoA and formate, which is excreted or oxidized by formate dehydrogenase to CO₂ and H₂. Acetyl-CoA is converted to either acetate or ethanol to satisfy ATP or redox demands, respectively (81). ¹³C-based metabolic flux analysis suggests that pyruvate dehydrogenase, recognized as mainly an aerobically-induced enzyme, is also active in *A. succinogenes* under anaerobic conditions to produce acetyl-CoA (46). In the C4 pathway, PEP is carboxylated to OAA by PEPCK. OAA is subsequently reduced to malate by

malate dehydrogenase, malate is dehydrogenated to fumarate by fumarase, and fumarate is finally reduced to succinate by fumarate reductase (Figure 1.5).

Early investigations of *A. succinogenes*'s fermentative metabolism suggested a simple metabolic map, where the C3 and C4 fermentation pathways branched at the level of PEP (81). This view was supported by the positive effects of increased CO₂ and H₂ availability on succinate yields, since both are substrates for the C4 pathway. However, detection of high in vitro activities of C4-decarboxylating enzymes (i.e., malic enzyme and OAA decarboxylase) as well as a significant in vivo C4-decarboxylating flux in *A. succinogenes* cultures suggest a more intricate mechanism for controlling product distribution (46, 81).

¹³C-MFA studies of glucose-grown cultures measured the intermediary metabolic fluxes responsible for the changes in product distribution in response to variations in CO₂ and H₂ concentrations (47). Compared to cultures grown with 25 mM NaHCO₃, cultures grown with 100 mM NaHCO₃ had increased fluxes in the C4 pathway. Surprisingly, while fluxes through PEPCCK and pyruvate kinase remained unchanged, C4-decarboxylating fluxes (i.e., through malic enzyme and/or OAA decarboxylase) decreased. The reducing power required for succinate production was supplied by a combination of a decreased flux to ethanol and an increased flux through C3 pathway dehydrogenases (formate dehydrogenase and/or pyruvate dehydrogenase) (47). H₂ is known to provide electrons for succinate production: H₂ oxidation is coupled to menaquinone reduction, and menaquinol oxidation is subsequently coupled to fumarate reduction to succinate by fumarate reductase (58). This mechanism is illustrated by the fact that in cultures grown in the presence of H₂, the C3 pathway does not need to produce as much

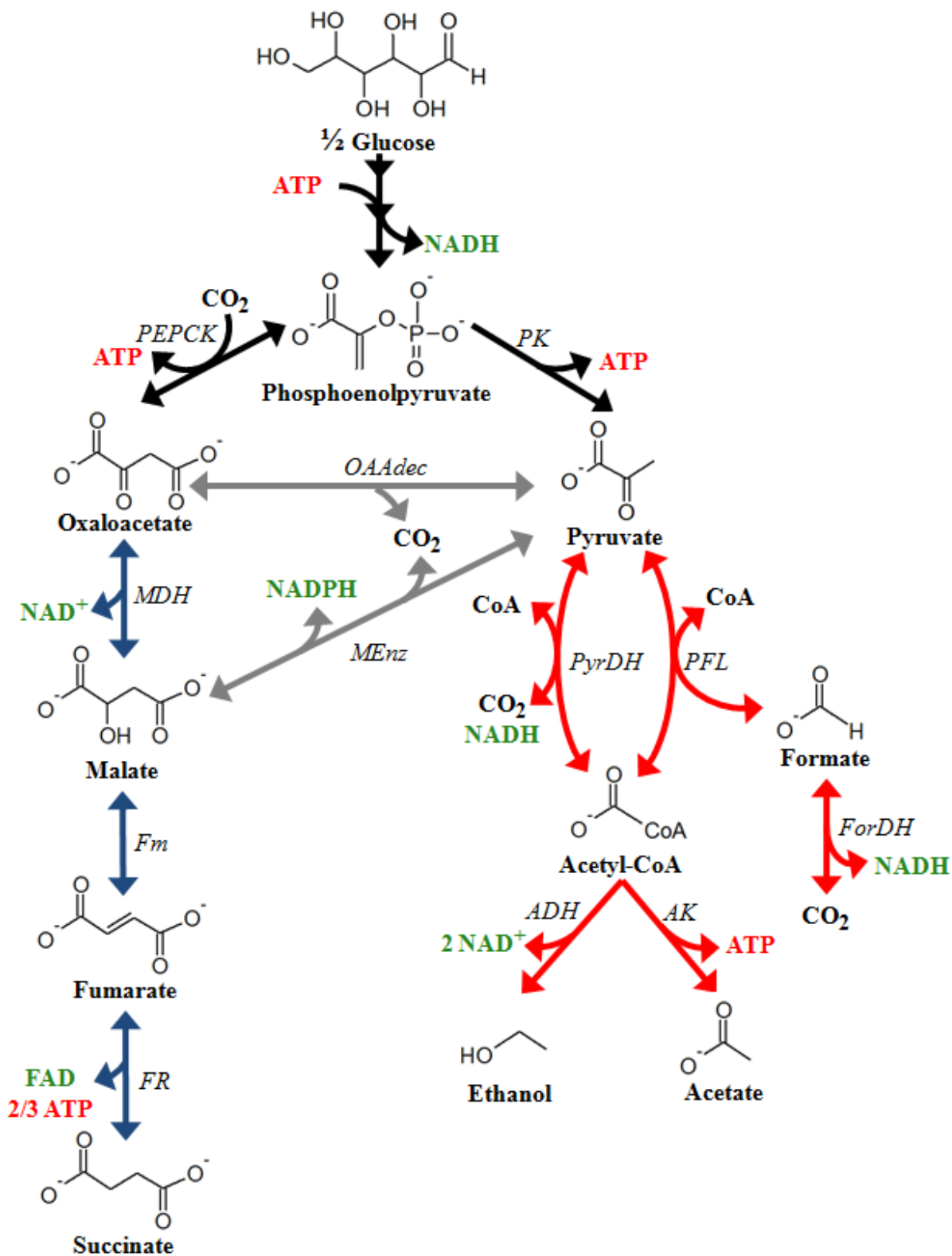


Figure 1.5. Simplified metabolic map of glucose metabolism in *A. succinogenes* during mixed acid fermentation.

Figure 1.5 (continued). Simplified metabolic map of glucose metabolism in *A. succinogenes* during mixed acid fermentation. Red lines, C3 pathway; Blue lines, C4 pathway; Grey lines, C4-decarboxylating reactions. Unidirectional arrows: fluxes considered to be unidirectional. 0.67 ATP is assumed to be produced by the reduction of fumarate by fumarate reductase (37). Reactions: *ADH*, alcohol dehydrogenase; *AK*, acetate kinase; *Fm*, fumarase; *ForDH*, formate dehydrogenase; *FR*, fumarate reductase; *MDH*, malate dehydrogenase; *ME_{enz}*, malic enzyme; *OAA_{dec}*, oxaloacetate decarboxylase; *PK*, pyruvate kinase; *PyrDH*, pyruvate dehydrogenase. ►►, multiple enzymatic steps. Figure and legend adapted from (46). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

reducing power to support succinate production. In these conditions, fluxes through alcohol dehydrogenase and the C3 dehydrogenases decrease (47). Lastly, in the presence of 100 mM NaHCO₃ and H₂, flux through PFL declined and acetate production decreased (47). Since acetate production is a main source of ATP during fermentative growth, ATP production is likely maintained by an increased flux through fumarate reductase, which has the reported ability to form an electrochemical proton potential to drive ATP synthesis (2/3 ATP per fumarate reduced) (37). These MFA studies illustrate the complexity of *A. succinogenes*'s central metabolism, which adjusts fluxes between the C3 and C4 pathways to satisfy demands for pyruvate, acetyl-CoA, ATP, and reducing power. It is important to note that nearly all the information on *A. succinogenes*'s metabolism comes from studies of glucose-grown cultures, and much less is known about its metabolism and ability to produce succinate from other sugars or from glycerol.

1.7 Critical open questions and specific aims

The main objective of this dissertation is to understand the respiratory glycerol metabolism of *A. succinogenes* and to exploit that knowledge to maximize succinate production.

Chapter 2 describes the experiments that were completed to determine the product distribution and activity of terminal oxidoreductases in cultures of *A. succinogenes* grown on glycerol with various terminal electron acceptors. In particular, nitrate and dimethylsulfoxide were examined as terminal electron acceptors for growth while microaerobic glycerol metabolism was also investigated.

Chapter 3 describes the development of genetic tools for *A. succinogenes* that will allow the construction of knockout mutants to test aspects of *A. succinogenes*'s metabolism and to increase succinate production.

Chapter 4 presents a final discussion of the advances made in understanding *A. succinogenes*'s glycerol metabolism, as well as possible future directions of research.

Appendix A is a genomic perspective on *A. succinogenes*'s potential as a biocatalyst for biobased succinate production.

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Chapter 2

Understanding and improving the respiratory glycerol metabolism of *Actinobacillus succinogenes* 130Z for succinate production

2.1 ABSTRACT

Actinobacillus succinogenes 130Z naturally produces among the highest levels of succinate from a variety of inexpensive carbon substrates. Previous reports of *A. succinogenes*'s metabolic capabilities mainly used glucose as a feedstock and provided insight into several key factors controlling succinate production. Conversely, little is known about how *A. succinogenes* metabolizes glycerol, a waste product of biodiesel manufacture and an inexpensive feedstock with potential application in biobased succinate production. As suggested by our manual annotation of its genome, *A. succinogenes* cannot ferment glycerol in defined minimal medium but it can metabolize glycerol aerobically or while respiring nitrate or dimethylsulfoxide anaerobically. We investigated *A. succinogenes*'s glycerol metabolism in a variety of respiratory conditions by comparing growth, metabolite production, and *in vitro* activity of terminal oxidoreductases. We also identified respiratory conditions most favorable for succinate production. Succinate yields were highest in microaerobic cultures, followed by dimethylsulfoxide-respiring, nitrate-respiring, and fully aerobic cultures. The highest succinate yield observed was 0.69 mol succinate/mol glycerol (69% of the maximum theoretical yield) under microaerobic conditions. A pyruvate formate-lyase deletion mutant (strain $\Delta pflB$) produced higher succinate yields than strain 130Z (0.85 mol/mol glycerol) under microaerobic conditions. Finally, we also show that *A. succinogenes* can grow and produce succinate on partially refined glycerols obtained directly from biodiesel manufacture. This is the first detailed report of glycerol metabolism in *A. succinogenes*. These results will likely be useful for understanding the glycerol metabolism of other succinate producers and *Pasteurellaceae*.

2.2 INTRODUCTION

Success of the new green economy will depend on the production of a variety of fuels, such as bioethanol (and higher alcohols), hydrogen, and biodiesel. One way to make biofuels more economically competitive with petroleum-based fuels is to create value-added products from biorefinery waste streams. Biodiesel production, for example, inevitably yields 10 lb of crude glycerol from the trans-esterification of every 100 lb of triglycerides. The growth of biodiesel production has caused a surge in the availability and a steep drop in price of crude glycerol, and it has prompted interest in glycerol as a feedstock for the production of valuable fuels and bulk chemicals (18).

Pure glycerol is marketed worldwide for the foodstuff and pharmaceutical industries, but this market is not projected to grow (18). Currently, crude glycerol is an attractive heating fuel (5) and animal feed supplement (8, 21), but research in these areas is ongoing. There is, however, growing interest in converting crude glycerol into specialty chemicals and fuels. Succinic acid tops the US Department of Energy's list of value-added products from biomass (51), because it has the potential, if produced economically, to become the feedstock for a bulk chemical industry currently based on maleic anhydride, a petrochemical. Derivatives of succinate, including 1,4-butanediol, diethyl succinate, and adipic acid, have applications in biodegradable plastics, solvent, and nylon markets, respectively (29, 55). The market size for succinic acid and related products could reach $245\text{--}270 \times 10^3$ tons yr⁻¹ (29, 52, 55), and that of succinic acid-derived polymers could reach 25×10^6 tons yr⁻¹ (7). In addition to the large market potential for succinate and its immediate derivatives, bio-based succinate production has the added environmental benefit of using CO₂, a greenhouse gas, as a substrate.

Actinobacillus succinogenes is one of the best-known natural succinate-producing microorganisms (29). *A. succinogenes* can ferment the most abundant sugars in hemicellulose to succinate as part of a mixed acid fermentation with acetate, formate, and ethanol as co-products. Succinate production by *A. succinogenes* is favored under conditions of high CO₂ availability (30) and reducing power, the latter of which can be provided as H₂ (28), as a reduced carbon source (49), or as electricity (33). Indeed, reduced carbon sources yield more succinate compared to more oxidized carbon sources. For example, *A. succinogenes* has succinate product ratios (mol succinate/[mol acetate + mol ethanol]) of 2.78 and 0.79 when grown on D-arabitol (oxidation state, -0.4) and D-glucose (oxidation state, 0.0), respectively (49). In addition to its availability and low cost, glycerol is an attractive feedstock for fermentative succinate production because of its highly reduced nature (oxidation state, -0.67). Succinate (C₄H₆O₄, oxidation state, 0.5) is less reduced than glycerol. However, if CO₂ is supplied in the growth medium, the succinate carbon skeleton coming from the main carbon source (e.g., glycerol) is C₃H₆O₂, with the same reduction level (oxidation state, -0.67) as glycerol. While succinate production from glycerol plus CO₂ is redox-balanced, production of biomass (oxidation state, -0.4, assuming a typical microbial biomass composition of CH₂O_{0.5}N_{0.2} [42]) from glycerol is not. Developing a glycerol-based succinate fermentation process is feasible only if the reducing power left over from biomass production is consumed in an additional redox reaction.

It is currently unclear if *A. succinogenes* can ferment glycerol as the sole carbon and energy source and produce high levels of succinate. When *A. succinogenes* was first isolated it was tested for acid production from a variety of carbon sources using a commercial rapid biochemical screening system. *A. succinogenes* did not produce acid from glycerol (14). The ability to ferment glycerol is not a ubiquitous trait of microorganisms (53). Glycerol entry into

glycolysis (at the level of dihydroxyacetone phosphate, DHAP) requires the recycling of one extra reducing equivalent (i.e., NADH) compared to pentose and hexose sugars (i.e., xylose, glucose). All known glycerol-fermenting organisms recycle this extra NADH by producing 1,3-propanediol (1,3-PD; oxidation state, 1.33). 1,3-PD is the best understood metabolic determinant of glycerol-fermenting bacteria, and it is produced by all glycerol-fermenting members of the genera *Citrobacter*, *Klebsiella*, *Clostridium*, *Enterobacter*, *Lactobacillus*, and *Bacillus* (2, 53). Production of 1,3-PD is completed by two parallel pathways, one oxidizing glycerol to DHAP (which is further metabolized through glycolysis), and the other reducing glycerol to 1,3-PD (which is excreted) (Figure 2.1) (2). The enzymes involved are glycerol dehydrogenase (encoded by *dhaD* or *gldA*) and dihydroxyacetone kinase (encoded by *dhaKLM*) in the oxidative branch and glycerol dehydratase (encoded by *dhaB1B2*) and 1,3-PD dehydrogenase (encoded by *dhaT*) in the reductive branch (4). According to Sun *et al.* (43), the genomes of all known glycerol-fermenting bacteria contain genes encoding these four enzymes.

Several 1,3-PD-independent glycerol fermentations have been described, but these processes seem to all fall short of being true fermentations. For example, *Anaerobiospirillum succiniciproducens* produced succinate as the main fermentation product from glycerol with minimal byproduct formation (23). However, the process required supplementation with an unidentified component of yeast extract. A strain of *Klebsiella planticola* produced predominantly ethanol and formate from glycerol in minimal medium (17). Besides H₂, no other reduced products were detected, including succinate, butyrate, propionate, or 1,3-PD. However, the conclusions of this article were limited by lack of explanation for low carbon recoveries (50-80%). Recently *Escherichia coli* (12, 32) and *Paenibacillus macerans* (15)—neither of which

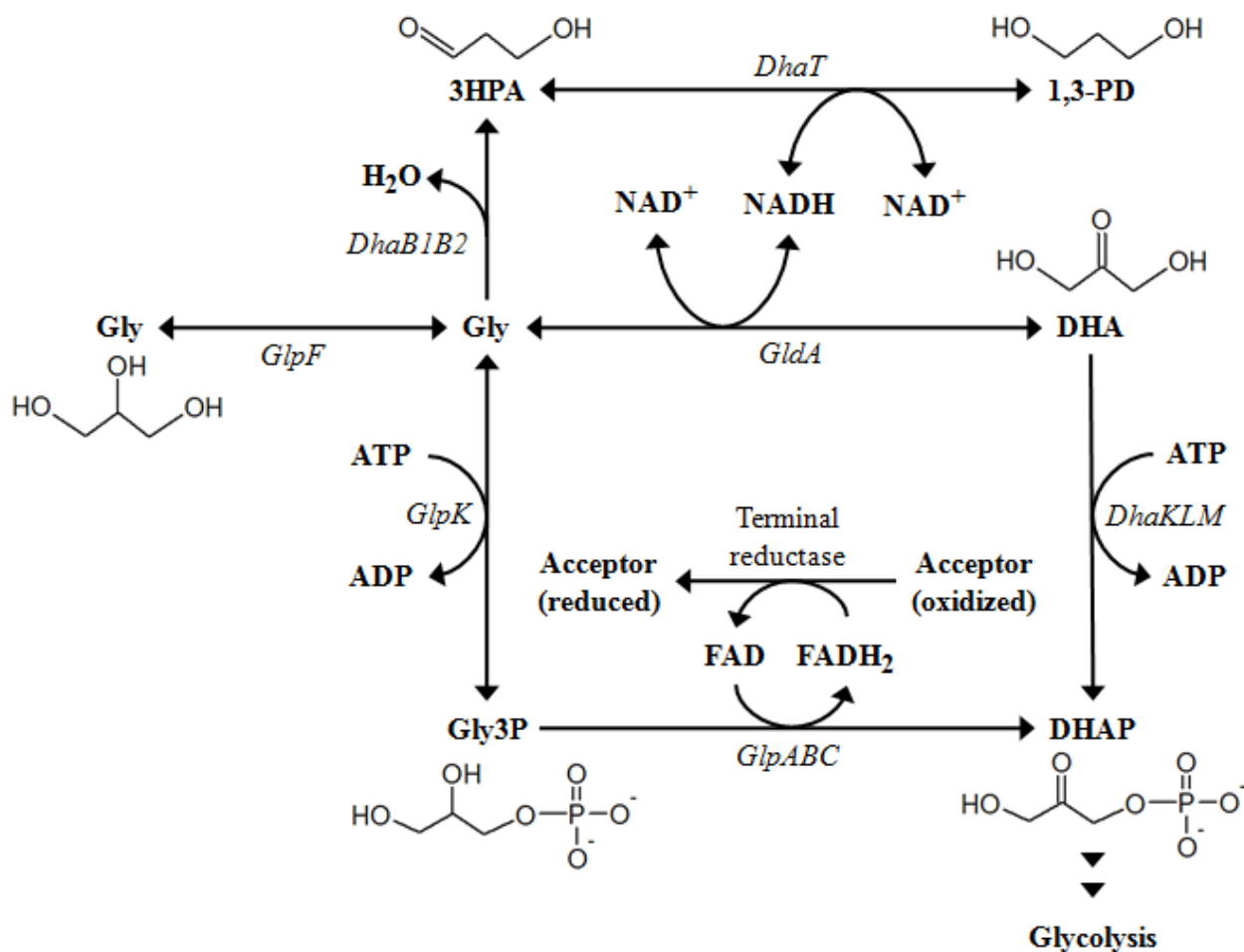


Figure 2.1. Metabolic pathways of glycerol-fermenting bacteria. Metabolites: 1,3-PD, 1,3-propanediol; 3HPA, 3-hydroxypropionaldehyde; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; Gly, glycerol; Gly3P, glycerol 3-phosphate. Transporters and reactions: *DhaB1B2*, glycerol dehydratase; *DhaKLM*, dihydroxyacetone kinase; *DhaT*, 1,3-propanediol dehydrogenase; *GldA*, glycerol dehydrogenase; *GlpABC*, glycerol 3-phosphate dehydrogenase; *GlpF*, glycerol facilitator, *GlpK*, glycerol kinase.

can produce 1,3-PD—were shown to metabolize glycerol in a pseudo-fermentative manner while producing mainly ethanol, CO₂, and H₂, as well as low levels of 1,2-propanediol (1,2-PD). This glycerol metabolism requires stringent environmental conditions, including a means to eliminate H₂ recycling, which was found to create a growth-inhibiting internal redox state (32). The low level of 1,2-PD produced was enough to oxidize excess reducing equivalents released in the synthesis of biomass derived from glycerol. Supplementation of the medium with tryptone was necessary for growth, though, suggesting that the process is not a true fermentation. Jantama *et al.* (16) engineered *E. coli* to ferment glycerol to succinate. Strain KJ073 (*E. coli* Δ *ldhA*, Δ *adhE*, Δ *ackA*, Δ *focA*, Δ *pflB*, Δ *mgsA*, Δ *poxB*) produced 0.89 mol succinate/mol glycerol, which approaches the maximum theoretical yield of 1.0. Unfortunately, the conditions used and alternative products produced were not described and cannot be discussed further. Zhang *et al.* (56) also engineered *E. coli* to produce succinate from glycerol. Although they described their process as a fermentation, the authors believed that dissolved O₂ from air leakage into the reactor vessel contributed to NADH oxidation. Thus, so far, bacteria that do not produce 1,3-PD seem to require an external electron acceptor such as nitrate, dimethylsulfoxide (DMSO), trimethylamine oxide (TMAO), or fumarate to grow on glycerol as the sole carbon and energy source.

