GLUCOSE IS TAKEN UP BY GALACTOSE PERMEASE IN METABOLIC ENGINEERED *ESCHERICHIA COLI* TO PRODUCE SUCCINATE

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Abstract

***E. coli* strain KJ073 was previously developed to produce succinate by a combination of metabolic engineering and metabolic evolution. The strain produces succinate as a major product under anaerobic fermentation with high titer and yield. Metabolic Flux Analysis (MFA) results revealed that about 70% of PEP (phosphoenolpyruvate) flux from glucose generated during glycolysis flowed to a succinate production route in strain KJ073. This event is not normally found in *E. coli* wild type since almost the entire PEP pool flows through PYK (pyruvate kinase) to generate ATP for growth and maintenance rather than flowing through PPC (pyruvate carboxylase) since this latter pathway does not produce ATP. However, strain KJ073 has been reported to show an increase of PCK (PEP carboxykinase) activity but a decrease in PPC activity. It is worth noting that the production of OAA (oxaloacetate) by PEP carboxylation via PCK produces an extra mole of ATP. In addition, the results from gene deletion analysis showed that *galP*$^-$ mutants of KJ073 exhibited severely low glucose consumption rates, while the deletions of genes involving in the classical PEP (phosphoenolpyruvate)-dependent PTS transporters (*ptsG* and *manX*) did not affect the rates of glucose consumption in the strains. This result could confirm that the activation of GalP (galactose permease) for glucose transporting in strain KJ073 would enhance the succinate production by preserving PEP pool size in which PEP is not used for phosphorylating glucose. The strain gained the benefit in which the elevated PEP level was used for OAA production simultaneously with increasing ATP production through PCK.**

Keywords: Succinate, *E. coli,* PTS systems, GalP, metabolic flux analysis

Introduction

Succinic acid is a potential source of many important intermediates and specialty benzene-derived chemicals such as food ingredients, green solvents, pharmaceutical products, biodegradable plastics and polymers, and amino acids (McKinlay *et al*., 2007). Succinate has been petrochemically produced by hydrogenation of maleic anhydride, which is derived from oil. The limitation of the world's supply of oil has made its price about $87 a barrel, thus it is not economical to make chemically-produced succinate. However, the

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succinate production from maleic anhydride could be substituted by its fermentation.

Fermentative production of succinate from low cost sugar substrates by microorganisms would be an economically favorable process. Succinate-producing bacteria such as *Actinobacillus succinogenes,* and *Anaerobiospirillum succiniciproducens* have been used to produce succinate in industry so far. However, they require complex media for enhancing the bacterial growth and gases for generating anaerobic conditions. Therefore, the fermentation processes of these microorganisms make the price of succinate not competitive enough compared to the petrochemically produced compound. *Escherichia coli* exhibits a high growth rate and its metabolic pathways can easily be manipulated by genetic engineering. As a result, succinate production by fermentation of *E. coli* has the potential to become practical in industry through strain improvement and process design. In the past decade, many research groups have been extensively studying methods to attain high production yield of succinate by metabolic engineered *E. coli* strains (Vemuri *et al*., 2002; Lin *et al*., 2005; Sanchez *et al*., 2005; Andersson *et al*., 2007). Nevertheless, most of the studies of succinate production from *E. coli* derivatives have been performed in nutrient-enriched media in an association with gas exhaustion. Then the costs related to media compositions, gases connected to the fermentor and downstream processing including product recovery and purification processes, which are required to remove impurities, have been very high. To become more attractive, the production of succinate in *E. coli* should be performed fermentatively under any simple processes without the requirements of complex nutrients and/or any gases during fermentation to minimize the purification steps and the production costs.

Recently, Jantama *et al*. (2008) reported the development of *E. coli* strain named KJ073 that produce succinate under simple anaerobic conditions in mineral salts medium without any requirements of rich nutrients for promoting growth and antibiotics for expressing heterologous genes. This was accomplished by combining metabolic engineering and metabolic evolution methods. The strain produced succinate near the theoretical maximum and in very high titer from glucose. It was implied that strain KJ073 utilized almost all PEP pool derived from glucose to succinate production with less carbon loss to pyruvate through the classical PEP-dependent PTS (phosphotransferase system) for transporting glucose. It was likely that strain KJ073 did not use PEP-dependent PTS transporters for glucose uptake. Therefore, it is very interesting to identify the sugar-transporting pathway utilized by strain KJ073.

In this paper, I used the gene deletion framework to elucidate the key sugar-transporting pathways that are utilized by the strain and the metabolic flux analysis (MFA) approach to explain how gene deletions would affect the succinate production. This would also reveal the changes in the intrinsic character of cultures producing succinate, the intracellular metabolic fluxes, internal redox balance, and energy production-consumption profile. The information obtained from this study could be useful in further strain improvements through engineering the sugar-transporting pathways to enhance the rate of succinate production in this strain.

Materials and Methods

Strains, Media and Growth Conditions

Strains used in this study are summarized in Table 1. Cultures were grown at 37°C in modified LB broth (per liter: 10 g tryptone, 5 g yeast extract, 5 g sodium chloride) (Miller, 1992) only during strain construction. Antibiotics were included as appropriate.

AM1 mineral salts medium (Matinez *et al*., 2007) supplemented with 100 mM $KHCO_3$, 1 mM betaine HCl, and 5% (w/v) glucose was used as a fermentation broth in most studies. No gene encoding antibiotic resistance and plasmid is present in strains developed except in intermediates during construction.

Table 1. ***Escherichia coli*** **strains, plasmids, and primers used in this study**

Strain	Relevant characteristics	Source
KJ073	*E. coli* B, Δ*ldhA* Δ*adhE* Δ*ackA* Δ*pflB* Δ*mgsA* Δ*poxB*	Jantama *et al*., 2007
KJ073P	KJ073, Δ*ptsG*	This study
KJ073G	KJ073, Δ*galP*	This study
KJ073M	KJ073, Δ*manX*	This study
KJ073GM	KJ073, Δ*galP* Δ*manX*	This study
KJ073PM	KJ073, Δ*ptsG* Δ*manX*	This study
KJ073GP	KJ073, Δ*galP* Δ*ptsG*	This study
Plasmid	**Relevant characteristics**	**Source**
pKD4	*bla* FRT-*kan*-FRT	Datsenko, 2000
pKD46	*bla* γ β *exo* (Red recombinase), temperature-conditional replicon	Datsenko, 2000
pFT-A	*bla flp* temperature-conditional replicon and FLP recombinase	Posfai, 1997
Primer	**Relevant characteristics**	**Source**
ptsG	**5'ATGTTTAAGAATGCATTTGCTAACCTGCAAAAGGTCGGTAAATCGTGTA**GGCTGGAGCTGCTTC3' 5'**TTAGTGGTTACGGATGTACTCATCCATCTCGGTTTTCAGGTTATC**CATATGAATATCCTCCTTAG3'	This study
galP	5'**ATGCCTGACGCTAAAAAACAGGGGCGGTCAAACAAGGCAATGACG**GTGTAGGCTGGAGCTGCTTC3' 5'**TTAATCGTGAGCGCCTATTTCGCGCAGTTTACGACCTTTCATCAG**CATATGAATATCCTCCTTAG3'	This study
manX	5'**GTGACCATTGCTATTGTTATAGGCACACATGGTTGGGCTGCAGAG**GTGTAGGCTGGAGCTGCTTC3' 5'**TTACTTATCGATTTTGCTGATCAGATCCATCATTTTCAGTTTCGG**CATATGAATATCCTCCTTAG3'	This study

Gene Deletion Techniques

Plasmids and primers used in this study are also summarized in Table 1. Methods for chromosomal deletions, integration, and removal of antibiotic resistance genes have been previously described in Datsenko and Wanner, 2000 (Figure 1). Briefly, sense primers contain sequences corresponding to the N-terminus of each targeted gene (boldface type) followed by 20 bp (underlined) corresponding to the FRT-*kan*-FRT cassette. Anti-sense primers contain sequences corresponding to the C-terminal region of each targeted gene (boldface type) followed by 20 bp (underlined) corresponding to the cassette. Amplified DNA fragments were electroporated into *E. coli* strains harboring Red recombinase (pKD46). In resulting recombinants, the FRT-*kan*-FRT cassette replaced the deleted region of the target gene by homologous recombination (double-crossover event). The resistance gene (FRT-*kan*-FRT) was subsequently excised from the chromosome with FLP recombinase using plasmid pFT-A, leaving a scar region containing one FRT site. Chromosomal deletions and integrations were verified by testing for antibiotic markers, PCR analysis, and analysis of fermentation products.

Fermentations

Seed cultures and fermentations were incubated at 37°C (100 rpm) in AM1 mineral salts medium containing 5% (w/v) glucose, 100 mM $KHCO_3$, and 1 mM betaine HCl. A mixture of 3M K_2CO_3 and 6N KOH (6:1 ratio) was added to maintain pH and supply CO_2. Fermentations were carried out in small vessels with a working volume of 350 ml. Fermentations were inoculated at an initial OD550 of 0.01. Anaerobiosis was rapidly achieved during growth. Added bicarbonate served to ensure an atmosphere of CO_2.

Analyses

Cell mass was estimated from the optical density at 550 nm (OD 1.0 = 333 mg CDW L^{-1}) by using a Bausch & Lomb Spectronic 70 spectrophotometer. Organic acids and sugars were determined by using high performance liquid chromatography with Biorad Aminex HPX-87H ion-exchange column.

Metabolic Flux Analysis

Calculation of Secreted Metabolite Specific Production Rates

The specific production rates of several excreted metabolites during exponential

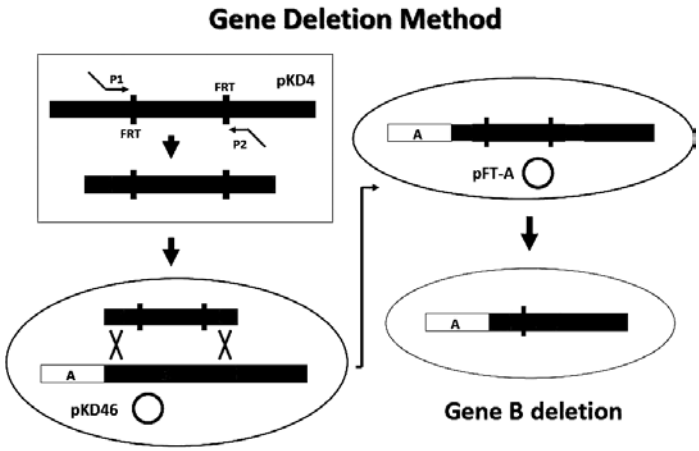

Figure 1. Summary of gene deletion method used in this study

growth were calculated using the measurements during stationary growth and based on the log mean average cell concentration during that time interval (Aristidou, 1995):

$$r_i = \frac{c_i(t+\Delta t) - c_i(t)}{\overline{X}\,\Delta t} \qquad (1)$$

where c_i is the concentration of metabolite *i* in the reaction (mmol L^{-1}) and $\overline{X}$ is the log mean concentration of biomass (gCDW L^{-1})

$$\overline{X} = \frac{X(t+\Delta t) - X(t)}{\ln\left(\frac{X(t+\Delta t)}{X(t)}\right)} \qquad (2)$$

Calculation of Metabolic Fluxes

The intracellular fluxes were calculated using a stoichiometric model and applying mass balances around intracellular metabolites (Aristidou *et al.*, 1999). Figure 2 shows the fermentation pathways of *E. coli*. The reactions involved in the fermentation network are presented in Table 2. The material balances result in a set of linear algebraic equations that can be expressed in matrix notation as:

$$r = \mathrm{M}\,v\,, \qquad (3)$$

where *r* is the vector for the net specific formation rates of 16 metabolites, *v* is the