Little is known about the glycerol metabolism of bacteria related to *A. succinogenes*, including other *Pasteurellaceae* and succinate producers. *Haemophilus influenzae* was reported to ferment glycerol in rich medium containing peptone and brain heart infusion but the fermentation products were not analyzed (26). *Mannheimia succiniciproducens*, *A. succinogenes*'s closest known relative and a well-characterized succinate producer, could not ferment glycerol in rich medium containing peptone and yeast extract (22). With low yeast extract supplementation (0.5 g L⁻¹), *Basfia succiniciproducens* converts glycerol to succinate at

80% of the maximum theoretical molar yield (39). The required supplementation with yeast extract and/or other rich nutrients for *H. influenzae* and *B. succiniciproducens* growth indicates that either glycerol is not consumed as the sole carbon and energy source or that some component in the medium acts as a terminal electron acceptor. In either case, the process is not a true fermentation. In this report we use a combination of genetic, physiological, and biochemical approaches to study the environmental determinants of *A. succinogenes*'s glycerol metabolism in defined minimal growth medium. We report here that *A. succinogenes* cannot ferment glycerol but can metabolize glycerol aerobically or while respiring nitrate or DMSO anaerobically. We also demonstrate that under microaerobic conditions, *A. succinogenes* conserves most of the reducing power in glycerol and produced succinate at high yields. Lastly, we show that a $\Delta pflB$ mutant produces succinate at higher yields than the wild-type strain under microaerobic conditions.

2.3 MATERIALS AND METHODS

2.3.1. Manual annotation of the *A. succinogenes* genome. The open reading frames (ORFs) described were annotated using the BLAST-based methods and criteria described in (27) based on similarity to ORFs with experimentally tested functions.

2.3.2 Chemicals, bacteria, and culture conditions. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. *A. succinogenes* type strain 130Z (ATCC 55618) was obtained from the American Type Culture Collection. *A. succinogenes* $\Delta pflB$ was constructed as described below and in Chapter 3. Pre-cultures for use in all experiments were grown in AM3 defined medium (30) with modifications (28). After natural transformations, *A. succinogenes* was grown in AM2 (AM3 minus glutamate) in the absence or presence of isocitrate (30 mM; medium AM2-isocitrate). AM3-glycerol contained 100 mM glycerol as the sole carbon source (replacing glucose) and 150 mM NaHCO₃ as the CO₂ source. Pure glycerol (> 99%) was from JT Baker (Phillipsburg, NJ). Vegetable and mixed glycerols were provided by Michigan Biodiesel, LLC (Bangor, MI). Vegetable and mixed glycerols originate from biodiesel production using either vegetable oils or a mixture of animal and vegetable oils, respectively. At Michigan Biodiesel, LLC, the glycerols were partially purified by: (i) treating crude glycerols with HCl to lower their pH to 4-5, (ii) storing in a settling tank (180°F) for 3-4 days to separate glycerol, fatty acids, and soap by density, and (iii) drawing off glycerol from the bottom of the tank and heating it (105°C) with 22-25 psi vacuum pressure to remove the majority of water and methanol. Before adding to AM3, mixed glycerol was autoclaved in a loosely-capped Wheaton bottle to decrease the methanol concentrations remaining after processing. Ten-mL and 60-mL cultures were grown in 28-mL anaerobic tubes and 150-mL serum vials (Bellco, Vineland, NJ),

respectively. Tubes and vials were flushed for 10 min with O₂-free N₂ gas (Airgas, Independence, OH), plugged with rubber bungs, flushed for an additional 10 min, and sealed with aluminum crimps. For all experiments, pre-cultures were collected during exponential phase growth, spun for 3 min at $4,500 \times g$, resuspended in 0.15 M phosphate buffer, pH 7.0, and transferred to AM3-glycerol with and without supplementation of electron acceptors (as indicated in the text). The starting OD_{660 nm} was between 0.05 and 0.10. Cultures were incubated with shaking (250 rpm) at 37°C.

Microaerobic cultures were grown in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). The gaseous atmosphere was maintained at either 1.0% or 0.1% O₂ by mixing CO₂ with air. 125-mL Erlenmeyer flasks containing 60-mL cultures were shaken on a Barnstead Lab Line MaxQ 2000 Benchtop Platform Shaker (Thermo Scientific, Asheville, NC) at 300 rpm for oxygenation. Dissolved O₂ levels were monitored using a Fluorometrix® Non-invasive DO Sensing System (CellPhaseTM DO-1, Fluorometrix Corp., Stow, MA). All experimental data are reported as the average of at least three biological replicates $\pm$ standard deviation. Samples were considered to be biological replicates after being separately serially transferred (1:40 dilution) to fresh medium at least twice.

2.3.3 Growth measurements and metabolic endproduct analysis. Samples (0.8 mL) were withdrawn periodically to quantify the biomass, glycerol, electron acceptors, and metabolites. Biomass was estimated by measuring OD_{660 nm} with a DU 650 spectrophotometer (Beckman, Fullerton, CA). Samples (0.1 mL) were diluted 10 times in 0.9% saline, and the values were used to calculate growth rates, as well as carbon and electron balances. Culture supernatants were analyzed on a Breeze high-performance liquid chromatograph (HPLC, Waters, Milford, MA)

equipped with an Aminex-87H ion-exchange column (Bio-Rad, Hercules, CA). Metabolites were measured as described (28) at 30°C using 4 mM H₂SO₄ as the mobile phase. Glycerol and ethanol were detected with a Waters 410 Differential Refractometer. Organic acids and DMSO were detected with a Waters 2478 Dual λ Absorbance UV detector at 210 nm. CO₂ production was calculated using the equation: CO₂ (mol) = Acetate (mol) – Formate (mol) (11).

2.3.4 Determination of nitrate and nitrite. The concentrations of nitrate and nitrite in culture supernatants were determined by ion chromatography coupled to suppressed conductivity with an IonPac AS 11-HC anion exchange column (2 × 250 mm, Dionex Corp., Sunnyvale, CA) in line with a Dionex IonPac AG11-HC Guard column (2 × 50 mm). Fifteen mM NaOH was used as the mobile phase and was pumped at a flow rate of 0.38 ml min⁻¹ using a Dionex P680 HPLC pump. The ions were quantified by a Dionex ED electrochemical detector that was suppressed by a 2-mm anion self-regenerating suppressor (ASRS Ultra II, Dionex) set in the external water mode and at 23 mA for analysis. Five μ L samples of culture supernatants were injected for analysis.

2.3.5 Preparation of cell extracts for enzyme assays: Growth of exponential-phase cultures was stopped by submerging in an ice-water bath for 10 min. A volume of cells equivalent to 8 OD₆₆₀ units was spun down for 10 min (5,000 × *g* at 4°C) and resuspended in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM DTT. The cells were centrifuged in microcentrifuge tubes for 5 min (5000 × *g* at room temperature) and finally resuspended in 500 μ L of the same buffer. Cell suspensions were disrupted by probe sonication (Branson S-450A probe sonifier, Danbury, CT) with four repetitions, each of 20 s, power level 2, and 50% duty

cycle. The cell extracts were centrifuged for 5 min ($2,000 \times g$) at room temperature to remove any bubbles introduced by sonication. The suspensions were stored on ice until used.

2.3.6 Enzyme activity assays for terminal oxidoreductases. Enzyme activity assays were conducted in 96-well plates using a TECAN SunriseTM Absorbance Reader (TECAN, Männedorf, Switzerland). Anaerobic conditions were maintained in a Coy anaerobic chamber. Two hundred microliter reactions contained the following: 5 mM benzyl viologen, 0.4 mM $\text{Na}_2\text{S}_2\text{O}_4$, 10 μL cell extracts, and 5 mM electron acceptor (NaNO_3 , NaNO_2 , sodium fumarate, or DMSO) (10, 49). All stock solutions were prepared in 50 mM potassium phosphate buffer (pH 7.0). Enzyme activity was calculated from the linear slope of benzyl viologen oxidation recorded at 595 nm. The extinction coefficient of benzyl viologen was $8.65 \text{ cm}^{-1} \text{ mM}^{-1}$ (49). Total cell protein was quantified using the Bio-Rad (Hercules, CA, USA) protein assay dye reagent concentrate with bovine serum albumin as the standard. Activities were reported as the average of at least three biological replicates, each with three technical replicates.

2.3.7 Construction of the *A. succinogenes* $\Delta pflB$ mutant. A detailed description of the construction of *A. succinogenes* $\Delta pflB$ can be found in Chapter 3. Briefly, the plasmid pCR2.1- $\Delta pflB::icd$ was constructed. It contained the chromosomal region surrounding the *A. succinogenes* *pflB* gene, in which the entire *pflB* gene was replaced by a selectable marker cassette. The marker cassette was composed of the *E. coli* isocitrate dehydrogenase gene (*icd*) downstream of the constitutive *A. succinogenes* phosphoenolpyruvate (PEP) carboxykinase promoter (p_{pckA}) and flanked by flippase recognition target (FRT) repeats for later excision. The

p_{pckA} -*icd* marker is used to select for growth in AM2-isocitrate medium. Linearized plasmid DNA was introduced into *A. succinogenes* by natural transformation based on the method described for *H. influenzae* (34). After confirmation of homologous recombination in *A. succinogenes*, the p_{pckA} -*icd* marker was removed by heterologous expression of the *Saccharomyces cerevisiae* flippase gene (*flp*) from plasmid pCV933 (Chapter 3). After confirmation of marker excision, plasmid pCV933 was cured by incubating the cells with 50 $\mu\text{g mL}^{-1}$ acridine orange (44).

2.4 RESULTS

2.4.1 Genetic reconstruction of the *A. succinogenes* glycerol metabolic pathway

A. succinogenes 130Z's genome is publicly available (Joint Genome Institute, 2007), making it possible to predict *A. succinogenes*'s ability to grow using glycerol as the sole carbon source. Based on manual annotation of its genome sequence *A. succinogenes* can uptake glycerol by facilitated transport through a glyceroporin (GlpF) and phosphorylate glycerol to glycerol-3-phosphate by glycerol kinase (GlpK). Since *A. succinogenes* lacks a glycerol dehydrogenase (GldA), phosphorylation by GlpK, followed by dehydrogenation to DHAP by glycerol 3-phosphate dehydrogenase (G3PDH), is the only possible route to glycolysis and central metabolism. Facultative anaerobic bacteria such as *E. coli* have an aerobic and anaerobic G3PDH, encoded by *glpD* and *glpABC*, respectively (6). *A. succinogenes* has only the anaerobic enzyme, GlpABC.

Manual annotation of the pathways known to be involved in glycerol metabolism suggests that *A. succinogenes* is unable to ferment glycerol, since it lacks the complete enzymatic pathway to synthesize 1,3-PD. Of the four enzymes required for 1,3-PD synthesis (glycerol dehydrogenase, dihydroxyacetone kinase, glycerol dehydratase, and 1,3-PD dehydrogenase), *A. succinogenes* lacks the genes encoding all but dihydroxyacetone kinase. The pathway to 1,2-PD is incomplete in *A. succinogenes* as well, since the *A. succinogenes* genome lacks the *mgsA* gene. *mgsA* encodes methylglyoxal synthase, which catalyzes DHAP conversion to methylglyoxal, the committing step of 1,2-PD synthesis. The assumption that *A. succinogenes* cannot ferment glycerol was confirmed by the absence of growth in AM3-glycerol under anaerobic conditions.

2.4.2 Genomic reconstruction of the *A. succinogenes* respiratory chains

The *A. succinogenes* genome encodes several terminal oxidoreductases (Table 2.1), suggesting that production of reducing equivalents by G3PDH can be linked to the reduction of external electron acceptors, and that *A. succinogenes* can grow on glycerol via respiration. Based on this annotation, fumarate, nitrate, nitrite, DMSO and oxygen were all tested for their ability to support growth of *A. succinogenes* in AM3-glycerol. As expected, *A. succinogenes* grew on glycerol by respiring all of these terminal electrons acceptors and produced a variety of mixed acids during growth. Since fumarate, like succinate, is a target value-added chemical for production from biomass (51), it was not studied further as an electron acceptor for growth and for succinate production from glycerol. Similarly, since the main source of trimethylamine *N*-oxide (TMAO) is marine fish and invertebrate tissue (31), TMAO was considered irrelevant for industrial applications and it was not tested in this study.

2.4.3 Nitrate respiration.

A. succinogenes was grown in AM3-glycerol with different initial concentrations of NaNO₃. Culture supernatants were sampled after 72 h of incubation (after all growth had stopped) and analyzed by HPLC. In Figure 2.2A, residual glycerol was used as a measure of total glycerol consumption. Acetate and CO₂ were the predominant metabolites produced in these growth conditions, followed by succinate. Ethanol is a typical product of *A. succinogenes* glucose fermentations (30), but we did not detect ethanol in these conditions. *A. succinogenes* produced less than 1 mM formate at all NaNO₃ concentrations tested except at 40 mM NaNO₃, at which it produced 3 ± 2 mM.

Table 2.1. Respiratory enzymes encoded by the *A. succinogenes* genome.

Enzyme	Redox couple	E _o ' (V)	Gene	Asuc_#	Location ^a
Primary dehydrogenases					
Formate dehydrogenase (anaerobic)	HCO ₃ ⁻ /HCO ₂ ⁻	-0.43	<i>fdnG</i> <i>fdnH</i> <i>fdnI</i>	1264 1263 1262	P
Hydrogenase II	H ⁺ /H ₂	-0.42	<i>hybA</i> <i>hybB</i> <i>hybC</i> <i>hybD</i> <i>hybE</i> <i>hybF</i> <i>hybG</i>	1278 1279 1280 1281 1282 1516 1283	P
NADH dehydrogenase II	NAD ⁺ /NADH	-0.32	<i>Ndh</i>	1980	C
Glycerol-3-P dehydrogenase (anaerobic)	DHAP/Gly-3-P	-0.19	<i>glpA</i> <i>glpB</i> <i>glpC</i>	0205 0204 0203	C
D-Lactate dehydrogenase	pyruvate/D-lactate	-0.19	<i>dld</i>	0005	C
Terminal reductases					
Quinol oxidase <i>bd</i>	1/2O ₂ /H ₂ O	+0.82	<i>cydA</i> <i>cydB</i>	1797 1796	C
Nitrate reductase	NO ₃ ⁻ /NO ₂ ⁻	+0.42	<i>napF</i> <i>napD</i> <i>napA</i> <i>napG</i> <i>napH</i> <i>napB</i> <i>napC</i>	2040 2039 2038 2037 2036 2035 2034	P
Nitrite reductase	NO ₂ ⁻ /NH ₄ ⁺	+0.36	<i>nrfA</i> <i>nrfB</i> <i>nrfC</i> <i>nrfD</i> <i>nrfE</i> <i>nrfF</i> <i>nrfG</i>	0704 0705 0706 0707 0708 0710 0711	P
DMSO reductase	DMSO/DMS ^u	+0.16	<i>dmsA</i> <i>dmsB</i> <i>dmsC</i>	1523 1522 1521	P
TMAO reductase	TMAO/TMA ^u	+0.13	<i>torC</i> <i>torA</i> <i>torD</i>	2034 0898 0899	P
Fumarate reductase	fumarate/succinate	+0.03	<i>frdA</i> <i>frdB</i> <i>frdC</i> <i>frdD</i>	1813 1814 1815 1816	C

^aC, cytoplasmic catalysis; P, periplasmic catalysis.^bDMS, dimethylsulfide; TMA, trimethylamine.

Table adapted from (47).

We found a direct relationship between the initial NaNO_3 concentration and glycerol consumption. Acetate, CO_2 , and succinate production also increased with increasing initial NaNO_3 concentrations. We did not, however, observe an increase in the production of succinate over the other products. The ratio of succinate to acetate (S/A ratio, mol succinate produced/mol acetate produced) is used throughout this report to compare the production of reduced products with that of oxidized products (23). A low S/A ratio indicates a loss of reducing power (e.g., electrons) to external terminal electron acceptors (i.e., nitrate or nitrite). A high S/A ratio indicates conservation of reducing power in reduced products (i.e., succinate). Regardless of the initial nitrate concentration the S/A ratio remained below 0.25, with an average of 0.15 ± 0.01 (Figure 2.2B). The average succinate yield for glycerol-nitrate cultures was 0.12 ± 0.03 mol succinate/mol glycerol. Since glycerol is more reduced than glucose we had expected a S/A ratio higher than that observed for *A. succinogenes* fermenting glucose in AM3 (S/A ratio of 1.09) (30).

Medium AM3 contains vitamins and the amino acids glutamate, cysteine, and methionine in concentrations sufficient to only satisfy the corresponding auxotrophies. Nitrate is the only abundant nitrogen source in AM3-glycerol- NaNO_3 grown cultures. Growth of *A. succinogenes* in this medium demonstrated that *A. succinogenes* is able to reduce nitrate to ammonia (41) to satisfy the cellular nitrogen demand. This result confirmed our genome annotation of *A. succinogenes*'s nitrate reduction pathway. We should note that *A. succinogenes* lacks the genes required for denitrification (data not shown). Nitrite alone also supported growth (data not shown). However, like in nitrate-respiring cultures, nitrite-respiring cultures produced acetate as the main product and nitrite was not tested further.

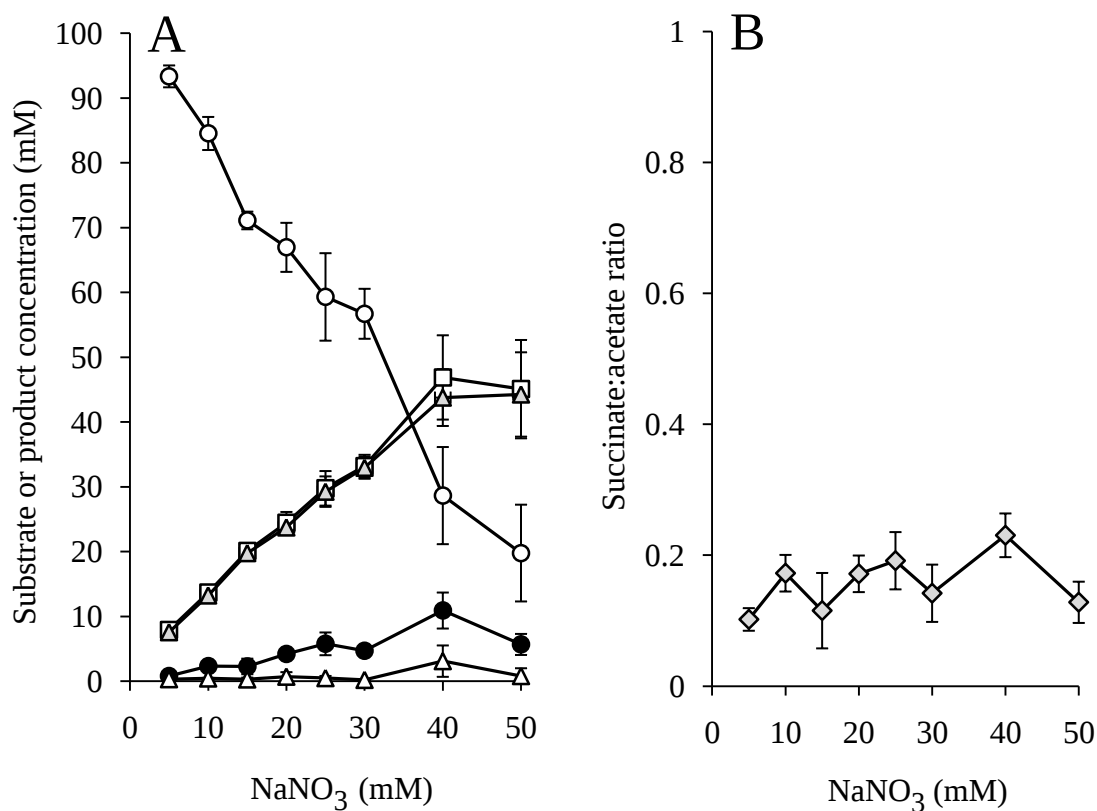


Figure 2.2 Effect of initial NaNO₃ concentration on glycerol consumption and final product distribution in AM3-glycerol-grown *A. succinogenes* 130Z cultures. Supernatant samples were collected 72 h after inoculation. (A) Glycerol consumption and product distribution. (B) Succinate:acetate product ratios of cells grown in the conditions depicted in panel A. Results are the average of three biological replicates $\pm$ standard deviation. Legend: ○, residual glycerol; ●, succinate; □, acetate; △, formate; ▲, CO₂; ◆, S/A ratio.

To further understand *A. succinogenes*'s metabolism under nitrate-respiring conditions, growth and products were tracked during a batch culture grown on AM3-glycerol with 20 mM NaNO_3 . As seen in Figure 2.3A, nitrate is first respired to nitrite, followed by a phase of nitrite reduction. We observed a biphasic growth pattern in which the growth rate was initially fast (generation time of 4.7 ± 0.2 h) during nitrate reduction. Once the nitrate was exhausted (at approx. 12 h), growth slowed down during nitrite reduction to a generation time of 11.7 ± 0.6 h (Figure 2.3B). Growth stopped when the nitrite was completely depleted. Succinate started accumulating only after 12 h of growth (Figure 2.3A), once the nitrate was completely reduced to nitrite. Since succinate production by *A. succinogenes* was low in nitrate and nitrite-respiring conditions, these conditions were not studied further.

Although nitrate-and nitrite-respiring cultures of *A. succinogenes* produce succinate, the yields were lower than expected given glycerol's reduced chemical nature. For that reason other electron acceptors were investigated to increase succinate yields.