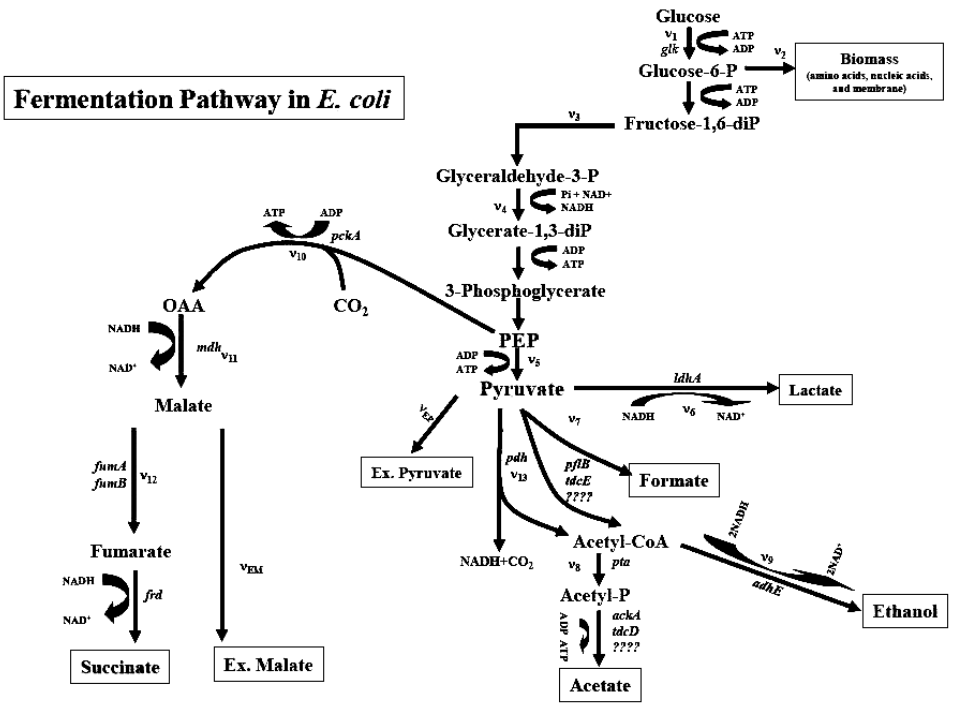

Figure 2. Fermentation Pathway of *E. coli* under anaerobic condition. v_i, represents the fluxes used in the calculation for metabolic flux analysis. The boxes represent the measured metabolites. Solid arrows represent central fermentative pathways. Enzymes listed are *pdh*, pyruvate dehydrogenase; *mdh*, malate dehydrogenase; *fumA* and *fumB*, fumarase isozymes; *frd*, fumarate reductase; *mgsA*, methylglyoxal synthase; *poxB*, pyruvate oxidase; *ppc*, phosphoenolpyruvate carboxylase; *ldhA*, lactate dehydrogenase; *pflB*, pyruvate-formate lyase; *adhE*, alcohol dehydrogenase, *ackA*; acetate kinase; *tdcD*, propionate kinase, *tdcE*; 2-ketobytyrate formate-lyase; *glk*, glucokinase; *pckA*, PEP carboxykinase

Table 2. Summary of the reactions used in Metabolic Flux Analysis (MFA) for succinate production under anaerobic condition

	Reaction	Enzyme (s)	EC number
1	Glucose + ATP ⇔ Glucose-6P + ADP	Galactose permease	-
2	Glucose-6P ⇔ Biomass		
3	Glucose-6P + ATP ⇔ 2Glyceraldehyde 3-P + ADP	Glucose-6P isomerase	5.3.1.9
	a. Glucose-6P ⇔ Fructose 6-P	Phosphofructokinase	2.7.1.11
	b. Fructose-6P+ATP ⇔ Fructose 1,6-diP+ADP	Fructose-diP aldolase	4.1.2.13
	c. Fructose 1,6-diP ⇔ 2Glyceraldehyde 3-P		
4	Glyceraldehyde-3P + NAD^+ + Pi + ADP □ PEP + ATP + NADH + H^+_+ H_2O		
	a. Glyceraldehyde-3P + NAD^+ + Pi ⇔ Glycerate-1,3-diP +NADH+H^+	3P-Glyceraldehyde dehydrogenase	1.2.1.12
	b. Glycerate-1,3-diP + ADP ⇔ 3-Phosphoglycerate + ATP	3P- Glycerate kinase	3.6.1.7
	c. 3-Phosphoglycerate ⇔ 2-Phosphoglycerate	Phosphoglycerate mutase	5.4.2.1
	d. 2-Phosphoglycerate ⇔ PEP + H_2O	Enolase	4.2.1.11
5	PEP + ADP ⇔ Pyruvate + ATP	Pyruvate kinase	2.7.1.40
6	Pyruvate + NADH ⇔ Lactate + NAD^+	Lactate dehydrogenase	1.1.1.28
7	Pyruvate + HSCoA ⇔ Formate + Acetyl-CoA	Pyruvate-formate lyase	2.3.1.54
8	Acetyl-CoA +Pi + ADP ⇔ Acetate + HSCoA + ATP		
	a. Acetyl-CoA+Pi ⇔ Acetyl-P + HSCoA	Acetate phosphotransferase	2.3.1.8
	b. Acetyl-P + ADP ⇔ Acetate + ATP	Acetate kinase	2.7.2.1
9	Acetyl-CoA + 2NADH + $2H^+$ ⇔ Ethanol + HSCoA + $2NAD^+$		
	a. Acetyl-CoA + $NADH^+$ H+ ⇔ Acetaldehyde + HSCoA + NAD^+	Aldehyde dehydrogenase	1.2.1.10
	b. Acetaldehyde + $NADH^+$ H+ ⇔ Ethanol + NAD+	Alcohol dehydrogenase	1.1.1.1
10	PEP + CO_2 + ADP ⇔ OAA + ATP	PEP carboxykinase	4.1.1.49
11	OAA + NADH ⇔ Malate + NAD^+	Malate dehydrogenase	1.1.1.37
12	Malate + NADH + MQ ⇔ Succinate + MH_2 + H_2O	Fumarase ABC	
	Malate ⇔ Fumarate + H_2O	Fumarate reductase	4.2.1.2
	Fumarate + NADH + MQ ⇔ Succinate + MH_2		1.3.5.-
13	Pyruvate + HSCoA + NAD^+ ⇔ Acetyl-CoA + CO_2 + NADH + H^+	Pyruvate Dehydrogenase (PDH) Complex:	
		AceF: lipoate acetyltransferase	2.3.1.12
		AceE: E1p subunit of PDH	1.2.4.1
		LpdA: lipoamide dehydrogenase	1.8.1.4
EM	Malate transport	Dicarboxylate DAACS transporter, DctA	-
EP	Pyruvate transport	-	-

vector of up to 15 metabolic fluxes (mmol $gCDW^{-1}$ h^{-1}) shown in Figure 2 ($\nu^T = [\nu_1, \nu_2, \ldots, \nu_{13}, \nu_{EM}, \nu_{EP}]$), and M is the matrix of stoic hiometric coefficients presented in Table 3. The concentrations of eight extracellular products (glucose, succinate, acetate, formate, lactate, ethanol, excreted malate, and excreted pyruvate) represented in the boxes of Figure 2 were measured, and their specific production rates were calculated using Equation (1). The net accumulation rates of intracellular metabolites were assumed to be zero. This is justified because pseudo-steady state can be assumed due to the high turnover of the metabolite pool or, alternatively, balanced growth, and because the dilution term due to growth can be neglected due to low intracellular levels (Stephanopoulos *et al.*, 1998). For the NADH balance, it is assumed that the NADH production and consumption must be equal and this is also included as a constraint for the calculations.

The solution of Equation (3) is obtained using the pseudo-inverse matrix since the system is overdetermined. This corresponds to the least squares estimate.

$$\nu = (M^T M)^{-1} M^T r \quad (4)$$

The net NADH specific production and utilization rates per mole of glucose consumed are expressed as follows. Since the balance of NADH was used as a constraint for calculation, the specific production and utilization rates of NADH would be equal.

$$NADH_P/G = \frac{(\nu_4 + \nu_{13})}{\nu_1} \quad (5)$$

$$NADH_U/G = \frac{(\nu_6 + 2\nu_9 + \nu_{11} + \nu_{12})}{\nu_1} \quad (6)$$

To calculate the split flux ratio at PEP node,

Split flux ratio through

$$PYK = \frac{\nu_5}{(\nu_5 + \nu_{10})} \quad (7)$$

Split flux ratio through

$$PCK = \frac{\nu_{10}}{(\nu_5 + \nu_{10})} \quad (8)$$

ATP Analysis

Reaction 5 is reversible (Table 2), and the direction affects ATP production or consumption. From flux analysis results (Table 5), all fluxes through pyruvate, ν_5 were greater than zero in strain KJ073 and its derivatives. Zhang *et al.* (2010) reported that KJ073 exhibited PEP carboxylation to OAA by PCK, which is normally involved in gluconeogenesis, instead PPC in reaction 10 (Table 2). The PEP carboxylation process by PCK yields one mole of ATP when every mole of OAA is produced. Then the following net of ATP produced per mole of glucose used is calculated as Equation (9).

$$ATP/G = \frac{(\nu_4 + \nu_5 + \nu_8 + \nu_{10} - \nu_1 - \nu_3)}{\nu_1} \quad (9)$$

Results and Discussion

Up-regulating the Galactose Permease for glucose uptake in *E. coli* KJ073

The formation of succinate during anaerobic conditions in *E. coli* occurs via carboxylation of PEP. Endogenous or exogenous carbon dioxide is combined with PEP. The OAA formed is reduced to malate and to further succinate by the activities of malate dehydrogenase and fumarate reductase, respectively. Simultaneously, the net of four reducing equivalents (NADH) is re-oxidized through this pathway to regenerate NAD^+ for carrying the electron in glycolysis (Cecchini *et al.*, 2002). However, succinate formed in the pathway is essentially irreversible because of the energy loss associated with the release of inorganic phosphate thus making this reaction not preferable. Then the assimilation of PEP to pyruvate with ATP production is more favored in which the carbon flux from PEP through acetate is found in a greater manner than those

Table 3. Stochiometric relationship between the metabolic intermediates and metabolites and the network reactions (matrix M represented) for an anaerobic succinate production in glucose minimal medium in *E. coli* KJ073 and its derivatives

Flux To	r1	r2	r3	r4	r5	r6	r7	r8	r9	r10	r11	r12	r13	rEM	rEP
Glucose used	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
succinate	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Acetate	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Formate	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Lactate	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Ethanol	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Excreted Malate	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Excreted Pyruvate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Biomass	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Pyruvate	0	0	0	0	1	-1	-1	0	0	0	0	0	-1	0	-1
Glucose-6P	1	-1	-1	0	0	0	0	0	0	0	0	0	0	0	0
Glyceraldehyde-3P	0	0	2	-1	0	0	0	0	0	0	0	0	0	0	0
Acetyl-CoA	0	0	0	0	0	0	1	-1	-1	0	0	0	1	0	0
PEP	0	0	0	1	-1	0	0	0	0	-1	0	0	0	0	0
OAA	0	0	0	0	0	0	0	0	0	1	-1	0	0	0	0
Malate	0	0	0	0	0	0	0	0	0	0	1	-1	0	-1	0
NADH	0	0	0	1	0	-1	0	0	-2	0	-1	-1	1	0	0

through succinate.

To enhance the succinate production in *E. coli*, strain KJ073 was engineered and developed by combining the metabolic engineering with the growth-based selection (metabolic evolution) approaches for both conserving the reducing equivalents and directing the carbon flow to PEP carboxylation generating OAA. This led us to produce succinate as a major fermentative product during anaerobic conditions (Figure 3). The succinate productions obtained from strain KJ073 were 1.2 mol/mol substrate consumed in yield, which is closed to theoretical maximum when glucose was used as a sole carbon source (Jantama *et al.*, 2008). It was likely that PEP pool produced from glucose flux was mostly directed to the carboxylation process to OAA, resulting in high rate of succinate production in strain KJ073, which was about 13 mmol $gCDW^{-1}$ h^{-1} (Figure 4). Unlike *E. coli* wild type, how strain KJ073 conserved and directed the PEP pool in higher fraction would be interesting.

It has been generally known that the phosphotransferase system (PTS) is the major sugar transporters that are responsible for the entry of various carbohydrates including glucose in gram-negative, and gram-positive bacteria including *E. coli* (Plumbridge, 2002). Glucose can specifically enter the cell by either of two PTS transporters. First, the glucose-specific EIIBC, product of *ptsG*, locate at 25 min on the *E. coli* chromosome. Second, the mannose PTS, EIIAB, IIC, IID^{Man}, which are encoded by the *manXYZ* genes, locates at 41 min (Plumbridge, 1998). The mannose PTS transports many sugars including glucose, mannose, fructose, and N-acetylglucosamine, and glucosamine

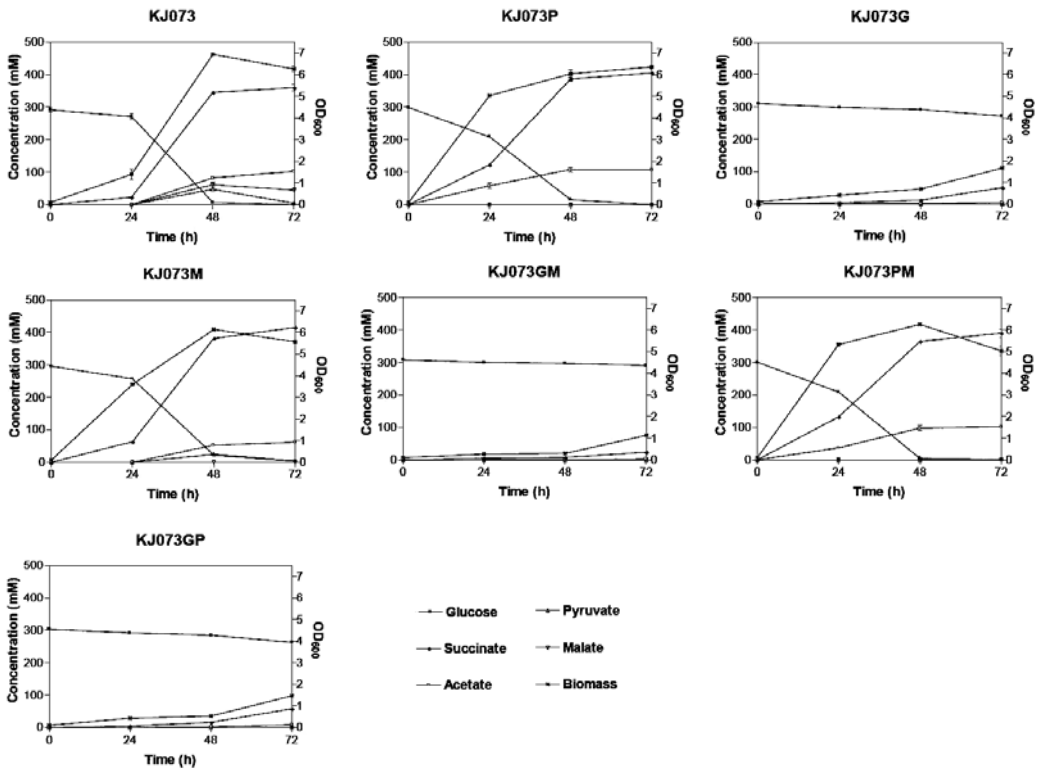

Figure 3. Fermentation profiles in 5% (w/v) glucose AM1 medium of various *E. coli* strains

(Postma *et al.*, 1996). Both PTS systems form a cascade of phosphorylated intermediates that transfer a phosphate from intracellular PEP to the incoming glucose. Glucose-6-phosphate (Glu-6-P) produced is the central compound of intermediary metabolism and the starting point for energy production by glycolysis. However, *E. coli* usually utilizes half of PEP produced from glucose to uptake and to phosphorylate exogeneous glucose into the cell during glucose transport via PTS systems. This has an effect to limit the redox balance and ATP production as well as to lower PEP pool size. Taking this to consideration, it is impossible for *E. coli* KJ073 to produce succinate such a high titer from almost all the glucose fluxes if PTS systems function in the strain. How the strain transported exogeneous glucose into the cell without affecting PEP pool for carboxylation to OAA was expected.