2.4.4. DMSO respiration.

A. succinogenes was grown in AM3-glycerol with different initial concentrations of DMSO and culture supernatants were sampled and analyzed by HPLC after 72 h incubation (after growth had stopped). As seen in Figure 2.4A, glycerol consumption and succinate production increased with increasing initial DMSO concentrations. Succinate was the main metabolite produced under these growth conditions, followed by acetate, formate, and CO_2 . As with nitrate-respiring cells, ethanol was not detected with DMSO as the electron acceptor. To test the requirement for bicarbonate in these respiratory conditions, we compared the glycerol consumption and metabolite production by cells grown in the presence of 25 mM and 150 mM

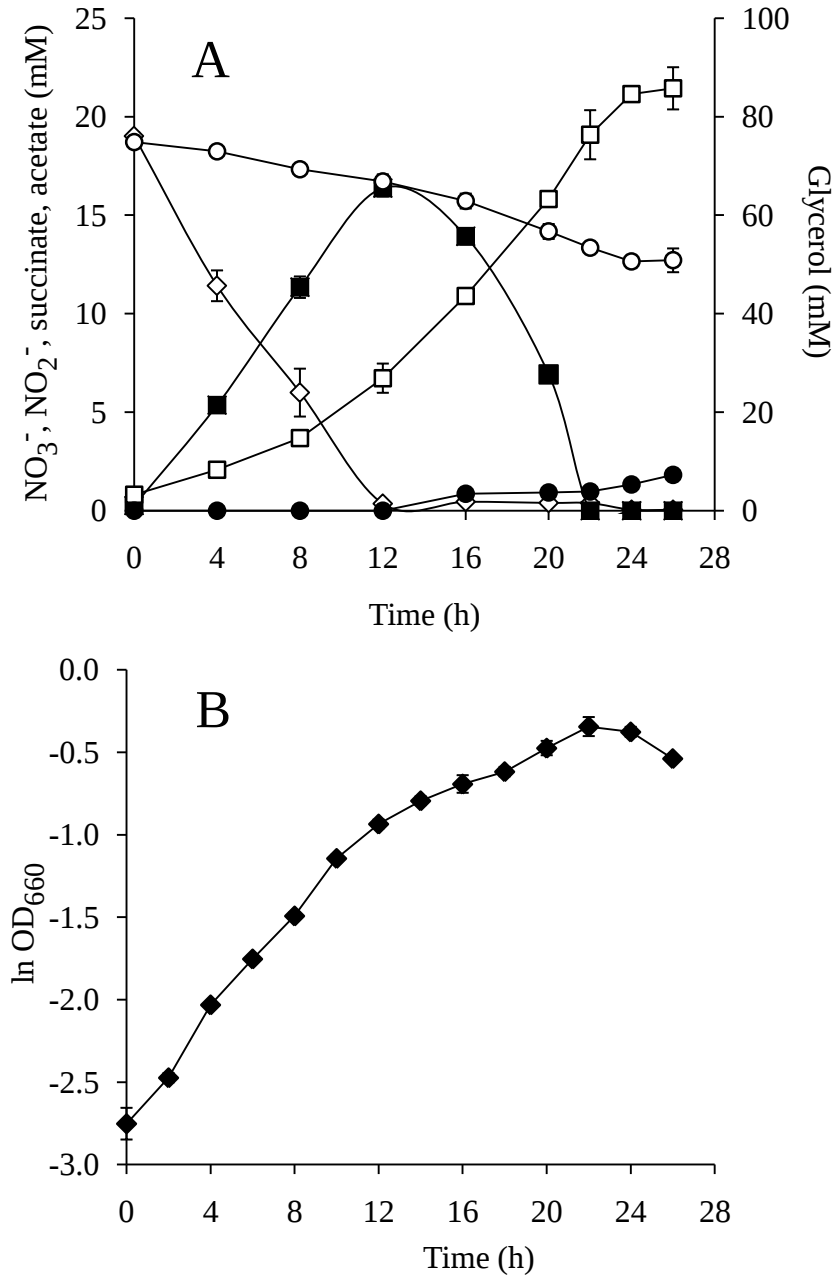


Figure 2.3 Time course of nitrate respiration by *A. succinogenes* grown in 60 mL AM3-glycerol with 20 mM NaNO₃. A) Metabolites. B) Cell growth. Data represent the average of three biological replicates $\pm$ standard deviation. Legend: ○, glycerol; ●, succinate; □, acetate; ◇, nitrate; ■, nitrite; ◆, biomass. CO₂ levels were equimolar to acetate and are not shown for simplicity.

NaHCO₃ (Figures 2.4A and 2.4B, respectively). Twenty five millimolar NaHCO₃ permitted the consumption of up to 45 mM glycerol and the production of up to 30 mM succinate. In contrast, the presence of 150 mM NaHCO₃ permitted the consumption of over 90 mM glycerol, the production of up to 53 mM succinate, and the reduction of 80 mM DMSO (Figure 2.4B). This bicarbonate-limited glycerol consumption and succinate production was also observed for nitrate-respiring cells (data not shown).

In contrast to nitrate-respiring cultures, where the final product distribution was independent of the initial NaNO₃ concentration, in DMSO-respiring cultures the products of glycerol metabolism varied with the initial DMSO concentration. At 150 mM NaHCO₃, the end-point S/A ratio of DMSO-respiring cells was highest with 10 mM DMSO (3.6) and decreased with increasing initial DMSO concentrations (Figure 2.4C). Surprisingly, with 10 mM DMSO initially present, the S/A ratio was higher with 25 mM NaHCO₃ than with 150 mM NaHCO₃.

To further understand *A. succinogenes*'s metabolism under DMSO-respiring conditions, growth and products were tracked during a batch culture grown on AM3-glycerol with 20 mM DMSO. As seen in Figure 2.5, from 0 h to 38 h, DMSO is respired while biomass and metabolites (i.e., succinate, formate, and acetate) accumulate. Growth and metabolite production stop after 38 h of incubation, when DMSO has been completely reduced. Based on the lack of growth after all DMSO was reduced we suggest that 130Z is not able to use dimethyl sulfide (DMS) as a carbon or energy source or terminal electron acceptor. DMSO is stable in liquid form and easily quantified by HPLC. In contrast, DMS is volatile and direct measurements were not included in our HPLC analyses. Regardless, we qualitatively observed DMSO reduction to DMS over time by HPLC (not shown). Figure 2.5B shows the S/A ratio throughout the batch culture. From 0 h to 20 h the S/A ratio increased from 1.1 to 3.8. After 20 h the S/A ratio remained near

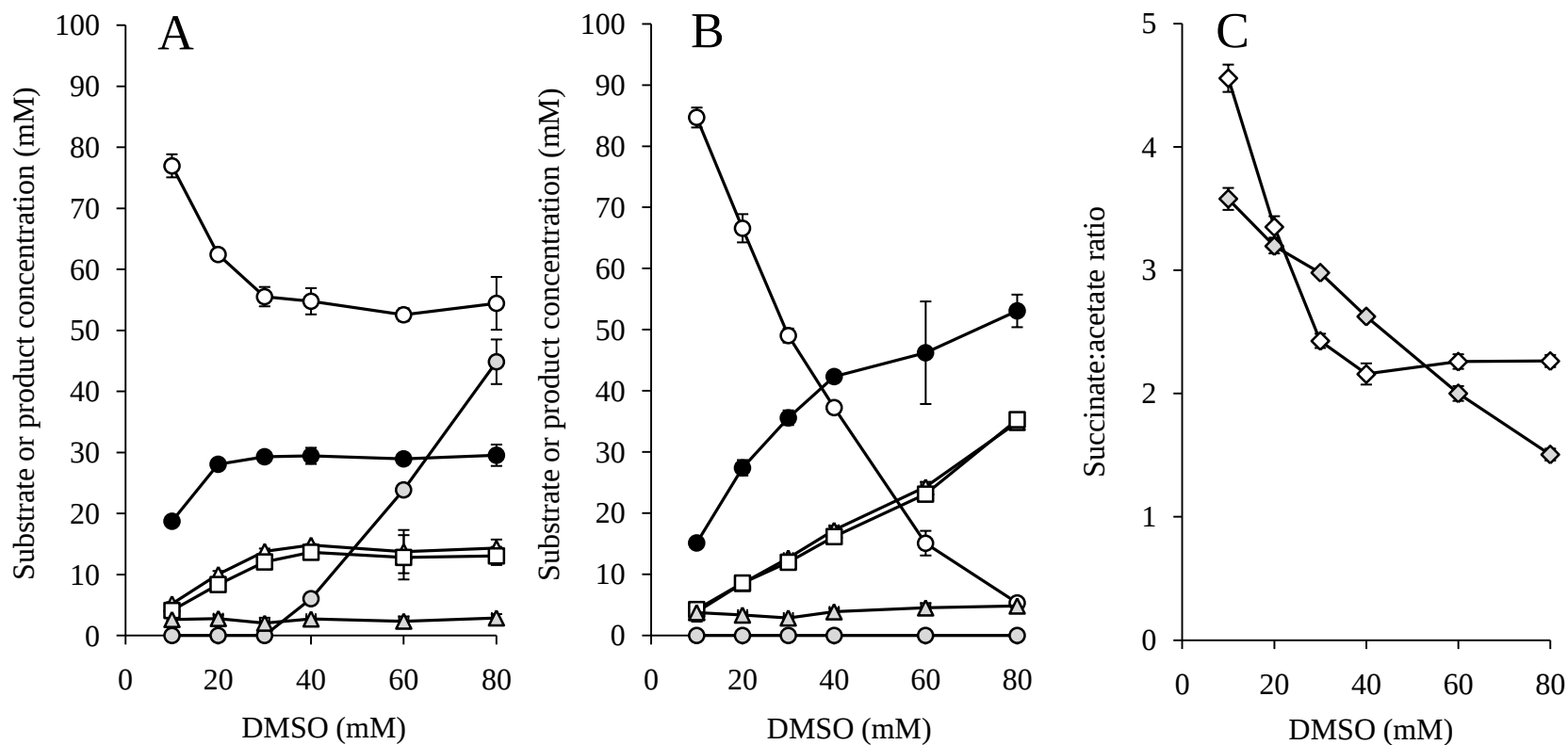


Figure 2.4 Effect of initial DMSO concentration on glycerol consumption and final product distribution in AM3-glycerol-grown *A. succinogenes* 130Z cultures. Supernatant samples were collected 72 h after inoculation. (A) Medium supplemented with 25 mM NaHCO₃. (B) Medium supplemented with 150 mM NaHCO₃. (C) Succinate:acetate product ratios of cells grown in the conditions depicted in panels A and B. Results are the average of three biological replicates $\pm$ standard deviation. Legend: ○, residual glycerol; ●, residual DMSO; ●, succinate; □, acetate; △, formate; ▲, CO₂; ◇, 25 mM NaHCO₃; ◆, 150 mM NaHCO₃.

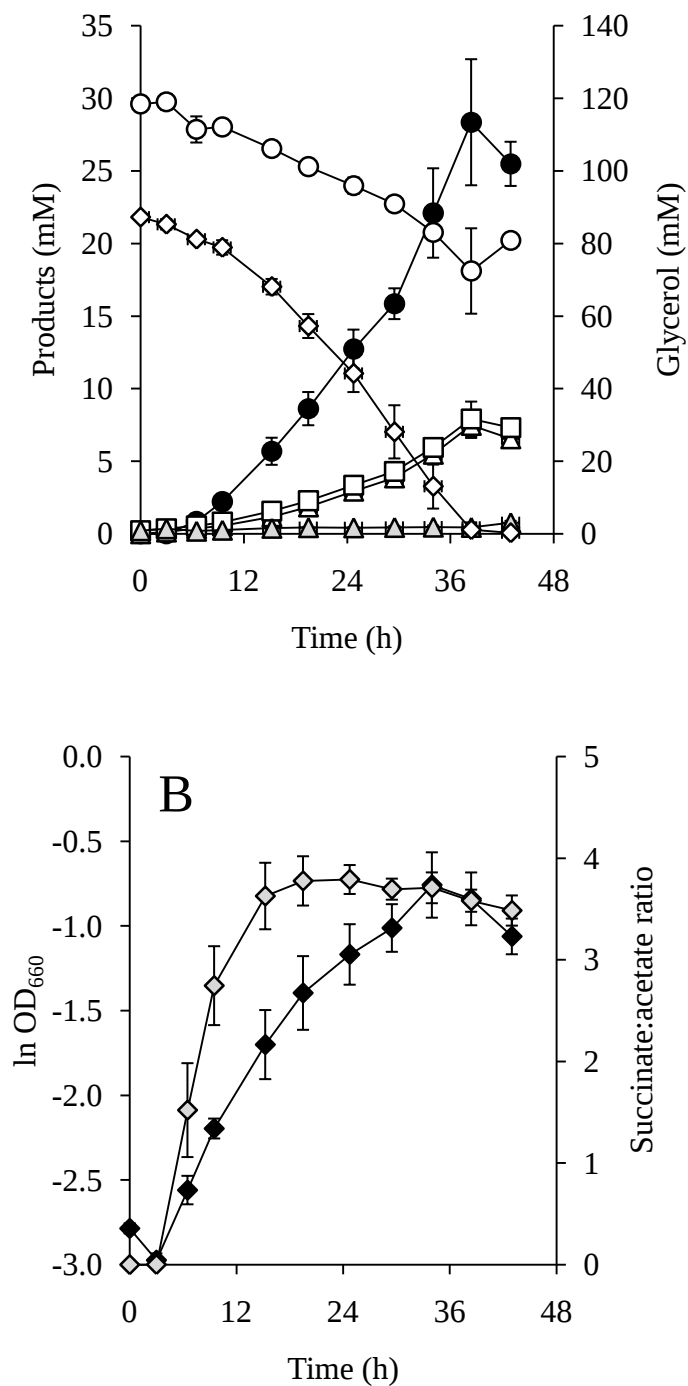


Figure 2.5 Time course of DMSO respiration by *A. succinogenes* grown in 60 mL AM3-glycerol with 20 mM DMSO. A) Metabolites B) Growth and S/A ratio. Data represent the average of three biological replicates $\pm$ standard deviation. Legend: ○, glycerol; ●, succinate; □, acetate; △, formate; ▲, CO₂; ◇, DMSO; ◆, growth; ◇, S/A ratio.

3.8 but slowly decreased to 3.5 by the end of incubation.

Having found DMSO respiration to be favorable to succinate production from glycerol, we compared pure glycerol and glycerols originating from biodiesel manufacture as carbon and energy sources for *A. succinogenes* growth and succinate production. Vegetable glycerol was obtained from the transesterification of vegetable oils. Mixed glycerol was obtained from the transesterification of vegetable and animal fats. Both vegetable and mixed glycerols have been partially purified to remove most of the impurities (i.e., methanol, fatty acids, and soaps). Due to the presence of water and impurities in the process glycerols, we first determined the exact glycerol concentration in these samples. Vegetable glycerol contained 0.67 g glycerol g⁻¹ and mixed glycerol contained 0.56 g glycerol g⁻¹. Growth curves and maximum OD₆₆₀ reached in 20 mM DMSO batch cultures were identical for *A. succinogenes* cultures grown on pure and on vegetable glycerols (not shown). In the presence of 20 mM DMSO, cultures grown on mixed glycerol grew faster than on pure glycerol (they reached their maximum OD₆₆₀ 3 h earlier) and their maximum OD₆₆₀ was 20% higher than on pure glycerol. Since growth never reaches a steady state in batch DMSO-respiring cultures, we are unable to report a specific growth rate. A time course study of the mixed glycerol batch culture revealed that the S/A ratio followed the same trend over time as that seen with pure glycerol (not shown).

The glycerol metabolism of *A. succinogenes* in DMSO-respiring cultures was similar with all three glycerols. Complete end-point carbon and electron balances were calculated using supernatant samples taken at the beginning and end of these cultures (0 h and 43 h). The results, listed in Table 2.2, reflect *A. succinogenes*'s metabolism throughout the entire DMSO-respiring growth period. Results for *A. succinogenes*'s metabolism on vegetable glycerol are not shown since they were nearly identical to those on pure glycerol. As listed in Table 2.2, the molar yields

Table 2.2 Products of glycerol metabolism in 60-mL cultures of *A. succinogenes* grown either with DMSO or microaerobically.

Strain	Electron acceptor	Gly ^a	mmol/100 mmol glycerol consumed							Carbon Recov. (%) ^c	Electron Recov. (%) ^d	S:A ratio
			Products						DMSO reduced			
			Succinate	Formate	Acetate	CO ₂	Pyruvate	Biomass ^b				
130Z	DMSO (20 mM)	P	66 ± 1	17 ± 1	19 ± 1	2.4 ± 0.4	0.0 ± 0.0	26 ± 4	53 ± 2	94 ± 2	96 ± 2	3.4 ± 0.1
130Z	DMSO (20 mM)	M	64 ± 4	21 ± 1	20 ± 1	0.0 ± 0.0	0.0 ± 0.0	52 ± 1	53 ± 2	102 ± 6	105 ± 6	3.3 ± 0.0
130Z	O ₂ (1%)	P	67 ± 4	0.4 ± 0.1	25 ± 0	25 ± 0	0.0 ± 0.0	15 ± 1	N/A ^f	98 ± 5	87 ± 4	2.7 ± 0.1
130Z	O ₂ (1%)	M	62 ± 3	0.8 ± 0.1	34 ± 5	33 ± 5	0.2 ± 0.2	17 ± 4	N/A	104 ± 5	90 ± 4	1.9 ± 0.4
<i>ΔpflB</i>	O ₂ (1%)	P	76 ± 3	0.0 ± 0.0	19 ± 2	19 ± 2	0.0 ± 0.0	15 ± 1	N/A	100 ± 5	93 ± 4	4.0 ± 0.2
<i>ΔpflB</i>	O ₂ (1%)	M	64 ± 6	0.0 ± 0.0	25 ± 3	25 ± 3	3.9 ± 4.5	11 ± 1	N/A	97 ± 7	86 ± 7	2.6 ± 0.3
<i>ΔpflB</i> ^e	O ₂ (0.1%)	P	85 ± 4	0.0 ± 0.0	10 ± 0	10 ± 0	0.0 ± 0.0	10 ± 1	N/A	97 ± 3	96 ± 3	8.7 ± 0.5

Except for 130Z cultures grown under 1% O₂ (two biological replicate cultures), data are means ± standard deviation of at least three biological replicate cultures

^a Glycerol: P, pure; M, mixed.

^b Biomass (mM) was determined using assumed values of 567 mg L⁻¹ dry cell weight per OD₆₆₀ and a cell composition of CH₂O_{0.5}N_{0.2} (24.967 g mol⁻¹) (49).

^c Carbon in product/carbon in glycerol consumed. The assumption was made that one mole of CO₂ was fixed per mole succinate produced (49). Therefore, C₃H₆O₂ was used as the chemical composition of succinate derived from glycerol consumed.

^d Electron recoveries are based on available hydrogen (13).

^e Data for strain $\Delta pflB$ grown under 0.1% O₂ use the time period during which the S/A ratio was highest (34 h to 73 h of incubation, see section 2.4.8 of text).

^f N/A, not applicable.

of succinate, formate, and acetate were very similar on pure and mixed glycerols, with succinate being the main acid produced in both cases. The CO₂ yield of 2.4 ± 0.4 mmol/100 mmol glycerol reported for cultures grown on pure glycerol is based on the difference between acetate and formate productions rather than on a direct measurement of CO₂. It is possible that this small CO₂ yield is an experimental artifact, and that acetate and formate are instead produced in equimolar concentrations (without CO₂ production) from pure glycerol as is observed on mixed glycerol. The ratio of DMSO reduced/glycerol consumed was also similar between the different glycerols. One seemingly large difference between the cultures grown on pure and mixed glycerol is the biomass yield on pure glycerol, which appears to be half of that on mixed glycerol. *A. succinogenes* cultures grown on pure glycerol with 20 mM DMSO typically reached a maximum OD₆₆₀ of 0.80 starting from an initial OD₆₆₀ of 0.05. In the experiment shown in Figure 2.5, the triplicate cultures grown on pure glycerol had growth curves similar to those of other biological replicates grown on other days until late in the culture. At the later stages of the cultures shown in Figure 2.5, cells started to aggregate and made OD₆₆₀ measurements highly variable. This observation is illustrated by the fact that average glycerol consumption and metabolite production had standard deviations below 3.5%, whereas the average biomass produced had a standard deviation above 15% (based on samples taken at times 0 h and 43 h). If we use the OD₆₆₀ values of cultures grown on days when cells did not aggregate (OD₆₆₀ 0.75), the biomass yield (45 ± 1 mmol/100 mmol glycerol) on pure glycerol becomes much closer to that on mixed glycerol (52 ± 2 mmol/100 mmol glycerol), and the carbon and electron recoveries increase to $100 \pm 2\%$ and $103 \pm 2\%$, respectively.

2.4.5 Activity of terminal oxidoreductases during anaerobic respiratory glycerol metabolism.

To explain the large differences in succinate production by nitrate- and DMSO-respiring *A. succinogenes* cultures, the *in vitro* activities of fumarate reductase and other terminal oxidoreductases were tested under these different respiring conditions. The activities of various terminal oxidoreductases are known to be transcriptionally regulated by the Fnr, ArcAB, Fis, and NarQP systems to maximize energy production (46). This hierarchical control of terminal reductases was evident in *A. succinogenes* cultures (Table 2.3). Maximal nitrate and DMSO reductase activities were measured in nitrate-grown and DMSO-grown cultures, respectively. Maximal fumarate reductase activity was observed in anaerobic glucose cultures, followed by glycerol-DMSO cultures. Surprisingly, nitrite reductase activity was higher in glycerol-DMSO cultures than in glycerol-nitrate cultures.

Table 2.3. Activity of terminal oxidoreductases in *A. succinogenes* crude cell extracts from various culture conditions.

Growth condition	Specific activity ($\mu\text{mol}/\text{min mg protein}$)			
	DMSO	Fumarate	Nitrate	Nitrite
	reductase	reductase	reductase	Reductase
Glucose (aerobic)	0.05 ± 0.03	1.10 ± 0.40	2.32 ± 0.50	7.71 ± 2.35
Glucose (anaerobic)	0.15 ± 0.01	3.97 ± 0.08	2.56 ± 0.29	10.1 ± 0.4
Glycerol-NaNO_3 (early)^a	0.22 ± 0.03	1.62 ± 0.05	13.8 ± 2.5	47.9 ± 9.0
Glycerol-NaNO_3 (late)^b	0.05 ± 0.03	1.35 ± 0.23	6.49 ± 0.72	28.3 ± 3.3
Glycerol-DMSO	0.32 ± 0.05	2.80 ± 0.43	12.7 ± 3.7	41.2 ± 11.5

^aEarly phase of culture, during which nitrate is reduced to nitrite (Concentrations at sampling time: $[\text{NO}_3^-] = 18.8 \pm 0.5 \text{ mM}$; $[\text{NO}_2^-] = 8.2 \pm 0.4 \text{ mM}$)

^bLate phase of culture, when all nitrate is depleted and nitrite is being reduced to ammonia (Concentrations at sampling time: $[\text{NO}_3^-] = 0.1 \pm 0.1 \text{ mM}$; $[\text{NO}_2^-] = 15.5 \pm 3.7 \text{ mM}$)

2.4.6. Microaerobic growth on glycerol.

While collecting preliminary data for nitrate-respiring *A. succinogenes*, cultures were grown in 96-well plates within an anaerobic chamber. To our surprise, control cultures containing 15 mM NH_4Cl but no NaNO_3 produced as much succinate as cultures grown with 10 mM NaNO_3 (1.3 mM), but showed negligible growth (i.e., 20 times less biomass production than with 10 mM NaNO_3). The S/A ratio in the absence of NaNO_3 was also much higher (3.4) than in the presence of 10 mM NaNO_3 (0.1). A gas chromatography measurement of the chamber's gas composition estimated the O_2 concentration to be under 2%, and an O_2 monitor in the chamber gave readings below 150 ppm (0.015%). To test if microaerobic conditions could support growth of *A. succinogenes* on glycerol, we inoculated *A. succinogenes* into a 28-mL serum tube with 10 mL AM3-glycerol and replaced 1.6 mL of the N_2 headspace with air to create a 2% O_2 concentration in the headspace. After 15 h of incubation, the culture density had increased from OD_{660} 0.03 to 0.15. Three additional exchanges of the headspace with air caused the cells to grow to OD_{660} 0.33 (data not shown). The S/A ratio after four air exchanges was 1.7 with 5 mM succinate produced. These results prompted us to study the aerobic glycerol metabolism of *A. succinogenes*.

A fermenter experiment was performed in which *A. succinogenes* was grown in AM3-glycerol under 2% O_2 . The inlet gas composition was 90% CO_2 and 10% air, with rapid gas inflow and agitation. Under these conditions, *A. succinogenes* grew almost as fast as when fermenting glucose (2-h doubling time), glycerol eventually became growth-limiting, and acetate and CO_2 were the only products formed (not shown). 130Z lacks a complete tricarboxylic acid cycle (27, 30) explaining why, even under aerobic conditions, acetate was excreted to produce

ATP. This series of preliminary experiments suggested that only microaerobic conditions (i.e., O₂ concentrations below 2%) promote succinate production by *A. succinogenes*.

Growth of *A. succinogenes* on glycerol at 1% O₂ was studied in a microaerobic chamber. The composition of the chamber was 5% air and 95% CO₂ (or 95% CO₂, 4% N₂, and 1% O₂). CO₂ was chosen as the background gas to prevent the loss of bicarbonate from the cultures. When N₂ was used as the background gas growth was limited by the availability of bicarbonate after it had been depleted from the medium as CO₂ (not shown).

A. succinogenes's growth and products were tracked during batch cultures under 1% O₂ with pure glycerol as the substrate (Figure 2.6). After a brief lag phase, growth was exponential between 3 h and 9 h (growth rate of 0.20 h⁻¹) but then steadily slowed down until 48 h (Figure 2.6B), where growth completely stopped. Monitoring of the dissolved O₂ concentration in these growth conditions showed that dissolved O₂ quickly dropped to undetectable levels within 5 h after inoculation (data not shown). As expected, succinate was the predominant product. Unlike DMSO-respiring cells, which produced nearly equal concentrations of acetate and formate, acetate and CO₂ were produced in near equimolar amounts under 1% O₂ with almost no formate production. As with the other respiratory conditions tested ethanol was not produced.

As observed with DMSO-respiring cultures, metabolite production changed over time under 1% O₂ (Figure 2.6A and Figure 2.6C). From time 0 h to time 40 h the S/A ratio gradually increased from 0 to 2.8. In other words, as the culture OD₆₆₀ increased, the cells produced more succinate and less acetate. Although we tested 4 biological replicates, for clarity, data shown in Figure 2.6 represent the average of only two biological replicates (cultures 1 and 4). During the first 24 h of incubation the four cultures had similar growth curves and similar metabolite outputs. However, beyond 24 h incubation we observed two different phenotypes. Cultures 1 and

4 consumed all of the available glycerol within 90 h (Figure 2.6A) and reached maximum S/A ratios of 2.7 and 3.1. Cultures 2 and 3 stopped consuming glycerol after 66 h of incubation, with 40 and 55 mM glycerol remaining, and reached maximum S/A ratios of 3.1 and 1.7, respectively. The fermentation balance of cultures 1 and 4 grown under 1% O₂ is shown in Table 2.2. With one exception, results from cultures grown under 1% O₂ were calculated from supernatant samples analyzed at the beginning of the culture (0 h) and after all glycerol was consumed (90 h). Since cultures of strain $\Delta pflB$ grown on pure glycerol consumed all glycerol after only 66 h, the 66 h supernatant sample was used instead. Supernatant samples from later time points were not used since succinate concentrations began decreasing after glycerol was depleted. The microaerobic succinate yield (0.67 ± 0.04 mol/mol glycerol) was not significantly different from DMSO-respiring cells. However, instead of equimolar acetate and formate production as with DMSO, *A. succinogenes* produced 98% less formate than acetate under microaerobic conditions and instead produced CO₂.

We next compared the microaerobic growths and metabolisms of *A. succinogenes* when grown on pure and mixed glycerols. Unlike DMSO-respiring cultures, in which *A. succinogenes*'s overall glycerol metabolism was similar regardless of glycerol purity, microaerobic cultures (under a 1% O₂ headspace) had slightly different product yields on pure and mixed glycerols (Table 2.2). The S/A ratio on pure glycerol (2.7 ± 0.1) was higher than that seen on mixed glycerol (1.9 ± 0.6): the succinate yield was ~8% lower and the acetate and CO₂ yields were ~30% higher in cultures grown on mixed glycerol than in cultures grown on pure glycerol.

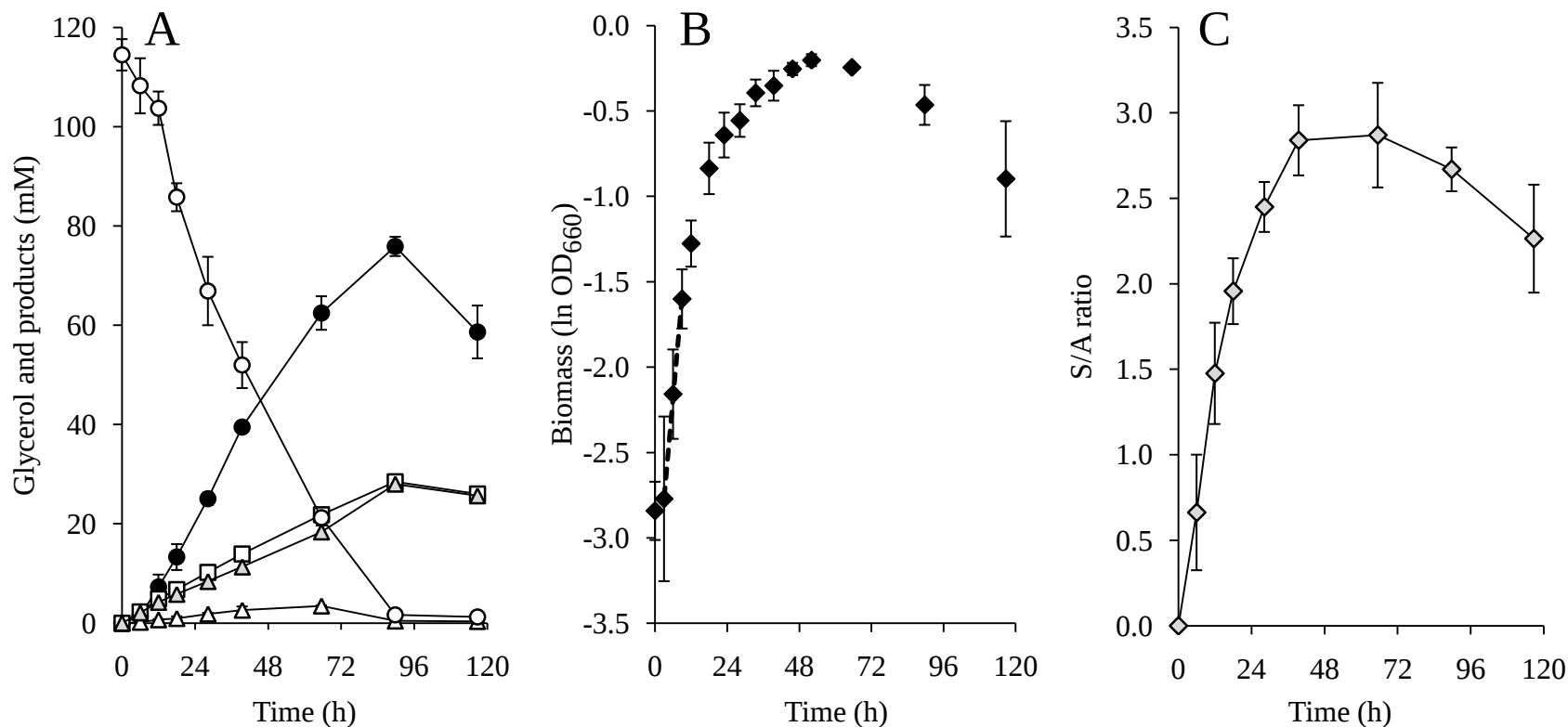


Figure 2.6 Time course of microaerobic respiration by *A. succinogenes* in AM3-glycerol with a 1.0% O₂ headspace. A) Metabolites, B) Cell growth, C) S/A ratio. Data were obtained from 60-mL cultures and are the averages of 2 biological replicates $\pm$ standard deviation. Legend: $\circ$, glycerol; $\bullet$, succinate; $\triangle$, formate; $\square$, acetate; $\blacktriangle$, CO₂; $\blacklozenge$, biomass; $\blacklozenge$, S/A ratio. The dotted line in panel B shows the linear fit of exponential-phase growth from 3 h to 9 h (least-squares method) (11). The equation for this line is $\ln OD_{660} = 0.20 t - 3.34$ ($R^2 = 0.999$).

2.4.7. Reducing co-products and increasing succinate yields by inactivating *pflB*.

Pyruvate formate lyase (PFL) was initially targeted for inactivation because the formate generated during *A. succinogenes* glycerol-DMSO metabolism contributed to a significant loss of carbon that would otherwise be available for succinate production. We also anticipated, based on studies with *M. succiniciproducens* (24) and *E. coli* (56) that PFL inactivation would reduce carbon flux to acetate. Indeed, *A. succinogenes* $\Delta pflB$ produced no formate, had an increased S/A ratio, and had an increased succinate yield from glycerol in both DMSO-respiring and microaerobic conditions (Table 2.2).

We were surprised to find that strain $\Delta pflB$ was sensitive to DMS production, as cells aggregated and lysed very early in batch cultures grown with 20 mM DMSO. This sensitivity made presenting full carbon balances difficult. However, in cultures grown with 10 mM DMSO, strain $\Delta pflB$ had a succinate yield of 0.70 ± 0.02 mol succinate/mol glycerol from pure glycerol. Under 1% O₂, strain $\Delta pflB$ had a succinate yield 13% higher and an S/A ratio 48% higher than strain 130Z (Table 2.2). As described earlier for strain 130Z, these values represent the strain's metabolism over a range of O₂ tensions in which *A. succinogenes* briefly reached an optimal condition for succinate production. The best succinate yield observed with strain $\Delta pflB$ was with cultures grown with pure glycerol under 0.1% O₂. After 34 h incubation the culture has an S/A ratio of 6.8. The S/A ratio reached 7.8 at 73 h (data not shown). The growth balance of this strain between 34 h and 73 h of incubation is shown in Table 2.2. During this time, the culture's acetate, CO₂, and biomass yields were the lowest observed in this work and the succinate yield was 85% of the maximum theoretical yield. Incredibly, of the glycerol consumed during this time period, only 3.3% was used for biomass production. These results suggest that cell density and O₂ tension could be optimized in a continuous culture to maximize succinate production.

2.5 DISCUSSION

Although not the first report of succinate production from glycerol by a natural succinate-producing bacterium (23, 38), this study is the first to use comparative genomics and a defined minimal medium to investigate the important role of respiration in the process. Manual annotation of the *A. succinogenes* genome justified the use of external electron acceptors, since *A. succinogenes* cannot produce 1,3-PD, the best-known metabolic determinant of glycerol fermentations. Furthermore, the near 100% carbon and electron recoveries of DMSO-respiring cultures grown in AM3 defined medium demonstrates the need for external electron acceptors.

A. succinogenes, like many facultative anaerobic bacteria, is able to reduce a variety of electron acceptors. Unlike *E. coli*, though, which has multiple enzyme complexes for the reduction of nitrate, nitrite, and oxygen (9, 35), we did not find redundancy in the respiratory chains of *A. succinogenes* (i.e., one complex for each electron acceptor). It is interesting to note that *A. succinogenes*'s genome encodes the high affinity cytochrome *bd* oxidase (*cydAB*), which is expressed by *E. coli* under hypoxic conditions (45), and not the low-affinity cytochrome *bo* oxidase, which is expressed under higher O₂ tensions. This observation is not surprising since *A. succinogenes* has only been found in the rumen, where O₂ tensions are below 2 μM dissolved O₂, (40). *A. succinogenes*'s genome encodes the enzymes for respiratory nitrate ammonification and not for denitrification. This observation was expected since respiratory nitrate ammonification is the predominant nitrate respiratory mechanism in the bovine rumen microbiota (19). As observed for cytochrome *bd* oxidase, *A. succinogenes*'s genome encodes the high affinity, nitrate-scavenging nitrate reductase (Nap), presumably giving *A. succinogenes* a growth advantage during nitrate-limiting conditions in the rumen (36). While Guettler *et al.* were

the first to demonstrate nitrate reductase and oxidase activities in *A. succinogenes* (14), this report is the first to show that *A. succinogenes* can respire DMSO.

The differences in succinate production between nitrate-respiring, DMSO-respiring, and microaerobic cultures is likely due to the combined effect of respiratory pathway stoichiometry, availability of reducing equivalents, and redox potentials. When grown in AM3-glycerol- NaNO_3 *A. succinogenes* produces succinate as a minor product. Most of the succinate produced in these growth conditions accumulates in the nitrite-reducing phase, after nitrate is completely depleted. The reduction of nitrate to ammonia is a two-step process that oxidizes two reducing equivalents. In the second, nitrite-reducing, growth phase, more reducing equivalents become available for succinate production compared to the first stage of growth. Unlike the reduction of nitrate to ammonia, the reductions of DMSO to DMS and of O_2 to H_2O are single reduction steps that consume only one reducing equivalent, thus more reducing equivalents are available for succinate production.

The redox potential of O_2 to H_2O ($E_o' = +820$) is higher than that of nitrate reduction to nitrite ($E_o' = +420$ mV) nitrite reduction to NH_4^+ ($E_o' = +360$), DMSO reduction to DMS ($E_o' = +160$), and fumarate reduction to succinate ($E_o' = +30$). The relative *in vitro* activities of terminal reductases in *A. succinogenes* grown with different terminal electron acceptors reflect the fact that *A. succinogenes* likely controls flux to terminal reductases to maximize growth efficiency. For example, in O_2 - and nitrate-respiring cultures, the activity of fumarate reductase was half of that found in DMSO-respiring cultures or in anaerobic glucose cultures (Table 2.3). This low fumarate reductase activity suggests that *A. succinogenes* represses expression of the *frdABCD* operon in the presence of electron acceptors with high redox potentials (i.e., O_2 , NO_3^- , and NO_2^-)

We expected that nitrite reductase activity in nitrate-grown cultures would be higher, or at least the same, in the absence of nitrate as it was in the presence of nitrate. However, the nitrite reductase activity was 59% lower after nitrate was depleted (Table 2.3). This result can be supported by earlier observations by Wang and Gunsalus (50) who found that *E. coli* nitrite reductase (*nrfA*) expression was highest at low nitrite levels (< 2 mM), decreased as nitrite concentration increased to 6 mM, and remained low at nitrite concentrations above 6 mM. We suspect that *A. succinogenes* has a similar response to nitrite concentrations, since nitrate-grown cultures showed higher nitrite reductase activity at 8.2 ± 0.4 mM nitrite (early growth phase) than at 15.5 ± 3.7 mM nitrite (late growth phase).

Using batch cultures to study *A. succinogenes*'s respiratory glycerol metabolism demonstrated the organism's ability to change its catabolism in response to the availability of external electron acceptors. It is generally understood that facultative anaerobic bacteria will modify their respiratory and catabolic capabilities to maximize energy production (48). DMSO and fumarate are both low-potential electron acceptors. While the transcription of DMSO and fumarate reductases is somewhat repressed in the presence of O_2 , nitrate, or nitrite, there is no evidence of preferential transcription of one versus the other in the absence of higher potential electron acceptors. For example, *E. coli* fumarate reductase and DMSO reductase activities are similar in cultures grown on (i) glycerol-DMSO and (ii) glycerol-DMSO-fumarate (3). We propose that the difference in the S/A ratio between conditions of high and low DMSO availability is due to the combined effect of the relative kinetics of fumarate reductase and DMSO reductase and of the relative redox potentials of DMSO/DMS and fumarate/succinate. High DMSO concentrations favor the recycling of excess reducing equivalents through DMSO reductase and decrease flux through the C4 pathway to succinate. As DMSO is depleted and

biomass accumulates, the availability of DMSO per cell decreases over time and more reducing equivalents get recycled through fumarate reductase, thus increasing the ratio of succinate production to DMSO reduction.

The behavior of *A. succinogenes* under microaerobic conditions is similar to its behavior in the presence of DMSO. For example, when the O₂ tension is high, as in the first 10 h of growth under a 1.0% O₂ headspace, the S/A ratio is below 1.0. As the cell density increases and less O₂ is available per cell, the cells presumably respire less O₂ to maintain redox balance and the S/A ratio increases to ~3.0. These changes in metabolite production at different O₂ tensions have been well characterized in *E. coli* by Alexeeva *et al.* (1), who highlighted the changes in fluxes to acetate, formate, and ethanol across a range of aeration conditions from anaerobic to aerobic.

We were surprised by the similarity in succinate yields between DMSO-respiring and microaerobic cultures since DMSO-respiring cultures had a higher S/A ratio. This discrepancy can be explained by the observation that under microaerobic conditions the biomass yield was 67% lower than during DMSO respiration (calculated from a 20 mM-DMSO-grown culture without cell aggregation), but the acetate yield was 32% higher. The difference in S/A ratio between the two growth conditions suggests that, while the S/A ratio is useful to compare succinate production in cultures grown with the same external electron acceptor, it may not be suitable to compare conditions with different acceptors. To explain the difference in biomass yields we propose that, compared to DMSO-respiring cultures, microaerobic cultures develop an internal redox state closer to that observed in fermentative conditions in which biomass yield is low compared to respiratory conditions.

One clear difference between DMSO-respiring and microaerobic cultures is in the formate and CO₂ production. DMSO-respiring cultures produced nearly equimolar concentrations of acetate and formate with minimal CO₂ production, whereas microaerobic cultures produced nearly equimolar concentrations of acetate and CO₂ with minimal formate production. Flux from pyruvate to acetyl-CoA can go through either pyruvate dehydrogenase (PyrDH) or PFL (and then possibly formate dehydrogenase, ForDH).

In *E. coli*, transcription and *in vivo* activity of PFL are both controlled by the amount of O₂ in the environment (20, 37). PFL is strongly sensitive to O₂. By comparing the growth of *E. coli* PFL and PyrDH mutants Durnin *et al.* (11) showed that PFL is the main pyruvate-dissimilating enzyme active under microaerobic conditions. The same results were observed by Zhang *et al.* (56). Both studies showed that PFL mutants consumed less glycerol and produced more lactate under microaerobic conditions compared to the wild-type strain. The difference in metabolite production between the wild-type and mutant strains demonstrated that PyrDH is not the only pyruvate-dissimilating enzyme active during microaerobic glycerol metabolism. It is important to note that it can be difficult to compare results from microaerobic studies due to the technical difficulty in replicating dissolved O₂ tensions (1).

PyrDH activity (and to a large degree its synthesis) is controlled by the cell's internal redox state (the NADH/NAD⁺ ratio) in *E. coli*. As the NADH/NAD⁺ ratio increases during the transition from aerobic to anaerobic conditions, PyrDH is inactivated and PFL becomes activated (37). Thus, under microaerobic conditions, depending on the O₂ tension, flux from pyruvate goes through both PyrDH and PFL (1).

The high formate/CO₂ ratio in DMSO-respiring *A. succinogenes* cultures suggests that nearly all pyruvate-dissimilating flux goes through PFL. The low formate/CO₂ ratio in

microaerobic cultures suggests three possibilities for the flux to acetyl-CoA: (i) it goes through PFL with subsequent ForDH flux, (ii) it goes mainly through PyrDH with little-to-no PFL flux, or (iii) it is shared between PFL and PyrDH with moderate ForDH flux. All three possibilities lead to CO₂ release and conservation of reducing power (i.e., NADH) for succinate production. Strain *ΔpflB* had higher S/A ratios than strain 130Z under both 0.1% and 1% O₂ atmospheres as well as with both pure and mixed glycerol. These results can be best explained by possibilities (i) and (iii), but not (ii). If flux through PFL is absent in strain 130Z grown under microaerobic conditions then the product distribution of strain *ΔpflB* would have been the same as strain 130Z. This was not the case. Regardless of the exact distribution of pyruvate dissimilating flux in strain 130Z, it is clear that PyrDH does not completely compensate for the loss of PFL activity in strain *ΔpflB*. The result is a ~25% lower pyruvate-dissimilating flux, less acetate production, and more succinate production in strain *ΔpflB*.

Both *A. succiniciproducens* and *B. succiniciproducens* required an unidentified component of yeast extract for growth on glycerol but produced high yields of succinate (23, 38, 39). *A. succinogenes* differs from these two organisms, since we did not observe significant glycerol consumption when AM3-glycerol was supplemented with 10 g L⁻¹ yeast extract in the absence of any added external electron acceptor.

A. succinogenes produces ethanol as a product of glucose fermentation (7–9 mmol ethanol/100 mmol glucose) (30, 49). In all conditions tested, *A. succinogenes*'s respiratory glycerol metabolism produced no detectable ethanol. *adhE* expression in *E. coli* is transcriptionally repressed under conditions of nitrate respiration, and it is likely influenced by the internal NADH/NAD⁺ ratio (25). On another hand, Alexeeva *et al.* (1) demonstrated ethanol production by *E. coli* in continuous culture systems maintained at O₂ tensions below 53%

aerobiosis (100% aerobiosis is the minimum O₂ concentration required for complete oxidation of substrate to CO₂). This observation may point to a difference in the control of *adhE* in *A. succinogenes* and *E. coli*, which produces ethanol as the main product of microaerobic glycerol metabolism (0.66 mol/mol glycerol) (11).