It was speculated that glucose might enter the cell with a non-phosphorylated form by a sugar-H^+ symport mechanism via galactose permease, product of *galP* (Curtis and Epstein, 1975) and might be further transformed to Glu-6-P inside the cell by the action of glucokinase activity with ATP utilization. To test the hypothesis, genes involved in glucose uptake including PTS (*ptsG*, and *manX*) and non-PTS (*galP*) dependent systems were deleted from strain KJ073. The results showed that the deletion of *galP* in strain KJ073 significantly decreased specific glucose consumption rates and fluxes, while the deletions of *ptsG*, *manX*, and even in double mutations of *ptsG* and *manX* did not considerably affect the specific glucose consumption rates and fluxes (Figure 4, and Table 4). Consequently, the decreases in specific glucose consumption fluxes in *galP*$^-$ strains also caused great lessening in the specific succinate production fluxes about 85-90% comparing to those of strain KJ073 (Table 4). Nevertheless, the deletion of *galP* did not abolish all abilities of glucose transport in the strains lacking *GalP*. It would indicate that the PTS systems would transport the glucose in some extent. The reduction in the specific glucose consumption rates and fluxes in *galP*$^-$ mutants was in the agreement with increased expression of this gene about 20-fold in strain KJ073 comparing to its parental strain of *E. coli* ATCC8739 wild type (Zhang *et al.*, 2010). How GalP evolved in glucose uptake instead of that by PEP-dependent PTS system in KJ073 could be explained as follows.

Strain KJ073 exhibited the loss of catabolite repression due to increasing levels of cyclic-AMP (cAMP) and cAMP receptor protein (CRP) even it grew on glucose (Zhang *et al.*, 2010). It should be noted that the loss of catabolite repression in strain KJ073 may activate transcription of *mlc* gene, which

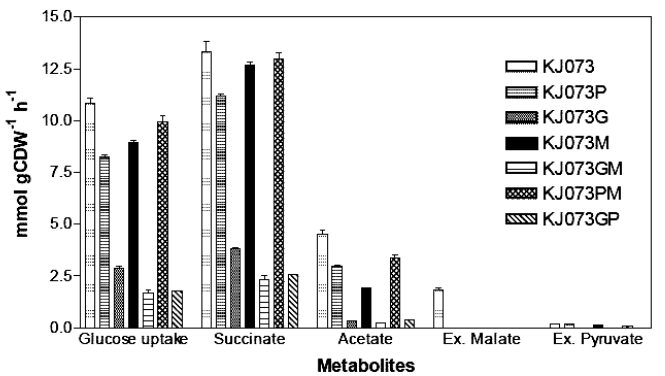

Figure 4. Specific production rate of extracellular metabolites

Table 4. Metabolic fluxes distribution of an anaerobic succinate production in 5% (w/v) glucose AM1 of various *E. coli* strains

Flux To[a] (mmol $gCDW^{-1}h^{-1}$)	Strains						
	KJ073	**KJ073P**	**KJ073G**	**KJ073M**	**KJ073GM**	**KJ073PM**	**KJ073GP**
Glucose, v_1	11.04 ± 0.33	8.37 ± 0.16	2.97 ± 0.13	9.16 ± 0.14	1.60 ± 0.29	9.39 ± 0.43	1.79 ± 0.02
Biomass, v_2	0.42 ± 0.01	0.43 ± 0.04	0.15 ± 0.01	0.45 ± 0.02	0.11 ± 0.03	0.39 ± 0.11	0.11 ± 0.02
Gly-3P, v_3	10.64 ± 0.10	8.05 ± 0.13	2.70 ± 0.10	8.90 ± 0.13	1.56 ± 0.29	9.05 ± 0.34	1.60 ± 0.02
PEP, v_4	21.34 ± 0.26	16.16 ± 0.25	5.45 ± 0.20	17.6 ± 10.26	2.98 ± 0.57	18.10 ± 0.66	3.27 ± 0.05
Pyruvate, v_5	6.16 ± 0.01	4.89 ± 0.05	1.84 ± 0.03	5.23 ± 0.07	1.05 ± 0.16	5.44 ± 0.21	1.07 ± 0.00
Lactate, v_6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Formate, v_7	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Acetyl-P, v_8	5.08 ± 0.13	3.67 ± 0.05	0.74 ± 0.01	3.07 ± 0.02	0.49 ± 0.06	4.20 ± 0.18	0.62 ± 0.02
Ethanol, v_9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
OAA, v_{10}	14.94 ± 0.13	11.35 ± 0.18	3.62 ± 0.13	12.32 ± 0.18	1.93 ± 0.02	12.67 ± 0.46	2.12 ± 0.05
Malate, v_{11}	14.70 ± 0.06	11.36 ± 0.16	3.43 ± 0.11	11.79 ± 0.16	2.00 ± 0.39	12.17 ± 0.44	2.21 ± 0.04
Succinate, v_{12}	12.97 ± 0.45	11.49 ± 0.17	3.43 ± 0.10	11.78 ± 0.16	2.00 ± 0.37	11.92 ± 0.46	2.24 ± 0.06
PDH, v_{13}	5.86 ± 0.13	4.71 ± 0.03	1.55 ± 0.04	5.16 ± 0.05	1.05 ± 0.13	5.41 ± 0.20	0.70 ± 0.01
EM, v_{EM}	1.85 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
EP, v_{EP}	0.19 ± 0.02	0.17 ± 0.00	0.00 ± 0.00	0.13 ± 0.02	0.00 ± 0.00	0.07 ± 0.04	0.00 ± 0.00
ATP/G	2.34 ± 0.03	2.35 ± 0.01	2.02 ± 0.02	2.20 ± 0.03	1.90 ± 0.04	2.34 ± 0.03	2.08 ± 0.01
$NADH_P$/G	2.46 ± 0.15	2.51 ± 0.21	2.36 ± 0.33	2.49 ± 0.26	2.52 ± 0.21	2.50 ± 0.11	2.36 ± 0.11
$NADH_U$/G	2.50 ± 0.22	2.73 ± 0.32	2.31 ± 0.32	2.57 ± 0.23	2.50 ± 0.28	2.56 ± 0.20	2.49 ± 0.09
Split Flux Ratio through PCK	0.71 ± 0.01	0.70 ± 0.05	0.66 ± 0.03	0.70 ± 0.07	0.65 ± 0.05	0.70 ± 0.15	0.66 ± 0.00
Split Flux Ratio through PYK	0.29 ± 0.07	0.30 ± 0.10	0.34 ± 0.08	0.30 ± 0.10	0.35 ± 0.04	0.30 ± 0.21	0.34 ± 0.03