The ultimate aim of this work is to develop a process for the conversion of crude glycerol to succinate. Crude glycerols of different origins can vary widely in composition, especially in methanol and fatty acid concentrations. For this reason, we focused first on mixed glycerol (from Michigan Biodiesel, LLC), the composition of which is more easily standardized than that of crude glycerol. Mixed glycerol is 99% free of fatty acids and methanol and costs \$0.04-0.12 lb⁻¹ (average \$0.05 lb⁻¹, John Oakley, Michigan Biodiesel, LLC). The S/A ratios of DMSO-respiring cultures were the same with both pure and mixed glycerol. To our surprise, though, the overall S/A ratios were higher on pure glycerol than on mixed glycerol in microaerobic cultures. This result is most clear in strain $\Delta pflB$ grown under both 1% and 0.1% O₂. The S/A ratios are nearly identical in samples taken 12 h after inoculation. After 12 h (1% O₂) and 22 h (0.1% O₂) the S/A ratio of cultures grown on pure glycerol increases significantly above the ratio of cultures grown on mixed glycerol (Table 2.2). Which components of the mixed glycerol affect succinate production in each of these conditions will be investigated in future studies. Because *A. succinogenes* did not grow on mixed glycerol in the absence of O₂ or DMSO, the unidentified components are unlikely to act as a terminal electron acceptor. However, they may be amino acids or other growth factors that allow cells to grow faster and to a higher OD₆₆₀ on mixed glycerol than on pure glycerol.

Under optimized conditions, with either DMSO or O₂ as the electron acceptor, *A. succinogenes* produces succinate from glycerol in high yields, highlighting the fact that *A.*

succinogenes's ability to produce succinate is intrinsic to this organism, and not just limited to fermentative conditions. *E. coli*, under conditions optimal for succinate production (i.e., 100 mM KHCO_3), produces only 0.19 mol succinate per mol glucose under anaerobic conditions (16) and 0.25 mol succinate per mol glycerol microaerobically (56). These differences suggest that the tendency of bacteria to produce succinate fermentatively can be extrapolated to their ability to produce succinate from glycerol via respiration.

The research groups of Dr. Ramon Gonzalez (Rice University) and Dr. Lonnie Ingram (University of Florida) have made significant progress in establishing *E. coli*-based platforms for the conversion of glycerol to ethanol and succinate, respectively (54, 56). In three genetic engineering steps, Zhang *et al.* (56) built *E. coli* strain XZ721, which is able to microaerobically produce succinate from glycerol with a 0.80 molar yield. First, the *pckA* promoter was mutated to increase PEP carboxykinase expression. Second, the *GldA*-*DhaKLM* route for glycerol oxidation was inactivated, forcing glycerol oxidation through the respiratory *GlpK*-*GlpABC* pathway. These first two engineering steps changed the *E. coli* central metabolism to more closely resemble that of *A. succinogenes*, which lacks the *gldA* gene (thus the *gldA-dhaKLM* pathway) and naturally has high PEP carboxykinase activity (49). Finally, *pflB* was inactivated to eliminate formate production and decrease ethanol and acetate production. This triple mutant's succinate yield resembles that of *A. succinogenes* strain $\Delta pflB$ under microaerobic conditions. Strain XZ721 required 6 days to metabolize 128 mM glycerol (56). This result is very similar to what we observed for strains 130Z and $\Delta pflB$ grown under a 0.1% O_2 headspace.

We demonstrated that genetic engineering of *A. succinogenes* can improve succinate yield from glycerol. The $\Delta pflB$ strain produced no formic acid, had an increased S/A ratio, and had an increased succinate yield from glycerol in both DMSO-respiring and microaerobic

cultures. We expect the $\Delta pflB$ mutation to be the first step in engineering an *A. succinogenes* strain that can produce succinate from glycerol at near theoretical yields.

This study is the first to demonstrate several metabolic features of *A. succinogenes* including: (1) *A. succinogenes* can use DMSO, nitrate, nitrite, and oxygen as external electron acceptors for respiratory growth with glycerol as the sole carbon source; (2) *A. succinogenes* can completely reduce nitrate to ammonia as the primary nitrogen source for biomass production; (3) the succinate yield on glycerol is strongly influenced by the nature and concentration of external electron acceptors; and (4) genetic manipulations can reduce flux in competing pathways and increase succinate yields. Although we used mainly pure glycerol in this work, we also demonstrated that *A. succinogenes* can microaerobically convert partially-refined crude glycerol, an inexpensive and abundant feedstock, to succinate in high yields. More work is required to demonstrate the industrial importance of these findings, namely, to demonstrate that the microaerobic process can be scaled up to increase productivity while maintaining high succinate yields. In all, we have demonstrated that with further engineering and process optimization studies, *A. succinogenes* is a promising biocatalyst for the conversion of glycerol to succinate.

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Chapter 3

A markerless gene knockout method for *Actinobacillus succinogenes* 130Z based on natural transformation

The work completed in this chapter is part of a collaboration between Rajasi Joshi (MMG professional masters graduate), Nikolas McPherson (Genetics Ph.D. student in Dr. Vieille's lab), and myself to develop genetic tools for *A. succinogenes*. Rajasi Joshi has previously submitted a report, "Development of a gene knockout method for *A. succinogenes* strain 130Z and engineering of *A. succinogenes* for aspartate production", to her committee to satisfy the professional master's degree requirements. Her report described the use of natural transformation to create a fumarate reductase knockout mutant, $\Delta frdAB$, and additional engineering efforts to create an *A. succinogenes* strain that produces aspartate during fermentation. I trained, supervised, and worked with Rajasi to complete her project.

My contributions to her report included:

- Testing the effectiveness of over-expressing the *E. coli* isocitrate dehydrogenase (*icd*) gene in *A. succinogenes* to complement its glutamate auxotrophy when grown in defined medium lacking glutamate and supplemented with isocitrate
- Training and mentoring Rajasi Joshi during her entire research project in the lab, as part of the completion of her Masters degree in Industrial Microbiology.
- Collaborating to construct an *FRT-icd-FRT* selectable marker cassette for insertion into genes targeted for knockout. The *FRT* sequences are the recognition sites for the *Saccharomyces cerevisiae* flippase recombination system, for selection marker removal.
- Supervising the design and construction of a truncated *A. succinogenes* fumarate reductase operon interrupted with the *FRT-icd-FRT* selectable marker cassette. In this construct, the *FRT-icd-FRT* cassette replaces the *frdAB* genes in the *frdABCD* operon. The $\Delta frdAB::icd$ construct was used to knockout the wild-type fumarate reductase operon

in *A. succinogenes* via natural transformation (the first knockout mutant of *A. succinogenes* constructed).

- Confirming the reproducibility of the knockout method and marker-removal techniques by constructing a markerless $\Delta pflB$ mutant in *A. succinogenes*.

The chapter below describes my use of the natural transformation approach to construct the *A. succinogenes* markerless $\Delta pflB$ mutant. Strain $\Delta pflB$ was characterized for succinate production from glycerol in Chapter 2.

3.1 ABSTRACT

Actinobacillus succinogenes is one of the best known natural succinate-producing organisms. Much is already known about its central metabolism, including how intracellular fluxes are distributed during fermentative growth on glucose and how these fluxes are influenced by environmental factors such as the availability of CO₂ and reducing power. The complete genome sequence of *A. succinogenes* is publically available and has been manually annotated. Currently, the design of genetic tools for *A. succinogenes* is the most pressing hurdle to overcome to gain a deeper understanding of the organism's metabolism and to enable metabolic engineering for increased succinate production. In this study we devised a gene knockout strategy for *A. succinogenes* using natural transformation to introduce linearized DNA into the cells. The isocitrate dehydrogenase gene (*icd*) from *Escherichia coli* was used as a selection marker, enabling positive selection of recombination events based on the glutamate auxotrophy of *A. succinogenes*. After successful deletion of the target gene, we employed the *Saccharomyces cerevisiae* flippase recombinase to remove the *icd* marker, enabling its re-use. With the aim of increasing fermentative succinate yields, the *A. succinogenes pflB* gene (encoding pyruvate formate-lyase, PFL) was targeted for deletion. Compared to the wild-type strain, the $\Delta pflB$ strain produced 69% more succinate (in mol/mol glucose) with reduced acetate and ethanol production, and no longer produced formate.

3.2 INTRODUCTION

Succinic acid tops the US's DOE list of value-added chemicals from biomass (44). If produced economically by fermentation, succinic acid could replace maleic anhydride as the precursor to many bulk and commodity chemicals, with a potential market value of \$15 billion (47). *Actinobacillus succinogenes* 130Z, a member of the *Pasteurellaceae* family, is one the best known natural succinate-producing bacteria, able to produce high levels of succinic acid as part of a mixed acid fermentation. Biobased succinate will only be price-competitive with oil-based maleic anhydride if alternative fermentation products can be reduced or eliminated, achieving close to a homosuccinate fermentation. Such fermentation would reduce the cost of downstream succinate purification.

Maximizing succinate production by *A. succinogenes* will require not only optimizing environmental conditions but also eliminating fermentation pathways that compete with succinate production (e.g., gene knockouts) and adjusting promoter strengths to control enzyme expression levels (e.g., gene insertions). Such genetic manipulations require genetic tools amenable to *A. succinogenes*. Until recently, genetic tools available for *A. succinogenes* were limited to an expression vector and electroporation to introduce the plasmid into cells (17). This plasmid, pLGZ920, was constructed from an *Escherichia coli*-*Actinobacillus pleuropneumoniae* shuttle vector, pGZRS-19 (45). pLGZ920 confers ampicillin resistance, replicates in *A. succinogenes*, is stable in *E. coli* after addition of the ColE1 origin of replication, and permits high-level expression of foreign genes in *A. succinogenes* from the strong *A. succinogenes* *pckA* promoter (*p_{pckA}*) (17). Electroporation of *A. succinogenes* with pLGZ920 yields transformants with an efficiency of 10^4 to 10^6 CFU/ μ g of plasmid, depending on the electroporation parameters ((11), Schindler and Vieille, unpublished results).

Several different approaches have been used to build gene knockouts in other *Pasteurellaceae*. Conjugation was used to create an unmarked gene knockout mutant of *A. pleuropneumoniae* (29). This method used a shuttle vector containing a partially deleted copy of the *A. pleuropneumoniae* target gene next to the Tn903 kanamycin resistance marker and the *Bacillus subtilis* levansucrase gene (*sacB*) under the control of the *A. pleuropneumoniae omlA* promoter. *sacB* is a powerful counterselectable marker commonly used on plasmid vectors to select for double recombinations and knockout mutations. In *sacB* counterselection bacteria harboring the *sacB* gene are killed in a medium containing sucrose (34).

Two approaches have been developed to construct gene knockout mutants in *Mannheimia succiniciproducens*, *A. succinogenes*'s closest known relative. In the first approach, suicide vectors containing the *sacB* gene plus a gene knockout construct interrupted by kanamycin, chloramphenicol, and spectinomycin resistance genes were electroporated into *M. succiniciproducens*. After positive selection for antibiotic resistance to select for recombination events, sucrose counterselection was used to select for double recombination events and loss of the native chromosomal gene (23).

In the second approach, the *sacB*-containing suicide vector electroporated into *M. succiniciproducens* contained gene knockout constructs interrupted by a *loxP*-Km^R-*loxP* cassette (16). Following sucrose counterselection of Km^R colonies, the mutant strains were electroporated with a temperature sensitive *E. coli*-*M. succiniciproducens* shuttle vector for expression of the P1 phage Cre recombinase (20) to remove the Km^R marker (16). When expressed, Cre excises the DNA sequence flanked by two *loxP* sites, leaving a single *loxP* site as a scar. Lastly, the shuttle vector was cured from *M. succiniciproducens* by growth at non-permissive temperatures.

A temperature sensitive shuttle vector has also been used to create gene knockouts in *Mannheimia haemolytica*, *Pasteurella multocida*, and *Haemophilus somnus* (40). The plasmid contained both a gene knockout construct interrupted by a Km^R marker and a temperature sensitive, *Pasteurellaceae*-specific, origin of replication (6). Double crossover mutants were selected for growth at temperatures not permissive to plasmid replication and in the presence of kanamycin.

Besides electroporation and conjugation, another method to introduce foreign DNA into bacterial cells is natural transformation. During natural transformation, bacteria take up DNA from the environment and either degrade the DNA as a nutrient source or incorporate it into their chromosome by homologous recombination. Transformation frequencies can be as high as 10⁻² (24). Natural transformation is well suited for strain engineering, since it works best with linear DNA (e.g., PCR constructs), which requires double recombination events for complete chromosome integration.

DNA uptake by natural transformation in *Pasteurellaceae* is dependent on the recognition of an uptake signal sequence (USS) by cell surface DNA uptake proteins (10). USS repeats are composed of a 9-bp conserved core sequence (5'-AAGTGCGGT-3') followed by two AT-rich regions (33). To date, natural competence is the only function known for USS repeats (24). Among the completed *Pasteurellaceae* genomes, the density of USS repeats ranges from 0.12 to 0.84 USS/kb (0.73 USS/kb in *A. succinogenes*) with the total number of USS repeats ranging from 41 to 1,760 (1,690 in *A. succinogenes*) (25). Although all *Pasteurellaceae* genomes contain USS repeats, not all *Pasteurellaceae* species are naturally competent (24).

Among the *Pasteurellaceae*, natural transformation is best understood in *H. influenzae* (reviewed in (24)). DNA uptake into the periplasm is initiated in *H. influenzae* when outer

membrane-bound DNA uptake complexes recognize and bind to a USS. A single strand of linearized DNA then enters the cytoplasm through a channel in the cytoplasmic membrane, while the other strand is degraded. Finally, the internalized DNA either integrates into the chromosome or is degraded. Starvation stress induces competence in *H. influenzae*. Competence genes are expressed when growing cells are transferred from a rich medium into an induction medium containing only salts and amino acids.

Since the discovery of natural transformation, it has been used in many genetic studies of *H. influenzae* and related *Pasteurellaceae* found to be naturally competent. For example, a transposon-mutagenesis screen was used to identify essential genes in *H. influenzae* (46). Natural transformation was also used to create a *pilA* mutant of nontypeable *H. influenzae* strain 86-028NP to understand the function of a previously uncharacterized *H. influenzae*-expressed type IV pilus (2) and to test the requirement for a novel *H. influenzae* cell-surface protein for adhesion to lung epithelial cells (35). Besides *H. influenzae*, natural transformation has been used for genetic manipulations in the following *Pasteurellaceae*: *A. pleuropneumoniae* (4), *Haemophilus parasuis* (3), and *Aggregatibacter actinomycetemcomitans* (43). Although not targeted for gene knockouts, many *Haemophilus parainfluenzae* strains are naturally competent (13, 28).

Early attempts to construct knockout mutants of *A. succinogenes* by allelic exchange used electroporation of cells with suicide vectors. The suicide vectors contained gene knockout constructs interrupted by ampicillin or chloramphenicol resistance genes. These attempts were hampered by the high frequency of spontaneous antibiotic resistance mutants compared to the low frequency of double recombination events, and no knockout mutants were isolated in these studies (McKinlay and Vieille, unpublished results). To develop a knockout method for *A. succinogenes*, it thus seemed to be important to use another selection method and another means,

besides electroporation, to introduce DNA into cells. Although useful in the construction of knockout mutants in *M. succiniciproducens* (23), the closest known relative to *A. succinogenes*, expression of *sacB* in *A. succinogenes* did not confer sensitivity to sucrose and thus cannot be used as a counterselection method in this organism (Schindler and Vieille, unpublished results).

A. succinogenes's genome contains twenty three of the twenty five genes found in *H. influenzae*'s natural competence regulon (25, 32). The missing two genes encode hypothetical proteins with unknown roles in natural competence (24). Among the genes present in *A. succinogenes* are those encoding the competence regulatory proteins Sxy and CRP (the cyclic AMP receptor protein) as well as other proteins that are known to participate in DNA uptake or recombination (i.e., RecA, TopA, AtpA, and DsbA). The frequency of USS repeats in *A. succinogenes*'s genome and the likely presence of a complete natural competence machinery suggest that *A. succinogenes* is naturally competent.

Metabolic engineering often requires producing multiple knockout and/or gene insertion mutations within the same strain, thus it is essential to have a way to remove the positive selection marker for its re-use. It may also be important for production strains to have markerless, non-polar mutations. To these ends, we used the Flp recombinase of the *S. cerevisiae* 2 μ M plasmid (encoded by *flp*) (9). This recombinase catalyzes the recombination between two Flp recognition target sites (*FRT* sites, 5'-

GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC-3') (48). When the *FRT* sequences are in the same orientation, recombination results in the excision of the DNA region between the sites. The Flp/*FRT* recombinase system has been used in many different bacteria including *E. coli* (7) and *Mycobacterium smegmatis* (38). This recombinase method is similar to the Cre-lox method used in the genetic engineering of *M. succiniciproducens* (16).

The goal of this study is to determine if natural competence can be used to create gene knockout mutants of *A. succinogenes*. Success will aid future engineering endeavors to improve the succinate-producing performance of the bacterium. The *A. succinogenes pflB* gene (encoding pyruvate formate-lyase, PFL) was targeted for deletion to increase succinate yields during fermentative growth. Production of acetate and ethanol by *A. succinogenes* begins with the cleavage of pyruvate into acetyl-coenzyme A (CoA) and either formate (via PFL) or CO₂ (coupled to the reduction of NAD⁺, via the pyruvate dehydrogenase complex, PDH). From acetyl-CoA, acetate is produced through phosphotransacetylase and acetate kinase (producing one ATP), and ethanol is produced through alcohol dehydrogenase (36). During fermentative growth of *A. succinogenes* on glucose, most of pyruvate conversion to acetyl-CoA is catalyzed by PFL (26). PDH activity under these growth conditions is low, further suggesting low flux through this metabolic pathway (26, 42). During growth of *A. succinogenes* in defined medium 35-61% of the carbon in glucose is excreted as formate, acetate, and ethanol (depending on the level of bicarbonate provided) (27). To reduce the flux to these products and increase succinate yields, PFL was targeted for inactivation.

3.3 MATERIALS AND METHODS

3.3.1 Strains, media, culture conditions, and chemicals. All strains used in this study are listed in Table 3.1. *E. coli* strains were cultivated in Luria-Bertani (LB) broth and on LB agar plates (1). *A. succinogenes* strains were cultivated in Medium B (in g L⁻¹: yeast extract, 5; bactotryptone, 10; NaH₂PO₄ · H₂O, 8.5; K₂HPO₄, 15.5; NaHCO₃, 2.1; and glucose, 9) or AM3 defined medium (27). AM3 is a phosphate-based medium containing glucose, NH₄⁺, ten vitamins, and the amino acids glutamate, cysteine, and methionine. After natural transformations, *A. succinogenes* was grown in AM2 (AM3 minus glutamate) in the presence or absence of isocitrate (30 mM; AM2-isocitrate). Unless stated otherwise, all liquid cultures were incubated at 37°C with shaking at 250 rpm. *A. succinogenes* was grown anaerobically in N₂-flushed Hungate tubes. To isolate single colonies, *A. succinogenes* strains were grown under a CO₂-enriched atmosphere on LB agar plates supplemented with glucose (10 g L⁻¹) (23). *A. succinogenes* is naturally resistant to kanamycin and 10 µg mL⁻¹ kanamycin was included in agar plates to maintain culture purity. In all growth media, ampicillin was added for plasmid maintenance when required (40 µg mL⁻¹ for *A. succinogenes* and 100 µg mL⁻¹ for *E. coli*). For the preparation of naturally competent cells, *A. succinogenes* was grown in Bacto brain heart infusion (BHI; Becton-Dickinson and Co., Franklin Lakes, NJ), washed in AM3 phosphate buffer (per liter: NaH₂PO₄ · H₂O, 8.5 g; K₂HPO₄, 15.5 g), and re-suspended in MIV medium (28). cAMP (2 mM) was added to MIV medium to induce competency genes. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

3.3.2 Plasmids, DNA manipulations, and transformations. All plasmids used in this study are listed in Table 3.1. PCR products were cloned into pCR2.1 TOPO using the TOPO-TA kit

Table 3.1 Strains and plasmids and primers used in this study

	Description	Source
<i>E. coli</i>		
K-12	Wild-type strain	Laboratory collection
TOP10	(rk ⁻ , mk ⁺) <i>phoA supE44</i> λ - thi-1 <i>gyrA96 relA1 F- mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139</i> Δ (<i>ara-leu</i>) 7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i> λ -	Invitrogen (Carlsbad, CA)
<i>A. succinogenes</i>		
130Z (ATCC 55618)	Wild-type strain	ATCC
<i>ΔpflB::icd</i>	<i>ΔpflB::icd</i> , 130Z derivative	This study
<i>ΔpflB</i>	<i>ΔpflB</i> , 130Z derivative	This study
Plasmids		
pCR2.1 TOPO	Amp ^R , Km ^R , <i>lacZα</i> , cloning vector	Invitrogen
pCR2.1- <i>ΔpflB</i>	pCR2.1 derivative, <i>pflB</i> deletion (2-kb fusion product of sequences directly upstream and downstream of <i>pflB</i>)	This study
pCR2.1- <i>icd</i>	pCR2.1 derivative, <i>E. coli icd</i> under control of the <i>A. succinogenes pckA</i> promoter, and flanked by <i>FRT</i> repeats	This study
pCR2.1- <i>ΔpflB::icd</i>	pCR2.1 derivative, <i>A. succinogenes</i> <i>ΔpflB::FRT-icd-FRT</i>	This study
pLGZ920	<i>E. coli-A. succinogenes</i> shuttle vector; Amp ^R ; <i>pckA</i> promoter	(17)
pLGZ924	pLGZ920 derivative, <i>E. coli icd</i> under control of the <i>pckA</i> promoter	This study
pCP20	pSC101 derivative, <i>flp</i> ⁺ , λ cI857 ⁺ , λ p _R Rep ^{ts} , Amp ^R , Cm ^R	(7)
pCV933	pLGZ920 derivative, <i>S. cerevisiae flp</i> under control of the <i>A. succinogenes pckA</i> promoter	Joshi <i>et al.</i> , unpublished

(Invitrogen, Carlsbad, CA). Plasmid pLGZ920, a shuttle vector between *A. succinogenes* and *E. coli*, was used to express foreign proteins in *A. succinogenes* under control of the strong, constitutive, *A. succinogenes* *pckA* promoter (17). One Shot TOP10 chemically competent *E. coli* cells were transformed as described by the manufacturer. Plasmids were introduced into *A. succinogenes* by electroporation. Electrocompetent *A. succinogenes* cells were prepared by a method modified from a 10-min microcentrifuge protocol (8). A 10-mL culture of actively growing *A. succinogenes* ($OD_{660} 0.5 \pm 0.2$) in Medium B or AM3 was incubated on ice for 10 min to stop growth. Six milliliters of culture were distributed into four pre-chilled microcentrifuge tubes and harvested by centrifugation (3 min at $4,500 \times g$) at 4°C . The cell pellets were each washed three times with 1 mL of cold 272 mM sucrose and finally re-suspended in a combined total volume of 100 μL of 272 mM sucrose. For electroporation, a mixture of DNA (1 μL , 100–300 ng) and 100 μL of electrocompetent cells was transferred to a 2-mm gap width electroporation cuvette (Bio-rad, Hercules, CA). After electroporation (settings: 25 μF , 400 Ω , and 2.5 kV on a Bio-Rad GenePulserTM), 0.5 mL of room-temperature super optimal broth with catabolite repression (SOC) outgrowth medium (New England Biolabs, Ipswich, MA) was added to the cuvette, and cells were transferred to a 1.5-mL microcentrifuge tube and shaken horizontally for 1 h at 37°C . The cells were then centrifuged (3 min at $4,500 \times g$), re-suspended in 100 μL supernatant, and spread on a single plate. Colonies were visible within 48 h at 37°C .

PCRs were performed with the Advantage HD polymerase kit (Clontech, Mountain View, CA) unless otherwise stated. Restriction enzymes were purchased from New England Biolabs. Genomic DNA extractions were performed using the Wizard genomic DNA purification kit, plasmid DNA was purified using the Wizard miniprep kit, and DNA fragments were

recovered from PCR mixtures and agarose gels using the Wizard SV gel and PCR Clean-Up System (Wizard kits purchased from Promega, Madison, WI). All oligonucleotide primers used in this work are listed in Table 3.2. Except for primers P7 and P8 (ordered from Integrated DNA Technologies, Coralville, IA), all primers were synthesized by the Michigan State University Research Technology Support Facility (MSU RTSF). PCR and cloning accuracy were confirmed by DNA sequencing performed by the MSU RTSF. DNA manipulations (e.g., restriction digests, PCR, ligations) used standard protocols (1). Colony PCR was performed to confirm cloning steps. The PCR mixture (20 μ L) contained 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 50 μ M of each dNTP, 1.25 μ M of each primer, 0.025 U/ μ L Taq polymerase (Invitrogen), and a bacterial colony as the source of template DNA.

3.3.3 Construction of pLGZ924 for expression of *E. coli* isocitrate dehydrogenase in *A. succinogenes*.

The isocitrate dehydrogenase gene (*icd*) was amplified from *E. coli* K-12 genomic DNA using primers P9 and P10 and Taq polymerase. Primer P9 included an *Xba*I restriction site for cloning into pLGZ920. The PCR product was first cloned into pCR2.1-TOPO, then subcloned into the *Xba*I and *Sac*I restriction sites of pLGZ920, downstream of the *A. succinogenes* *pckA* promoter (*p_{pckA}*), yielding plasmid pLGZ924 (Table 3.1).

3.3.4 Construction of a Δ *pflB::icd* mutant.

The *E. coli* *icd* gene under control of *p_{pckA}* was amplified with primers P7 and P8 using pLGZ924 as the template. The primers were designed to flank the *p_{pckA}-icd* cassette with *Asc*I restriction sites plus *S. cerevisiae* *FRT* sequences in the same 5'-3' orientation. The PCR mixture (20 μ L) contained 1X Advantage HD buffer, 50 μ M of each dNTP, 1.25 μ M of each primer, 100 ng of pLGZ924, and 0.025 U/ μ L polymerase. PCR

Table 3.2 Oligonucleotide primers used in this study

Primer	Sequence ^a (restriction site)	Specificity (direction ^b)
P1	CGTTAACCGTGGGAATCAGT	<i>pflB</i> -up (F)
P2	TTACGTTACCCCAGGCGCGCCCTTCCTTTTGCTAGTATTGATAATGA (<i>AscI</i>)	<i>pflB</i> -up (R)
P3	GCGCGCCTGGGGTAACGTAATAAAAATGTAATG (<i>AscI</i>)	<i>pflB</i> -down (F)
P4	TCTCTCCTTCGCGGAATAAA	<i>pflB</i> -down (R)
P5	TGAGCCTGACTGGTAAATCCA	<i>pflB</i> -fusion nested (F)
P6	CACATCGACCCCGATAACTT	<i>pflB</i> -fusion nested (R)
P7	GAGGCGCGCCGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGA ACTTCTCGATAAATTGAAAATGCAGCAA (<i>AscI</i>)	<i>AscI</i> - <i>FRT</i> - <i>p_{pckA}</i> (F)
P8	CTGGCGCGCCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGA ACTTCTTACATGTTTTTCGATGATCGC (<i>AscI</i>)	<i>icd</i> - <i>FRT</i> - <i>AscI</i> (R)
P9	GTTCTAGAGATGGAAAGTAAAGTAGTTG (<i>XbaI</i>)	<i>E. coli</i> K-12 <i>icd</i> gene (F)
P10	CTGAATTCATTACATGTTTTTCGATGATC (<i>EcoRI</i>)	<i>E. coli</i> K-12 <i>icd</i> gene (R)
P11	AATTTGCGGATCTGGATGTC	217 bp upstream of <i>pflB</i> (F)
P12	TGGTGTATTCCGTCAGTTCG	146 bp downstream of <i>pflB</i> (R)
P13	GAAGGTGATGGAATCGGTGT	<i>E. coli</i> K-12 <i>icd</i> (F)
P14	GTTACGGTTTTTCGCGTTGAT	<i>E. coli</i> K-12 <i>icd</i> (R)
P15	ATGAGGTGATCTAGATGCCACAATTTGGTATATTATGTAAA (<i>XbaI</i>)	<i>S. cerevisiae flp</i> (F)
P16	CGGCCAGTGAATTCGAGCTCTTATATGCGTCTATTTATGTAGG (<i>SacI</i>)	<i>S. cerevisiae flp</i> (R)
P17	CGTTGTAAAACGACGGCC	pLGZ920 (F)
P18	AATTTTAAATTATCAATGAGGTG	pLGZ920 (R)

^a 5' to 3' direction; restriction sites are underlined and named at the end of the primer sequences

^b F, forward primer; R, reverse primer

cycling was as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 2 min; and a final extension at 72°C for 5 min. The PCR product (1.6 kb, *AscI*-*FRT*-*p_{pckA}*-*icd*-*FRT*-*AscI*) was cloned into pCR2.1, yielding pCR2.1-*icd*. Since Advantage HD Polymerase has 3'-5' exonuclease activity, PCR products are not amenable to TA (TOPO) cloning. Therefore, prior to TOPO cloning, the PCR reaction was heated to 95°C for 20 min, and 6 µL of 2 mM dATP, and 0.05 U of Taq polymerase were added, and the mixture was incubated at 70°C for 15 min. This adenine-adding protocol was repeated as needed for other cloning steps.

A $\Delta pflB$ construct was assembled in pCR2.1 using a two-step PCR-fusion approach modified from (39). In the first step, two 1.4-kb DNA fragments, each containing the genomic regions upstream and downstream of the *A. succinogenes pflB* gene (*pflB*-up and *pflB*-down, respectively) were amplified separately using primer pairs P1-P2 and P3-P4 respectively. As depicted in Figure 3.1, Primer P2 shares sequences with both the *pflB*-up and *pflB*-down regions to allow fusion of the two regions in the second round of PCR (fusion PCR). After agarose gel electrophoresis, the *pflB*-up and *pflB*-down PCR fragments were purified and used as templates in the fusion PCR reaction, together with nested primers P5 and P6. The 2.1-kb PCR product contained *pflB*-up and *pflB*-down linked by an *AscI* site. This product was cloned into pCR2.1, yielding plasmid pCR2.1- $\Delta pflB$. Finally, the *AscI*-*FRT*-*p_{pckA}*-*icd*-*FRT*-*AscI* fragment from pCR2.1-*icd* was cloned into the *AscI* restriction site of pCR2.1- $\Delta pflB$, yielding pCR2.1- $\Delta pflB::icd$. After restriction digest with *XmnI*, the 7.6-kb linear plasmid DNA was naturally transformed into *A. succinogenes* to create a *pflB* knockout mutant.

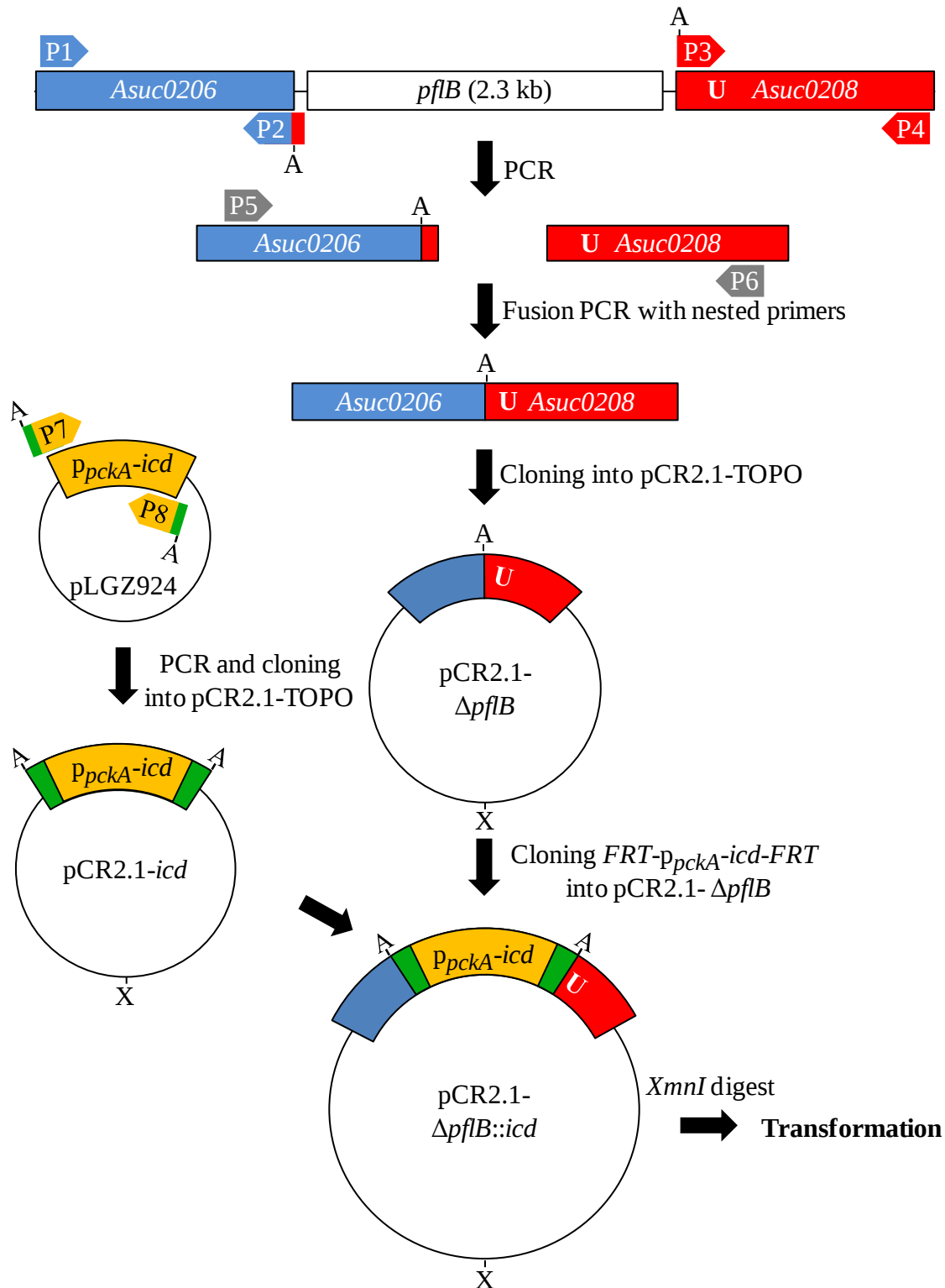


Figure 3.1. Construction of plasmid pCR2.1- $\Delta pflB::icd$ for *pflB* mutagenesis. Not drawn to scale. A, *AscI* restriction site; X, unique *XmnI* restriction site, U, USS repeat. Green bands represent *FRT* sites.

3.3.5 Natural transformation. The natural transformation protocol for *A. succinogenes* closely resembles those for *H. influenzae* (31) and *A. pleuropneumoniae* (5). Four hundred microliters of an overnight culture of *A. succinogenes* in BHI were transferred to fresh BHI (35 mL) and grown to an OD₆₆₀ of 0.2–0.3. Cells were washed twice with AM3 phosphate buffer, re-suspended in 10 mL of anaerobic MIV medium containing 2 mM cAMP, and incubated at 37°C for 100 min with shaking at 100 rpm to induce competence. A mixture of 1 µg DNA (< 75 µL) and 1 mL competent cells was incubated in a 37°C with shaking for 25 min. A separate transformation without DNA was included as a negative control. Two volumes of BHI were added to the transformation mixtures and incubated at 37°C with shaking (250 rpm) for 100 min. Cells were then harvested (4,500 × *g*, 4°C, 15 min) and washed twice with 0.75 mL AM3 phosphate buffer. Finally, 0.25 mL of cell suspension was inoculated into one tube each of AM3 (positive control), AM2 (negative control), and AM2-isocitrate (selective medium) liquid medium. All tubes were incubated at 37°C with shaking until growth was observed in AM2-isocitrate medium.

3.3.6 Removal of the *icd* marker. The *S. cerevisiae flp* gene was cloned into pLGZ920 for expression in *A. succinogenes*. Primers P15 and P16 were used to amplify the *flp* gene using plasmid pCP20 as the template. PCR cycling was as follows: an initial denaturation step at 95°C for 5 min was followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 2 min; and a final extension step at 72°C for 5 min. The PCR product was purified and cloned between the *Xba*I and *Sac*I sites of pLGZ920 using the In-Fusion cloning system (Clontech). The cloning reaction mixture was diluted 5 times and 2 µL was transformed into *E. coli* TOP10. The resulting plasmid, pCV933, was purified and electroporated into the *A. succinogenes* $\Delta pflB::icd$ strain. Transformants were screened for

excision of the *icd* marker (and one *FRT* site) by colony PCR using primer pairs P11-P12 (specific for sequences outside of the *pflB* gene) and P13-P14 (specific for internal sequences in *icd*). Excision of the *icd* marker yielded strain $\Delta pflB$ (pCV933), which contains a single *FRT* scar in the chromosome.

3.3.7 Curing of pCV933 from *A. succinogenes* $\Delta pflB$. An overnight culture of *A. succinogenes* $\Delta pflB$ (pCV933) in medium B was used to inoculate a series of medium B tubes supplemented with acridine orange at 0, 10, 50, 100, and 200 $\mu\text{g mL}^{-1}$. After 6.5 h of incubation (37°C), isolation streaks were performed from each tube onto LB-glucose-Kanamycin agar plates. The resulting colonies were patched onto LB-glucose-ampicillin plates to screen for the loss of the plasmid (pCV933 contains an Amp^R marker). Amp^s colonies were screened for plasmid curing by PCR using primers P17 and P18, specific for the pLGZ920 backbone sequences upstream and downstream of the multiple cloning site. PCR cycling conditions were the same as used to amplify the *flp* gene.

3.3.8 Measuring growth rates and determining fermentation balances. Cells (0.25 mL) from overnight cultures of *A. succinogenes* grown in AM3 (containing 25 mM NaHCO₃) were inoculated into fresh AM3 medium (10 mL final volume in 28 mL test tubes, containing 150 mM NaHCO₃). Growth was tracked by measuring OD₆₆₀ with a DU650 spectrophotometer (Beckman, Fullerton, CA). OD₆₆₀ values were used to determine growth rates and to calculate biomass concentrations for use in carbon and electron balances. Glucose and fermentation product concentrations in culture supernatants were determined by HPLC (Waters, Milford, MA) using a 300 × 7.8 mm Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 30°C with 4 mM

H₂SO₄ as the mobile phase (flow rate of 0.6 mL/min). Glucose and ethanol were detected with a Waters 410 differential refractometer. Organic acids were detected with a Waters 2478 UV detector at 210 nm.

3.4 RESULTS

3.4.1 Developing a reliable positive selection method for recombination events. Initial efforts to delete *A. succinogenes* chromosomal genes involved electroporating cells with suicide vectors and detecting recombination events by selection for antibiotic resistance acquired from the gene knockout construct. Unfortunately, no knockout mutants were isolated, likely due to the high frequency of spontaneous ampicillin or chloramphenicol resistance mutants, which hindered the detection of low frequency double recombination events (McKinlay and Vieille, unpublished results). To overcome this obstacle we sought to develop a cleaner, more reliable positive selection method for *A. succinogenes* based on its metabolism.

A. succinogenes is auxotrophic for the amino acids glutamate, cysteine, and methionine (27). *A. succinogenes* is missing two enzymes of the oxidative tricarboxylic acid cycle, citrate synthase and isocitrate dehydrogenase (ICD), and is unable to synthesize α -ketoglutarate (α KG), the precursor to glutamate. *A. succinogenes* has glutamate dehydrogenase activity and is able to grow on α KG instead of glutamate, though (27). We proposed that if *A. succinogenes* had ICD activity, the bacterium would grow when glutamate in AM3 medium was replaced with isocitrate. To test this hypothesis, the *E. coli icd* gene was cloned into pLGZ920 to create plasmid pLGZ924. 130Z(pLGZ924) did grow in AM2-isocitrate (Figure 3.2), while cells lacking pLGZ924 did not (data not shown). As shown in Figure 3.2, higher concentrations of isocitrate were required to achieve growth yields in the same range as that of *A. succinogenes* 130Z in AM3. The concentration of glutamate in AM3 is 1.4 mM. Even with 5.7 mM or 11.4 mM L-isocitrate (provided as 11.4 mM and 22.8 mM DL-isocitrate, respectively), growth yields in AM2-isocitrate were still 25% lower than that of strain 130Z in AM3. Figure 3.2 is a representation of the isocitrate concentration-dependent growth of 130Z(pLGZ924) in AM2-

isocitrate. It should be noted that while this trend was observed for three independent experiments performed on a single day, growth experiments performed on other days led to different growth yields from each initial concentration of DL-isocitrate (data not shown). This variability could reflect differences in *icd* expression levels based on plasmid copy number. We suspect that a higher growth yield resulted from high expression of *icd* and higher in vivo ICD activity, reducing the amount of isocitrate being diverted away from glutamate synthesis.

Besides a difference in growth yield, we also observed a difference in metabolite production between strains 130Z and 130Z(pLGZ924). The molar ratio of succinate to acetate produced (S/A product ratio) is a commonly used measure of succinate production performance and an estimate of the internal redox state of succinate-producing organisms (22, 23). During growth in AM3 containing 25 mM NaHCO₃, the S/A product ratios of strains 130Z and 130Z(pLGZ924) are both 0.8 ± 0.0 . However, in AM2-isocitrate containing 11.36 mM DL-isocitrate, strain 130Z(pLGZ924) has an S/A product ratio of 0.4 ± 0.0 . This result is surprising considering that the pathway from isocitrate to glutamate is redox neutral and CO₂-generating (Figure 3.2), which should have no impact on the internal redox state of the cells. Regardless of the mechanism, the decrease in S/A ratio in AM2-isocitrate with strain 130Z(pLGZ924) is evidence that, while AM2-isocitrate is appropriate for selection of homologous recombination events in *A. succinogenes*, this medium is not appropriate for maximum succinate production.

3.4.2 Natural transformation of *A. succinogenes* to interrupt the *pflB* gene. Unlike many other naturally competent bacteria, which efficiently take up DNA from various sources, members of the *Pasteurellaceae* preferentially take up DNA from their own species over unrelated DNA (33, 37). This mechanism is due to specific binding of the membrane DNA

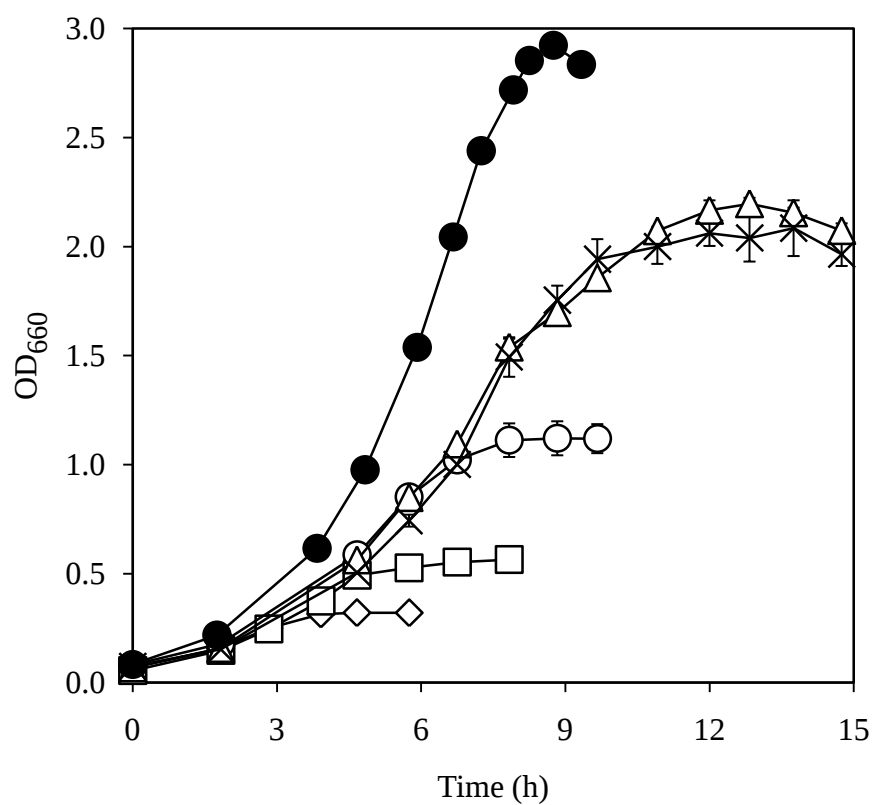


Figure 3.2 Growth of 130Z(pLGZ924) in AM2-isocitrate. Symbols: ◇, 1.4 mM DL-isocitrate; □, 2.8 mM DL-isocitrate; ○, 5.7 mM DL-isocitrate; △, 11.4 mM DL-isocitrate; X, 22.7 mM DL-isocitrate; ●, *A. succinogenes* 130Z grown in AM3 medium.