[a] All fluxes were calculated during anaerobic fermentation. The fermentations were performed in triplicate.

encodes the repressor responsible for the glucose induction of sugar PTS genes including *ptsG* and *ptsHI* as well as *manXYZ* operons. Mlc represses the transcriptions by binding its DNA-binding sites of upstream promoters of PTS genes (Shin *et al*., 2001; Tanaka *et al*., 2000). It implies that Mlc proteins prevent the recruitments of their transcriptional factors and RNA polymerase to promoter sites of PTS regulons. In addition, KJ073 containedthe frame-shift mutation at the carboxyl terminus of *ptsI* gene. Ryu (1995) suggested that the mRNA transcribed from *ptsHI*p0 promoter was shown to be post-transcriptional regulation by RNaseE-dependent degradation when *ptsI* mutants were present. This would prevent unnecessary synthesis of PtsI (Enzyme I; EI) and PtsH (HPr) that are common for PTS conserved domains to most sugars, hence prevent uptake of glucose when the cell is no longer able to utilize PEP-dependent PTS efficiently due to mutations. To conclude, strain KJ073 might inactivate the PEP-dependent PTS system by the combinations of loss of catabolite repression, gain of Mlc repression, presences of *ptsI* mutation, and regulation of RNaseE-dependent cleavage. It may result that alternative glucose uptake systems such as GalP would be activated and could functionally replace the glucose PEP-dependent PTS to restore glucose uptake.

Validating the Fermentation Pathway for Metabolic Flux Analysis

In this study, succinate production in strain KJ073 was assumed to be produced via the reductive TCA route only during anaerobic fermentation (Figure 1). The strain exhibited PCK (phosphoenolpyruvate carboxykinase) activity to carboxylate PEP to OAA with one mole of ATP production instead of PPC (phosphoenolpyruvate carboxylase) (personal communication). In addition, *E. coli* can also perform the glyoxylate bypass, which is an alternative pathway producing succinate. However, KJ073 was confirmed that it did not exhibit a detectable enzymatic activity of isocitrate lyase (Jantama *et al*., 2008). Therefore, the glyoxylate bypass was not considered in this metabolic flux analysis.

As known, the PDH (pyruvate dehydrogenase) complex for pyruvate conversion is inactive under anaerobic conditions (Guest *et al*., 1989; Unden *et al*., 2002). However, de Graef *et al*. (1999) showed low but significant PDH activity when *E. coli* grows under anaerobic conditions. Kim *et al*. (2007) further showed that the PDH activity in the anaerobic cell could be significantly elevated by a single mutation in *lpdA* gene encoding lipoamide dehydrogenase enzyme. The PDH complex provides an alternative route to acetyl-CoA, NADH, and CO_2 from pyruvate. An extra NADH gained from the PDH activity can supply the additional requirement of reducing power to produce succinate. Also, the enzymatic activity of this complex was very high in strain KJ073 even when it grew under anaerobic conditions (Jantama *et al*., 2008). Hence, the PDH pathway was included in the metabolic flux analysis. Consequently, the matrix M was constructed based on the conditions defined above (Table 3). This work firstly demonstrated the metabolic flux calculation based on the enzymatic activities of key enzymes for succinate production in which the wild type strains (not employed metabolic evolution) of *E. coli* do not usually exhibit in the certain direction under anaerobic conditions.

Metabolic Flux Analysis of KJ073

For MFA of KJ073, the important notice was a branch point at PEP node, where the carbon flux from catabolism (v_4) splits to pyruvate (v_5) and to OAA (v_{10}). The split flux ratio at this node was about 30% of PEP flux flowing to pyruvate (Table 4). This phenomenon was opposed to the wild type *E. coli* since the majority of the flux to pyruvate about 90% was observed while the flux to OAA was less (unpublished data). Note that the apparent K_m (Michaelis-Menten constant) for PEP of PYK (0.2 mM, Kornberg and Malcovati, 1973) is considerably lower than that of PPC (0.8 mM, Smith *et al*., 1980).

Strain KJ073 was previously reported

that an enzymatic activity of PPC was down-regulated, however, PCK (phosphoenolpyruvate carboxykinase) activity was increased comparing to those of wild type (Zhang *et al.*, 2010). The increase of PCK activity found in KJ073 was similar to an enhancement of succinate production in the presence of bicarbonate found in ruminant bacteria exhibiting PEP carboxylation via PCK (Samuelov *et al.*, 1991). High bicarbonate concentration promotes growth in ruminant bacteria because succinic acid is synthesized via CO_2 fixation of C_3 metabolites. However, it was reported that the K_m value of PCK for HCO_3^- is 13 mM, whereas that of PPC for HCO_3^- is 0.15 mM (Izui *et al.*, 1981). Thus, in the presence of both PPC and PCK enzymes (KJ073 and its derivatives), PPC would perform PEP carboxylation at a low concentration of HCO_3^-. On the other hand, PCK would be able to catalyze the reaction at a high concentration of HCO_3^-. It would be likely since HCO_3^- was provided at high concentration (about 100 mM) in the starting medium used in this study. Also, HCO_3^- was additionally supplied to the medium during fermentation as a neutralizing agent simultaneously when acids were produced. As a result, PCK would be responsible for OAA formation in strain KJ073 under conditions studied. In addition, the PCK reaction appears to be more suitable for succinic acid production because it generates nucleotide triphosphate (ATP), thus conserving the energy, whereas the PPC reaction generates no nucleotide triphosphate.

Strain KJ073 produced the high amount of succinate, which was splitting the flux at the PEP node to almost about 70% succinate (Table 4). It seemed that the redox balance probably controls this split, with higher NADH production through PDH favoring succinate. Strain KJ073 exhibited higher PDH activity (Jantama *et al.*, 2008) resulting in the greater flux out of the pyruvate node through PDH (v_{13}) leading to the production of acetate than that of *E. coli* wild type. The increase in the PDH flux is in agreement with the rate of succinate production (Table 4). This suggests that NADH produced from the PDH activity can increase the flux to succinate and balance the NADH production and consumption in strain KJ073. From Table 4, the redox balance is accomplished by having $v_4 + v_{13} \approx v_{11} + v_{12}$ in the strains lacking the ability to produce ethanol and lactate (e.g. Strain KJ073). The increased pyruvate flux through PDH would also result from compensating the production of acetyl-CoA as source of C_2 for biosynthesis in the strain KJ073, which also lacked PFL-B (pyruvate-formate lyase).