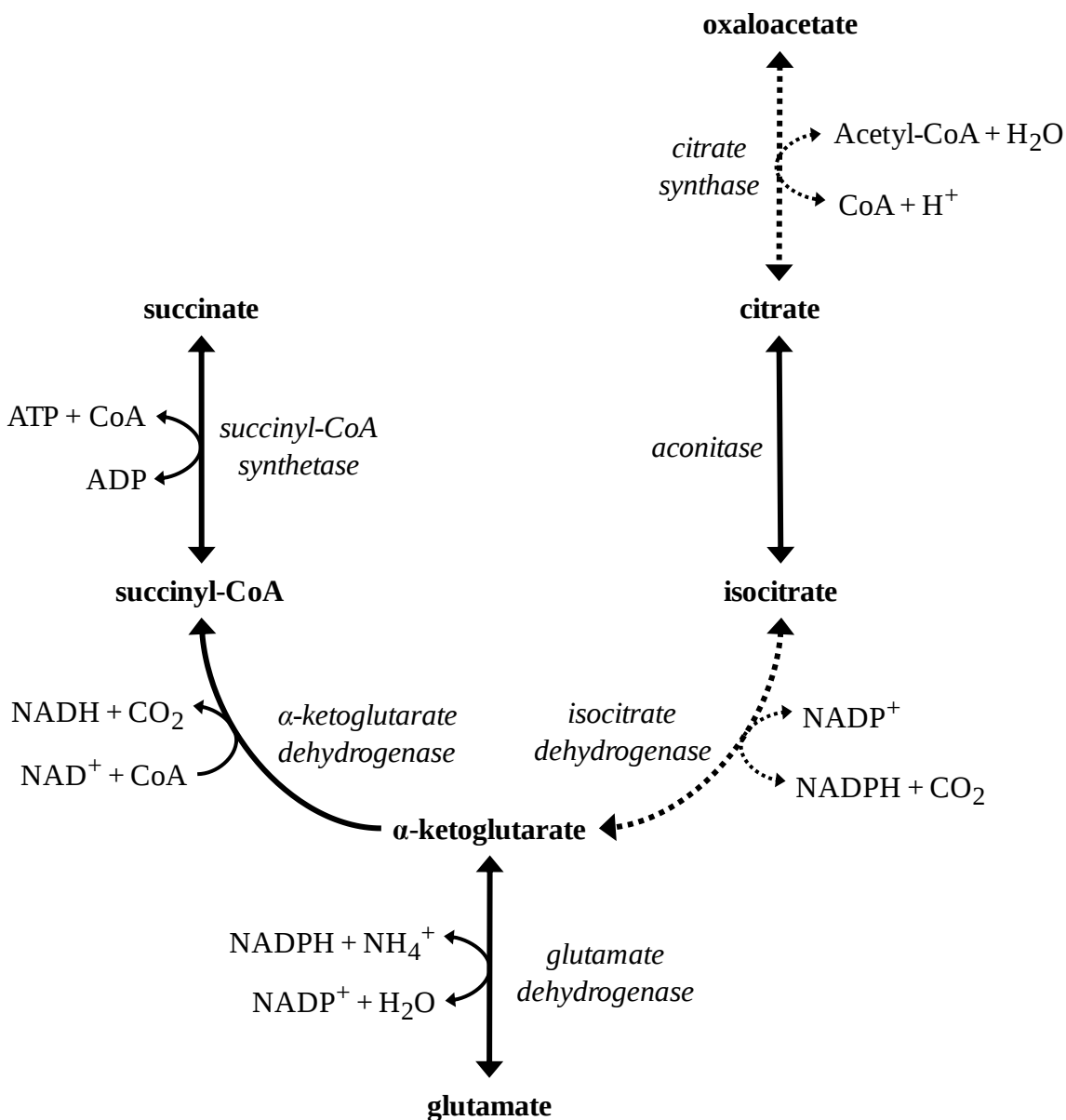


Figure 3.3 Pathways of isocitrate and glutamate metabolism in *A. succinogenes*. Solid lines, pathways assumed to be active based on genome annotation and growth tests with glutamate precursors (25, 27); dashed lines, reactions absent in *A. succinogenes*, based on genome annotation and *in vitro* enzyme activity assays (Joshi and Vieille, data not shown). *In vitro* glutamate dehydrogenase activity was detected in *A. succinogenes* cells (27).

uptake machinery to short DNA sequences, the USSs. The high density of USS repeats in *A. succinogenes* suggests the importance of including a USS repeat in the $\Delta pflB$ knockout construct. One USS was included in plasmid pCR2.1- $\Delta pflB::FRT$ -p_{pckA}-*icd*-*FRT*, 33 bp downstream of the *FRT* sequence and *AscI* restriction site on the 3' end of the *icd* marker. The entire linearized plasmid was used for transformation to ensure that the homologous DNA sequences (~1 kb on each side of the marker) were not degraded by exonucleases prior to recombination (the knockout construct was flanked by ~2 kb of pCR2.1 DNA on each side).

Growth was observed in AM2-isocitrate medium 4 days after transformation. Cells also grew in AM3 medium after overnight incubation but no growth was observed in AM2 medium. The first culture in AM2-isocitrate (0.25 mL) was used to inoculate a tube of fresh AM2-isocitrate medium to enrich for recombinants able to grow on isocitrate. This second culture was streaked onto LB-glucose-kanamycin agar plates to isolate putative recombinants. Colony PCR (primers P11 and P12) was performed to identify recombinants. Thirty-five of the 36 colonies screened were identified as recombinants (strains with interrupted *pflB* genes) (Figure 3.4).

3.4.3 Removal of the selectable marker. To test if Flp recombinase is active in *A.*

succinogenes, strain $\Delta pflB::icd$ was transformed with plasmid pCV933 (pLGZ920 expressing FLP). After transformation, twelve transformants were immediately screened by colony PCR to identify recombination events resulting in the loss of the *icd* marker along with one *FRT* site.

While all transformants contained plasmid pCV933, none of them showed a recombination event. To allow the cells more time for recombination to occur, one of these transformant colonies was re-isolated on LB-glucose-ampicillin and the resulting colonies were screened by PCR. Of the 38 colonies tested, 18 had lost the *icd* marker, 18 still contained the *icd* marker, and

2 showed a mixed population of cells with and without the marker. Figure 3.4 depicts the PCR confirmation of both the replacement of the *A. succinogenes pflB* gene with the *icd* marker and the excision of the marker with pCV933.

3.4.4 Curing plasmid pCV933 from *A. succinogenes* $\Delta pflB$ with acridine orange. Acridine orange (AO) is a DNA intercalating dye commonly used to inhibit plasmid replication (41). After AO treatment, colonies isolated on LB-glucose-kanamycin were replica-plated onto LB-glucose agar plates containing either kanamycin or ampicillin to identify ampicillin sensitive (i.e., cured) colonies. Thirty colonies were screened from each different concentration of AO used. Of the different AO concentrations used, $50 \mu\text{g mL}^{-1}$ was the most efficient, with 10 colonies not growing on ampicillin-containing plates, 16 colonies growing on both ampicillin- and kanamycin-containing plates, and 4 colonies growing on neither plate after patch plating. $10 \mu\text{g mL}^{-1}$ AO produced 4 colonies that only grew on kanamycin-containing plates, the no AO control produced one colony that had possibly lost pCV933, and the $100 \mu\text{g mL}^{-1}$ and $200 \mu\text{g mL}^{-1}$ AO treatments produced no colonies that grew on kanamycin-containing plates only. PCR was used to confirm the loss of pCV933 with primers P17 and P18. Of the 14 total colonies that had possibly been cured of pCV933 (1 from no AO treatment, 4 from $10 \mu\text{g mL}^{-1}$ AO, and 10 from $50 \mu\text{g mL}^{-1}$ AO), only 1 from $10 \mu\text{g mL}^{-1}$ AO and 2 from $50 \mu\text{g mL}^{-1}$ AO treatments lacked the plasmid after two replicate PCR screens. One colony from the $50 \mu\text{g mL}^{-1}$ AO treatment was designated *A. succinogenes* $\Delta pflB$ and subjected to further characterization.

3.4.5 Fermentative metabolism of *A. succinogenes* $\Delta pflB$.

To demonstrate that the *A. succinogenes pflB* gene was deleted and PFL activity was

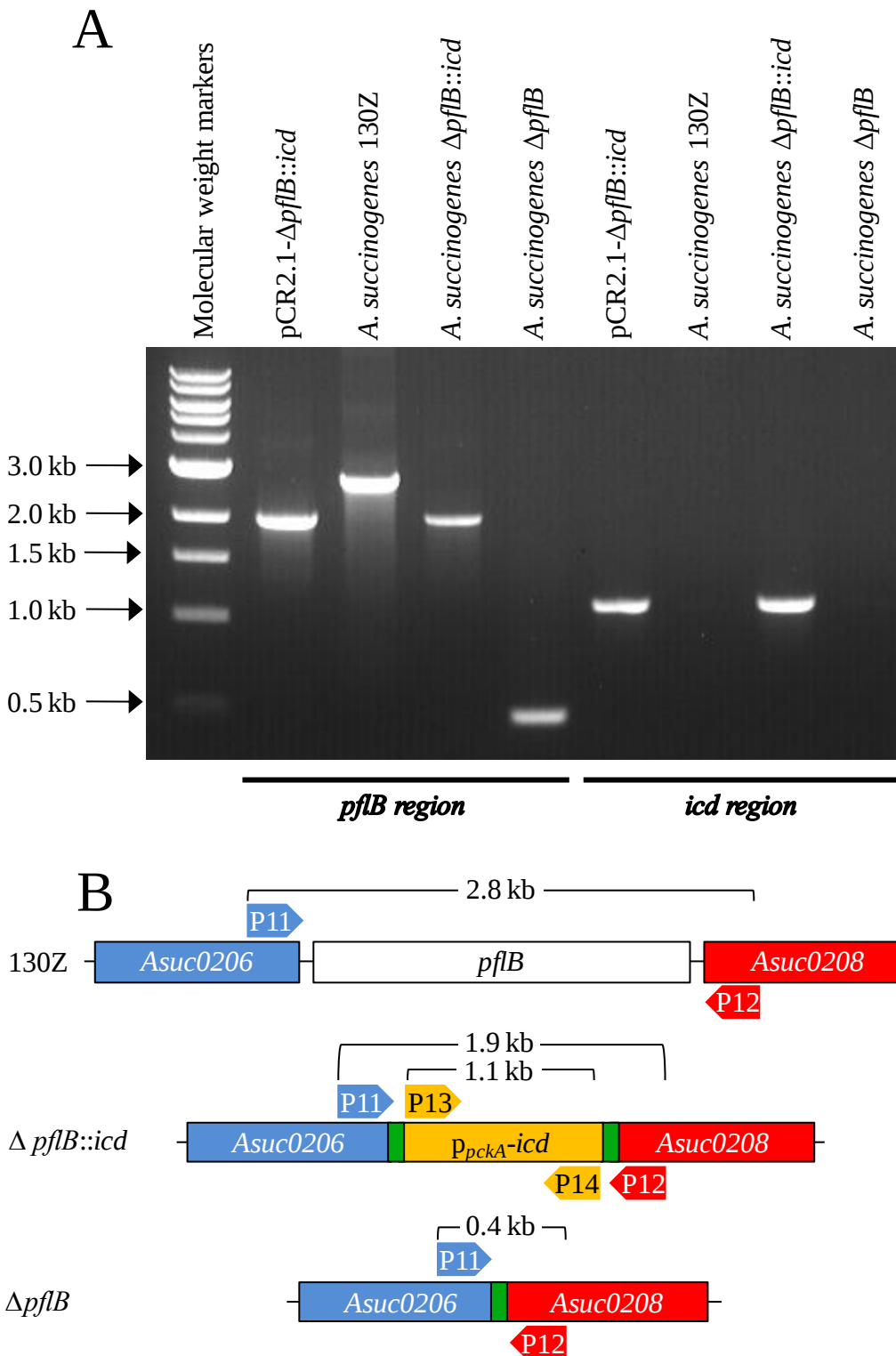


Figure 3.4. PCR verification of the *A. succinogenes pflB* gene deletion. Primer pairs P11-P12 and P13-P14 were used to amplify the sequences surrounding *pflB* and a sequence internal to the *icd* marker, respectively. Green bands represent *FRT* sites. A. Agarose gel electrophoresis of the PCR fragments. B. Physical maps of the $\Delta pflB$ region in strains 130Z, $\Delta pflB::icd$, and $\Delta pflB$.

eliminated we compared the HPLC profiles of fermentation supernatants of strains 130Z and $\Delta pflB$. To clearly show that the $\Delta pflB$ mutant no longer produces formate, we grew the strains under conditions of low CO₂ availability (e.g., 25 mM NaHCO₃). In these conditions *A. succinogenes* produces low concentrations of succinate and produces high concentrations of formate, acetate, and ethanol (27, 42). Figure 3.5 shows the fermentation supernatants of strains 130Z (panel A) and $\Delta pflB$ (panel B) grown in AM3 glucose containing 25 mM NaHCO₃. Panel B clearly demonstrates that strain $\Delta pflB$ no longer produces any formate.

The *pflB* gene was deleted to eliminate the production of formate and to reduce the carbon flow to acetate and ethanol. We observed both of these effects in strain $\Delta pflB$ during glucose fermentation (Table 3.3). In conditions where succinate production was not limited by CO₂ availability (i.e., in the presence of 150 mM NaHCO₃) strain $\Delta pflB$'s succinate yield was 69% higher than that of strain 130Z. When CO₂ was limiting, strain $\Delta pflB$ still produced nearly 1 mol succinate per mole glucose (the maximum theoretical yield is 1.71). Since strain $\Delta pflB$ still grows well in AM3 and still produces acetate and ethanol, we suspect that pyruvate dehydrogenase (PyrDH) activity is responsible for the remaining flux from pyruvate to acetyl-CoA.

Besides metabolite production, the growth phenotypes of strains 130Z and $\Delta pflB$ were also different. Regardless of the availability of CO₂, strain 130Z grew faster than strain $\Delta pflB$ (Table 3.3). In the presence of 25 mM NaHCO₃, strain $\Delta pflB$ grew to a lower maximum OD₆₆₀ (1.7 ± 0.0) than strain 130Z did (2.8 ± 0.0). Strain $\Delta pflB$'s growth yield was restored to wild-type levels (2.7 ± 0.1) by adding excess (150 mM) NaHCO₃. Regardless of NaHCO₃ availability (i.e., 25 mM or 150 mM), strain 130Z produces approximately 1 mol formate per mol of acetate or ethanol produced (Table 3.3, (27, 42)). This observation suggests that PFL is the primary

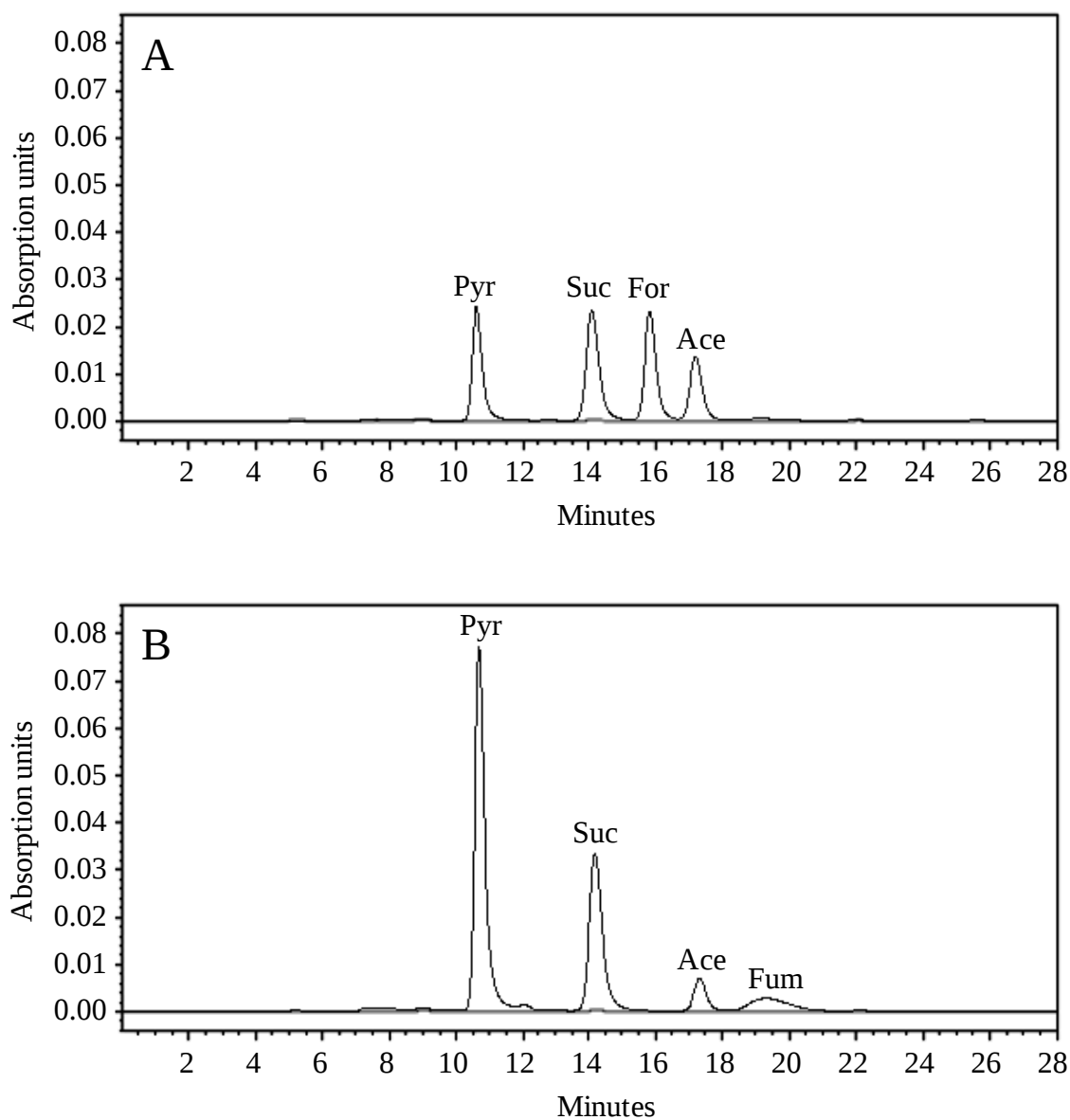


Figure 3.5. HPLC profiles of fermentation supernatants of *A. succinogenes* strains 130Z and $\Delta pflB$ grown in AM3 glucose containing 25 mM NaHCO_3 . Supernatant samples were taken immediately after inoculation (baseline) and after 12 h of growth. Strain 130Z, panel A; strain $\Delta pflB$, panel B.

Strain	NaHCO ₃ (mM)	mmol product/100 mmol glucose consumed						Carbon Recovery	Electron Recovery	Generation time (h)
		Succinate	Formate	Acetate	Ethanol	Pyruvate	Biomass ^a	(%) ^b	(%) ^c	
130Z	25	57 ± 2	80 ± 5	66 ± 3	25 ± 4	0 ± 0	165 ± 11	100 ± 6	109 ± 6	1.66 ± 0.01
130Z	150	64 ± 4	71 ± 3	64 ± 1	7 ± 2	0 ± 0	183 ± 3	98 ± 2	107 ± 2	1.68 ± 0.03
$\Delta pflB$	25	97 ± 2	0 ± 0	42 ± 2	7 ± 2	1 ± 1	139 ± 4	89 ± 2 ^d	104 ± 3	2.04 ± 0.02
$\Delta pflB$	150	108 ± 2	0 ± 0	50 ± 1	1 ± 1	0 ± 0	162 ± 9	100 ± 3	114 ± 4	1.91 ± 0.03

Table 3.3 Log phase fermentation balances of *A. succinogenes* strains 130Z and $\Delta pflB$ in AM3 medium with glucose as the sole carbon source. Data are means ± standard deviation from three biological replicates.

^a mmol Biomass was calculated based on assumed values of 567 mg dry cell weight L⁻¹ per OD₆₆₀ and a cell composition of CH₂O_{0.5}N_{0.2} (24.967 g/mol) (42).

^b Carbon in product/carbon in glucose consumed. The assumption was made that one mole of CO₂ was fixed per mol succinate produced (18). Therefore, C₃H₆O₂ was used as the chemical composition of succinate derived from glucose.

^c Electron recoveries are based on available hydrogen (12).

^d We assume that the true carbon recovery for strain $\Delta pflB$ in CO₂-limiting conditions is somewhere in between 89 and 104% since CO₂ for succinate production comes from both PyrDH activity and exogenous NaHCO₃.

pyruvate-dissimilating enzyme active under glucose fermentation conditions. In strain $\Delta pflB$ all flux to acetyl-CoA must go through PyrDH, which, unlike PFL, produces 1 mol NADH per mol acetyl-CoA. Based on the fermentation profile of strain $\Delta pflB$ (Table 3.3), the amount of ethanol produced by strain $\Delta pflB$ is not enough to recycle the NADH produced by PyrDH. The limited CO₂ availability limits the flux through the C₄ pathway (i.e., to succinate), which would otherwise consume the excess reducing equivalents produced by PyrDH and produce ATP for growth (the reduction of one mol fumarate produces 0.67 mol ATP (19)).

In conditions where succinate production was limited by CO₂ availability (i.e., in the presence of 25 mM NaHCO₃), strain $\Delta pflB$ produced more pyruvate than strain 130Z did. The fermentation profiles in Table 3.3 are based on culture supernatants taken during exponential growth and the small difference in pyruvate production between strains 130Z and $\Delta pflB$ most likely reflects non-CO₂-limiting conditions. However, analysis of endpoint fermentation samples from cultures grown with 25 mM NaHCO₃ shows that strain $\Delta pflB$ has produced four times as much pyruvate (0.20 ± 0.01 mol pyruvate/mol glucose) as strain 130Z did in stationary phase (0.05 ± 0.00 mol pyruvate/mol glucose). This difference suggests that once CO₂ becomes limiting strain $\Delta pflB$ balances its reducing equivalents by excreting pyruvate instead of making acetate or ethanol.

3.5 DISCUSSION

While significant work has been done to understand the fermentative metabolism of wild type *A. succinogenes* using genome annotation, *in vitro* enzyme activity assays, and metabolic flux analysis, a deeper understanding can come from the study of mutant strains. A hindrance to these studies is the lack of means to produce mutants by allele exchange. We demonstrated for the first time a genetic method that allowed the generation of defined mutants in *A. succinogenes*. Although we demonstrated the method by creating a gene deletion, the method is also applicable to the insertion of new genes. Producing point mutations would require some modification of the protocol described for producing gene deletions due to the *FRT* scar (48 bp) left behind after marker removal. For example, the mutation construct would be arranged with the *icd* marker placed outside of the target gene allele (containing the point mutation) and in an intergenic region to avoid interfering with transcription of the target gene or adjacent genes.

Several important features should be noted about the genetic tools reported here. First, natural transformation was used to introduce DNA into the cell. This is the first report demonstrating that *A. succinogenes* is naturally competent (although natural transformation only occurred when cAMP was added to the competency induction medium). Nik McPherson in the laboratory is currently testing whether electroporation can be used in place of natural transformation to introduce linear DNA into *A. succinogenes* for gene insertion or deletion. Second, linear DNA was introduced into *A. succinogenes*. The advantage to targeting chromosomal genes with linear DNA is that only double recombination events will be selected. With this method, a second counterselection step to excise an integrated plasmid, such as sensitivity to sucrose in the presence of *sacB*, is not needed. Third, we also demonstrated that the *S. cerevisiae* flippase recombinase, expressed from the pLGZ920 plasmid, is active in *A.*

succinogenes and can be used to excise the *icd* marker after allele exchange. Removal of the marker leaves a single 48-bp *FRT* site, minimizing the presence of foreign DNA in *A. succinogenes* mutants. Efforts are currently underway to produce double knockout mutants in *A. succinogenes* using this approach. We are also developing a knockout approach that will eliminate all foreign DNA fragments (e.g., *FRT* sites) from the chromosome. Fourth, we showed for the first time that a plasmid can be cured from *A. succinogenes* using the classic approach of AO treatment.

Neither *A. succinogenes* 130Z(pLGZ924) nor 130Z $\Delta pflB::icd$ grows on AM2-isocitrate agar plates. This observation probably correlates with the fact that 130Z(pLGZ924) cultures grow slower and to lower ODs in AM2-isocitrate than in AM3. It is possible that intracellular isocitrate is being diverted away from glutamate production at either the isocitrate or α KG metabolic nodes (Figure 3.3). *A. succinogenes*'s genome does not encode the genes required for the glyoxylate bypass (i.e., *aceA*, *aceB*, and *aceK*), which converts isocitrate into malate with the production of one mol succinate and consumption of one mol acetyl-CoA (18). In *E. coli*, isocitrate dehydrogenase activity is controlled by the phosphorylation (inactivating) and dephosphorylation (activation) of the enzyme by isocitrate dehydrogenase kinase/phosphatase (encoded by *aceK*) (21). The absence of *aceK* from the *A. succinogenes* genome suggests that *E. coli* ICD is un-phosphorylated and constitutively active in *A. succinogenes*. *A. succinogenes*'s genome contains the genes required for aconitase (*acnB*) and citrate synthase (*gltA*), suggesting that isocitrate may be isomerized into citrate and further converted to acetyl-CoA and oxaloacetate. This pathway is unlikely since synthesis of aconitase is significantly repressed under anaerobic conditions in *E. coli* (14). Alternatively, after isocitrate is converted to α KG, α KG could possibly be irreversibly decarboxylated to succinyl-CoA by α KG dehydrogenase

instead of being converted to glutamate via glutamate dehydrogenase. Overall, identifying the reasons for slower growth and lower biomass yields of strain 130Z(pLGZ924) in AM2-isocitrate medium is beyond the scope of this chapter and was not pursued further since we do not intend to leave the *icd* marker in place in our knockout mutant constructs.

Absence of growth of strain 130Z (pLGZ924) and 130Z $\Delta pflB::icd$ on AM2-isocitrate plates limits our selection of recombination to AM2-isocitrate liquid medium at this stage. For this reason, we are unable to calculate transformation efficiency at this stage. Nik McPherson has developed an enriched defined medium that contains all amino acids but glutamate and its derivatives plus high concentrations of vitamins. The medium is called AM16-isocitrate, and we are now able to select for double recombination events by growing directly on AM16-isocitrate plates. Work is now in progress with AM16-isocitrate plates to determine double recombination efficiencies with this natural transformation method.

We targeted the enzyme PFL for inactivation in this study for two reasons. First, PFL was expected to be non-essential for growth. This expectation is based on the viability of *A. succinogenes* strain FZ6 (15). Strain FZ6 is a fluoroacetate-resistant mutant isolated in a chemical mutagenesis screen, and it produces more succinate than strain 130Z. Park *et al.* showed that strain FZ6, which produces no formate during glucose fermentation in rich medium, has no PFL activity (30). Second, formate generated during *A. succinogenes*'s glucose fermentation contributes to a significant loss of carbon that would otherwise be available for succinate production. Formate is often one of the two most abundant products (mol product/mol sugar) from sugar fermentations (42). Therefore, a *pflB* deletion was expected to eliminate formate production and reduce the production of the other C3 pathway products (acetate and ethanol) while increasing succinate production. Indeed, *A. succinogenes* $\Delta pflB$ produced no

formic acid, had an increased succinate product ratio, and had an increased succinate yield from glucose fermentations. These results are similar to those observed with *M. succiniciproducens* strain LPK, a $\Delta ldhA$ - $\Delta pflB$ double mutant (*A. succinogenes* does not have an *ldhA* homologue) (23) and with *A. succinogenes* strain FZ6 (15). When grown in glucose complex medium *A. succinogenes* FZ6 (with corn steep liquor and yeast extract) and *M. succiniciproducens* LPK (with yeast extract) produce higher levels of pyruvate than the respective wild-type strains (15, 23). Here, *A. succinogenes* $\Delta pflB$ produced pyruvate when grown in AM3, but the levels were smaller than with *A. succinogenes* FZ6 or *M. succiniciproducens* LPK. We expect the $\Delta pflB$ mutation to be the first step in engineering an *A. succinogenes* strain that can produce succinate from glycerol and other sugars at near theoretical yields. The ability to remove and reuse the *icd* selection marker is invaluable for achieving this goal.

Combined with the completed manual genome sequence annotation (25), the development of genetic tools for *A. succinogenes* will allow future studies to understand the genetic control of *A. succinogenes*'s fermentative metabolism and facilitate future metabolic engineering endeavors.

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Chapter 4

Conclusions and future directions

4.1 Introduction

The increase in U.S. domestic biodiesel production has caused an influx of glycerol into the market, dropping its price and making its disposal a burden for biodiesel producers. In addition to its availability and low price, glycerol is a highly reduced molecule making it an attractive feedstock for production of reduced fuels and chemicals. The work in this dissertation has demonstrated that, under optimized conditions, glycerol may be a useful substrate for biobased succinate production by *Actinobacillus succinogenes*. For instance, in conditions of limited external electron acceptor *A. succinogenes* produces succinate in high yields while using DMSO or O₂ as an electron sink. In these conditions, reducing power is conserved for succinate production and not lost as dimethyl sulfide or H₂O. Succinate was produced from glycerol in yields higher than those observed during sugar fermentations (10, 14).

4.2 The current understanding of *A. succinogenes*'s glycerol metabolism for succinate production

We have shown that *A. succinogenes* cannot ferment glycerol, but that it can grow on glycerol via respiration. This demonstration stemmed from the manual genome annotation of *A. succinogenes*'s glycerol catabolic pathway and of its respiratory chain. Testing anaerobic and aerobic respiratory conditions for maximal succinate production from glycerol allowed us to make the following observations: (i) Nitrate-respiring cultures produce mainly acetate and CO₂ with succinate as a minor product; (ii) Succinate is the major product of DMSO-respiring cells and succinate yield is maximized when DMSO is limited; (iii) *A. succinogenes* grown under microaerobic, CO₂-rich, conditions produces succinate in yields similar to those on DMSO but with less formate and biomass production; (iv) *A. succinogenes*'s succinate yield from glycerol

(under DMSO-respiring and microaerobic conditions) can be increased by inactivating pyruvate formate lyase.

4.3 Future research to improve succinate production from glycerol by *A. succinogenes*

In Chapter 2 we showed that microaerobic conditions are favorable to high succinate yields from glycerol. Because O₂ availability decreases as cell density increases in batch cultures, microaerobic conditions optimum for succinate production cannot be maintained in batch cultures. In contrast, future studies using continuous culture systems should allow the optimization of microaerobic conditions that maximize succinate production. The work by Alexeeva *et al.* (1) is a good model to follow for this type of study. The authors grew *Escherichia coli* in glucose-limited chemostats under different O₂ tensions using a constant dilution rate but varying the O₂ flow rate. If similar conditions are investigated for *A. succinogenes* strains engineered for increased succinate production, I am confident that succinate yields will increase to above 90% of the maximum theoretical yield.

4.4 Thoughts for future genetic engineering

In both DMSO-respiring and microaerobic conditions, acetate is the next most abundant metabolite (after succinate) produced by *A. succinogenes* from glycerol. Reducing flux to acetate should be the next goal for genetic engineering of *A. succinogenes*. Fermentative acetate production was significantly reduced in both *E. coli* and *Mannheimia succiniciproducens*, *A. succinogenes*'s closest relative, by disrupting the *pta* and *ackA* genes, encoding phosphotransacetylase (EC 2.3.1.8) and acetate kinase (EC2.7.2.1), respectively (6). Phosphotransacetylase converts acetyl-CoA and inorganic phosphate to acetyl phosphate and

CoA. Acetate kinase converts acetyl phosphate and ADP to acetate and ATP (12). Like in *E. coli*, *A. succinogenes* has the *pta-ackA* operon (Asuc1661-1662) (5). This operon could be deleted using the same knockout methodology used for the *A. succinogenes pflB* gene (Chapter 3). This mutation should be introduced into the wild-type strain to study its effect in a wild-type background, but it should also be introduced in the $\Delta pflB$ mutant to capitalize on a strain that no longer produces formate to maximize succinate production. Jantama *et al.* (4) found that a *pta-ackA* mutant of *E. coli* still produced 0.27 mol acetate/mol glucose during fermentation. Jantama *et al.* further reduced acetate production to 0.18 mol/mol glucose by inactivating *tdcD*, encoding a propionate kinase with acetate kinase activity. As in *E. coli*, inactivating *pta-ackA* in *M. succiniciproducens* reduced but did not eliminate acetate production (6). The pathways responsible for the remaining acetate produced by these strains were not investigated. *A. succinogenes* contains a single copy of the *pta-ackA* genes but, based on the results in *E. coli* and *M. succiniciproducens*, we suspect that acetate production will not be completely eliminated with a single engineering step. Nik McPherson in the Vieille lab is currently constructing the $\Delta pta-ackA$ mutant of *A. succinogenes*. Since acetate production is coupled to ATP production, a $\Delta pflB$, $\Delta pta-ackA$ double mutant of *A. succinogenes* may grow very poorly in AM3-glycerol. In this case the strain could first be evolved on AM3 (with glucose) to find a derivative with improved growth. The derivative would then be tested in AM3-glycerol.

4.5 Design of reactors to improve succinate production from glycerol

Since bacteria respond quickly to changes in O₂ tension by changing their metabolism, the most critical challenge of microaerobic bioreactors, specifically on a pilot and commercial scale, is satisfying the requirement for a “defined and uniform O₂ supply” that is often easier to

maintain in bench-scale experiments (16). Zeng and Deckwer (16) discuss these challenges in detail. Time constants of the specific reactions, mixing parameters, mass transfer, and hydrodynamics must all be considered when scaling-up microaerobic technology. For instance, too rapid or slow mixing of the bacteria between zones of varying O₂ tension may be detrimental to cell health and impair performance. Furthermore, maintaining conditions for optimal product yield is important, but maximum product yield cannot be achieved at the cost of titer, volumetric productivity, and the energy efficiency of the bioreactor. These processes may even require novel bioreactor designs. All of these parameters must be considered to make the process economically viable.

An anaerobic microbial fuel cell (MFC) may be used as an alternative to using a microaerobic bioreactor (8). Park and Zeikus (11) used electrically reduced neutral red (NR) in a MFC to enhance succinate production from glucose by *A. succinogenes*. In this MFC, electrically reduced NR was a source of reducing power for the bacterium's growth and fermentation in the cathode compartment. NR seemed to function as a menaquinone, donating electrons to fumarate reductase (11). Since *A. succinogenes* requires an electron sink to grow anaerobically on glycerol, the Vieille lab is currently testing whether the reverse MFC can be used to allow *A. succinogenes* to grow fermentatively on glycerol. Instead of using reduced NR as a source of reducing power for the bacteria, the new MFC system would use electrically oxidized NR to extract electrons from an *A. succinogenes* culture in the anode compartment. If successfully demonstrated, this system can be optimized for succinate production by varying the applied potential.

4.6 Identification of crude glycerol components that influence succinate yield

Once a viable reactor set-up is established, it will be very important to identify a source of inexpensive crude glycerol feedstock with a reproducible chemical composition. Based on the results in Chapter 2, succinate yield was lower on mixed glycerol than on that from pure glycerol (Based on conditions where biological replicates grew similarly [i.e., strain $\Delta pflB$ under 1% O₂, and both strains 130Z and $\Delta pflB$ under 0.1% O₂]). It will be important to identify component(s) in the crude glycerol that decrease yield so that purification steps can be developed to remove those impurities in a cost-effective way. Based on the succinate yield from mixed glycerol and assuming that impurities are used as carbon and energy sources, these impurities are less reduced than glycerol. Alternatively, the decreased yield may also be due to *A. succinogenes* using these impurities as terminal electron acceptors, reducing available reducing power for succinate production. NMR was used to compare mixed glycerol to pure glycerol. Methanol was the only impurity detected by NMR in mixed glycerol but methanol supplementation of cultures grown on pure glycerol did not affect on *A. succinogenes*'s growth or product distribution. Because of its higher sensitivity compared to NMR, GC-MS could be a useful tool to identify other impurities. Candidate compounds would be added to AM3-pure glycerol cultures to test their effect on growth and metabolite production.

We did conduct preliminary experiments using crude, unprocessed glycerol, but these studies were complicated by the glycerol's physical properties and inconsistent composition. For example, AM3 containing crude glycerol is opaque, making biomass measurements by optical density impossible. The inconsistent composition of crude glycerol caused inconsistent results in microaerobic cultures. For instance, compared to pure glycerol, *A. succinogenes* produced a significantly higher S/A ratio and succinate yield from one batch of crude glycerol but lower

values from another batch. We also found that *A. succinogenes* did not grow in AM3-DMSO when either batch of crude glycerol was used. Because the cheapest, least refined glycerol may be the best for succinate production, the Vieille lab plans to continue collaborating with Michigan Biodiesel, LLC to study which components are most favorable for succinate production.

4.7 Further development and applications of genetic tools for *A. succinogenes*

In this dissertation we used a new set of genetic tools developed specifically for *A. succinogenes*. Manual genome annotation (9) suggested that *A. succinogenes* is naturally competent and can take up linear DNA containing uptake signal sequence (USS) repeats. *A. succinogenes*'s natural competence was demonstrated first by Rajasi Joshi (MMG professional masters graduate) who constructed the first knockout mutant of *A. succinogenes*. Natural competence was then used in Chapters 2 and 3 of this dissertation to construct *A. succinogenes* strain $\Delta pflB$. The knockout method used a novel positive selection method based on *A. succinogenes*'s auxotrophy for alpha-ketoglutarate (10). The positive selection marker is the *E. coli icd* gene, which allows *A. succinogenes* to grow on isocitrate instead of glutamate. We also developed a marker removal system based on the flippase recombinase of the *Saccharomyces cerevisiae* 2 μ M plasmid (3). After removal of the positive selection gene, a single flippase recognition target (FRT) site remained in place of the target chromosomal gene.

In the future, our lab wishes to make improvements to these genetic tools. First, we want to test if electroporation can be used instead of natural transformation to introduce linear DNA into cells targeted for homologous recombination. Preparing electrocompetent *A. succinogenes* is less time-consuming than preparing naturally competent cells. Furthermore, natural

transformation requires a USS in the linear DNA fragment for uptake into the cell. In the construction of strain $\Delta pflB$ there was a USS conveniently located in the gene downstream of *pflB*. In the absence of a proximal USS, one would have to be engineered into the linear DNA and may cause unintentional changes in the expression of neighboring genes.

We also wish to simplify the knockout and marker removal method for *A. succinogenes*. An improved method would be one that (i) avoids leaving the *FRT* scar, which would otherwise accumulate in multiple knockout mutants; and (ii) avoids using acridine orange, a mutagen. One promising alternative to our current method is the *pyrF*-based selection. *pyrF* encodes orotidine-5-phosphate decarboxylase (OMPdec), a required step in pyrimidine biosynthesis. In the presence of the analogue 5-fluoroorotic acid (FOA), OMPdec converts FOA to fluorodeoxyuridine, a toxic intermediate (2). The method would be applied to *A. succinogenes* by first transforming the bacterium with linear DNA containing the flanking regions of *pyrF* (Asuc1548) and selecting on AM3 agar plates supplemented with uracil and FAO (AM3 + uracil + FAO). Resistant colonies should lack the *pyrF* gene. The resulting strain, $\Delta pyrF$, could then be used as the recipient for knockout constructs. The genes targeted for knockout would be replaced by the *pyrF* marker and recombination would be selected on AM3 for uracil prototrophy. The *pyrF* marker can be removed simply by a second transformation with a similar DNA construct containing the unmarked gene knockout and selection on AM3 + uracil + FAO. The latter two steps can be repeated as desired to create multiple mutations within the same strain. At the end of construction, the *pyrF* gene would be added back into the chromosome. This method has been used in other organisms (7, 13) and should shorten the time needed for genetic engineering of *A. succinogenes*.

Biobased succinate production is of great interest because it offers the enormous opportunity to replace petrochemically-derived maleic anhydride as a chemical building block (15). As the world's oil reserves rapidly deplete, we need to develop alternative methods, using renewable resources, to produce existing commodity chemicals. In addition to using renewable resources as a feedstock, biobased succinate production consumes CO₂, a greenhouse gas, and some products derived from succinate are biodegradable.

After almost two decades of research, significant improvements in biobased succinate production are still required. To that end, we strongly believe that increasing succinate yields through further strain engineering and process development to levels beyond those observed in this dissertation is possible and will undoubtedly be useful in making biobased succinate cost competitive with maleic anhydride. Lastly, producing value-added chemicals from crude glycerol will improve the economics of not only succinate production, but also biodiesel production, pushing biodiesel closer to being cost-competitive with traditional petrochemical fuels.

4.8 REFERENCES

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APPENDICES

Appendix A

A genomic perspective on the potential of *Actinobacillus succinogenes* for industrial succinate production

The work completed in this appendix is part of a collaboration between Dr. James McKinlay (primary author, a former member of the Zeikus/Vieille lab), Dr. Vieille, current and past members of the Zeikus/Vieille lab, the Department of Energy's Joint Genome Institute, and Los Alamos National Laboratory. A detailed description of the author's individual contributions follows the main text.

My contributions to this report included:

- Manually annotating *A. succinogenes*'s vitamin biosynthesis pathways (see Table S4 and Figure S6)
- Determining *A. succinogenes*'s vitamin auxotrophies in AM3 defined minimal medium
- Testing *A. succinogenes*'s growth on candidate carbon sources chosen based on genome annotation of potential transporters and catabolic enzymes
- Drafting parts of the manuscript related to the first three bullet points above
- Reading and editing the entire manuscript

This article can be viewed at the following URL:

<http://www.biomedcentral.com/1471-2164/11/680>

Abstract

Background

Succinate is produced petrochemically from maleic anhydride to satisfy a small specialty chemical market. If succinate could be produced fermentatively at a price competitive with that of maleic anhydride, though, it could replace maleic anhydride as the precursor of many bulk chemicals, transforming a multi-billion dollar petrochemical market into one based on renewable resources. *Actinobacillus succinogenes* naturally converts sugars and CO₂ into high concentrations of succinic acid as part of a mixed-acid fermentation. Efforts are ongoing to maximize carbon flux to succinate to achieve an industrial process.

Results

Described here is the 2.3 Mb *A. succinogenes* genome sequence with emphasis on *A. succinogenes*'s potential for genetic engineering, its metabolic attributes and capabilities, and its lack of pathogenicity. The genome sequence contains 1,690 DNA uptake signal sequence repeats and a nearly complete set of natural competence proteins, suggesting that *A. succinogenes* is capable of natural transformation. *A. succinogenes* lacks a complete tricarboxylic acid cycle as well as a glyoxylate pathway, and it appears to be able to transport and degrade about twenty different carbohydrates. The genomes of *A. succinogenes* and its closest known relative, *Mannheimia succiniciproducens*, were compared for the presence of known Pasteurellaceae virulence factors. Both species appear to lack the virulence traits of toxin production, sialic acid and choline incorporation into lipopolysaccharide, and utilization of hemoglobin and transferrin

as iron sources. Perspectives are also given on the conservation of *A. succinogenes* genomic features in other sequenced Pasteurellaceae.

Conclusions

Both *A. succinogenes* and *M. succiniciproducens* genome sequences lack many of the virulence genes used by their pathogenic Pasteurellaceae relatives. The lack of pathogenicity of these two succinogens is an exciting prospect, because comparisons with pathogenic Pasteurellaceae could lead to a better understanding of Pasteurellaceae virulence. The fact that the *A. succinogenes* genome encodes uptake and degradation pathways for a variety of carbohydrates reflects the variety of carbohydrate substrates available in the rumen, *A. succinogenes*'s natural habitat. It also suggests that many different carbon sources can be used as feedstock for succinate production by *A. succinogenes*.