Besides the effects of internal redox state balance and permitting the acetyl-CoA production, PDH complex activity observed during anaerobic fermentation in KJ073 could be explained by the regulation of FNR (fumarate-nitrate regulatory protein) and intracellular pyruvate. FNR and PdhR repressor proteins control *pdh* transcription (Quail and Guest, 1995). In the absence of pyruvate, PdhR binds to the promoter of the *pdh* operon and inhibits transcription. Increased intracellular pyruvate levels because of the cell's inability to dissimilate pyruvate might result in binding of pyruvate with PdhR, thus releasing PdhR from the promoter region of the *pdh* operon. Control by FNR alone might not be enough to repress *pdh* transcription, reflecting a great increased flux through the PDH complex in the strain lacking PFL-B activity. The benefit of activation of the PDH activity to succinate production is that the flux via the PDH complex provides an extra mole of NADH that can be used to reduce malate to succinate.

Metabolic Flux Analysis in *galP*⁻ mutants

In all *galP*⁻ strains, the split flux ratio at PEP node through PYK was increased up to 5% compared among those of *galP*⁺ strains (Table 4). It might imply that the strains did not only require ATP for growth and maintenance, but also needed pyruvate and acetyl-CoA for biosynthesis and biomass. Under anaerobic conditions, *E. coli* usually shows the majority of the flux going to glyceraldehydes-3-P (v_3) resulting in decreasing the flux to biomass (v_2) (Tempest and Neijssel,

1987). It is indicated that the cells are less capable to dealing with energy production under anaerobic conditions. This also applied to strain KJ073 and its *gal*⁻ mutant derivatives (Table 4). The increase in intracellular pyruvate pool as precursors was expected to further maintain biomass level rather than losing the carbon skeleton by expensing to succinate when the glucose consumption fluxes were severely low due to only presence of down-regulated PTS systems in the *galP*⁻ strains.

The activation of GalP led the strain KJ073 exhibiting the high fluxes to acetyl-P or acetate (v_8) up to almost 50% of acetate per glucose consumed. It demonstrated that the *galP*⁻ mutants could lower the flux through PTA (phosphotransacetylase) up to 25% of acetate per glucose consumed (Table 4) due to a decrease in the glucose consumption flux. However, the high excretion of acetate in strain KJ073 might be due to the high glycolytic flux over a critical limit caused by GalP activation. Since strain KJ073 exhibited high flux through PCK to OAA, the flux through PDH was higher to maintain the redox balance required for succinate production. As a result, acetyl-P was produced from pyruvate via PDH, then accumulated and secreted as acetate. This phenomenon resembled to the overflow metabolism that usually happens when growing *E. coli* at a high rate of glucose consumption under aerobic conditions. Acetyl-CoA generated through PDH complex by pyruvate is secreted out of the cell directly to acetate instead of entering TCA cycle (Vemuri *et al.*, 2006).

In addition, the strain KJ073 excreted some amounts of pyruvate (v_{EP}). But the level of pyruvate excreted was even lower in *ptsG*⁻ and/or *manX*⁻ mutants, while v_{EP} was not observed in *galP*⁻ strains. It indicated that the rate of glucose consumption was greater than the capacity to re-oxidize the reduced equivalents (NADH) via succinate production route. To conserve the redox balance, the level of intracellular pyruvate has to be maintained. Therefore, an excess of intracellular pyruvate would be excreted in which the flux was found only in strain KJ073 and *ptsG*⁻ and/or *manX*⁻ mutants, but not in the strains lacking GalP that exhibited low glycolytic fluxes. Furthermore, the high glycolytic flux in strain KJ073 did not only contribute to accumulations of acetate and pyruvate in medium, but also malate. This demonstrated that the high glycolytic flux in strain KJ073 did not limit the flux through PCK to OAA. In contrast, the flux through PDH to acetyl-CoA to generate extra NADH was limited resulting in secretion of malate (v_{EM}) to prevent accumulation of malate in the cells.

ATP Analysis

In wild type *E. coli*, acetyl-CoA is produced and converted into approximately equal molars of ethanol and acetate. This process yields an additional mole of ATP per mole of glucose consumed. Fifty percent of PEP is required for the PTS systems to transport extracellular glucose that does not produce ATP. Then a maximum net ATP/G of 3 is achieved if another two net moles of ATP are gained from glycolysis (Tempest and Neijssel, 1987). This is true when the carbon loss through OAA is neglected. However, during fermentation the wild type strain also produces succinate. The carbon flux to the succinate production pathway does not produce ATP, resulting in production of ATP_B/G less than 3, approximately 2.5 (Jantama *et al.*, 2008). The amount of ATP produced depends on the split flux ratio from PEP to pyruvate (v_5) and to OAA (v_{10}) in wild type strain. The net maximum ATP/G produced in the strain lacking ACK-A and PFL-B should be less than that of wild type produced about one (approximately 1.5) because it could produce ATP only from glycolysis under anaerobic conditions (Tempest and Neijssel, 1987). As seen, the production of ATP in the strain lacking ACK-A and PFL-B would be low. How the strain resolving the energy shortage to maintain a certain intracellular ATP level used for promoting cell growth and metabolic activity is attractive.

From the flux analysis, strain KJ073 and its mutants (strains without ACK-A and PFL-B) produced ATP/G about 2-2.35

(Table 4) that was approximately the same level as shown in wild type strain. This confirmed that the inactivation of PEP-dependent PTS transporters in strain KJ073 conserved the PEP pool. Consequently, strain KJ073 utilized the conserved PEP pool and gained the benefit of generating ATP concomitantly with equivalent production of succinate through PCK activity (Figure 2). The net gain would be one extra mole of ATP per mole of OAA produced (Krebs and Bridger, 1980). However, the ATP/G was lower in *galP*$^-$ strains about 15-20% compared to those of *galP*$^+$ strains. It might be caused by the lower split flux ratio to OAA at PEP node due to lower glucose consumption fluxes in *galP*$^-$ strains.

Conclusions

The metabolism of *E. coli* is remarkably adaptive. The combination of gene deletions and metabolic evolution can result in significant changes in metabolite fluxes. Some interesting changes identified in this study are the significantly decreased glucose consumption fluxes by the PEP-dependent PTS transporters, the activation of GalP activity, and increase in split flux ratio through PYK at PEP node in *galP*$^-$ mutants. The better understanding of what mechanism the strains use for glucose transport and of how the strains metabolize glucose obtained in this study would allow us to engineer the strains for higher rates of glucose catabolism, resulting in increases in succinate productivities. In addition, MFA also provided us with insights and preliminary information on how the intracellular metabolite fluxes change at branch points as a result of the activation of GalP. However, metabolic flux analysis with ^{13}C-isotope experiments would be investigated further to follow the carbon partitioning and the patterns of metabolite formation, and to measure the intracellular metabolites in more accurate level. The method requires GC-MS, NMR, and high-order mathematics and algorithms to obtain simulated data that are corrected for a natural abundance, non-steady state conditions, and skewing effects. This information may guide us in future genetic modifications with the goal of directing more carbon flux to succinate production and minimizing the flux to byproducts such as acetate, pyruvate and malate.

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References

Andersson, C., Hodge, D., Berglund, K.A., and Rova, U. (2007). Effect of different carbon sources on the production of succinic acid using metabolically engineered *Escherichia coli*. Biotechnol Prog., 23(2):381-388.

Aristidou, A.A., San, K.Y., and Bennett, G.N. (1995). Metabolic engineering of *Escherichia coli* to enhance recombinant protein production through acetate reduction. Biotechnol Prog., 11:475-478.

Aristidou, A.A., San, K.Y., and Bennett, G.N. (1999). Metabolic flux analysis of *Escherichia coli* expressing the *Bacillus subtilis* acetolactate synthase in batch and continuous cultures. Biotech Bioeng., 63(6):737-749.

Cecchini, G., Schroder, I., Gunsalus, R.P., and Maklashima, E. (2002). Succinate dehydrogenase and fumarate reductase from *Escherichia coli*. Biochimica et Biophysica Acta., 1553:140-157.

Curtis, S.J., and Epstein, W. (1975). Phosphorylation of D-glucose in *Escherichia coli* mutants defective in glucosephosphotransferase,mannosepho sphotransferase, and glucokinase. J Bacteriol., 122:1189-1199.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA., 97:6640-6645.

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

Guest, J.R., Angier, S.J., and Russell, G.C. (1989). Structure, expression, and protein engineering in the pyruvate dehydrogenase complex of *Escherichia coli*. Ann N.Y. Acad Sci., 573:76-99.

Izui, K., Taguchi, M., Morikawa, M., and Katsuki, H. (1981). Regulation of *Escherichia coli* phosphoenolpyruvate carboxylase by multiple effectors in vivo. II. Kinetic studies with a reaction system containing physiological concentrations of ligands. J. Biochem. (Tokyo)., 90:1321-1331.

Jantama, K., Haupt, M.J., Svoronos, S.A., Zhang, X., Moore, J.C., Shanmugam, K.T., and Ingram, L.O. (2008). Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of *E. coli* C that produce succinate and malate. Biotechnol Bioeng., 99(5): 1140-1153.

Karp, P.D., Keseler, I.M., Shearer, A., Latendresse, M., Krummenacker, M., Paley, S.M., Paulsen, I,T., Collado-Vides, J., Gamma-Castro, S., Peralta-Gil, M., Santos-Zavaleta, A., Penaloza-Spinola, M., Bonavides-Martinez, C., and Ingraham, J. (2007). Multidimensional annotation of *Escherichia coli* K-12 genome. Nucl Acids Res., 35(22):7577-7590.

Kim, Y., Ingram, L.O., and Shanmugam, K.T. (2007). Construction of an *Escherichia coli* K-12 mutant for homoethanologenic fermentation of glucose or xylose without foreign genes. Appl Environ Microbiol.,73:1766-1771.

Kornberg, H.L., and Malcovati, M. (1973). Control of in situ of the pyruvate kinase activity of *Escherichia coli*. FEBS Letters., 32(2):257-259

Krebs, A., and Bridger, W.A. (1980). The kinetic properties of phosphoenolpyruvate carboxykinase of *Escherichia coli*. Can J Biochem., 58(4):309-318.

Lin, H., Bennett, G.N., and San, K.Y. (2005). Chemostat culture characterization of *Esherichia coli* mutant strains metabolically engineered for aerobic succinate production: A study of the modified metabolic network based on metabolite profile, enzyme activity, and gene expression profile. Metab Eng., 7:337-352.

Martinez, A., Grabar, T.B., Shanmugam, K.T., Yomano, L.P., York, S.W., and Ingram, L.O. (2007). Low salt medium for lactate and ethanol production by recombinant *Escherichia coli* B. Biotechnol Lett., 29:397-404.

McKinlay, J.B., Vieille, C., and Zeikus, J.G. (2007). Prospects for a bio-based succinate industry. Appl Microbiol Biotechnol., 76:726-740.

Miller, J.H. (1992). A short course in bacterial genetics. A laboratory manual and handbook for *Escherichia coli* and related bacteria. Plainview: Cold Spring Harbor Laboratory Press. p.456.

Phue, J.N., and Shiloach, J. (2004). Transcription levels of key metabolic genes are the cause for different glucose utilization pathways in *E. coli* B (BL21) and E. coli K (JM109). J. Biotechnol., 109: 21-30.

Plumbridge, J. (1998). Expression of *ptsG*, the gene for the major glucose PTS transporter in *Escherichia coli*, is repressed by Mlc and induced by growth on glucose. Mol. Microbiol., 29(4): 1053-1063.

Plumbridge, J. (2002). Regulation of gene expression in the PTS in *Escherichia coli*: the role and interactions of Mlc. Curr. Opi. Microbiol., 5:187-193.

Posfai, G., Koob, M.D., Kirkpatrick, H.A., and Blattner, F.R. (1997). Versatile

insertion plasmidsfor targeted genome manipulations in bacteria: Isolation, deletion, and rescue of the pathogenicity island LEE of the *Escherichia coli* O157:H7 genome. J Bacteriol., 179:4426-4428.

Postma, P.W., Lengeler, J.W., and Jacobson, G.R. (1996). Phosphoenolpyruvate: carbohydrate phosphotransferase systems. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*. Edited by Neidhardt, F.C., Curtis, R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M., Umbargo, H.E., Washington, DC: ASM Press: p.1149-1174.

Quail, M.A., and Guest, J.R. (1995). Purification, characterization and mode of action of PdhR, the transcriptional repressor of the pdhR-aceEF-lpd operon of *Escherichia coli*. Mol Microbiol., 15(3):519-29.

Ryu, S. (1995). Gene expression and mRNA. Nature., 376:560.

Samuelov, N.S., Lamed, R., Lowe, S., and Zeikus, J.G. (1991). Influence of CO_2-HCO_3 levels and pH on growth, succinate production, and enzyme activities of *Anaerobiospirillum succiniciproducens*. Appl. Environ. Microbiol., 57:3013-3019.

Sanchez, A.M., Bennett, G.N., and San, K.Y. (2005). Novel pathway engineering design of the anaerobic central metabolic pathway in *Escherichia coli* to increase succinate yield and productivity. Metab Eng., 7:229-239.

Smith, T.E., Balasubramanian, K.A., and Beezley, A. (1980). *Escherichia coli* Phosphoenolpyruvate carboxylase: Studies on the mechanism of synergistic activation by nucleotides. J Biol Chemistry., 255(4):1635-1642.

Stephanopoulos, G., Aristidou, A.A., and Nielsen, J. (1998). Metabolic engineering: Principles and Methodologies. Acadamic Press. Sandiego, CA, 206p.

Tanaka, Y., Kimata, K., and Aiba, H. (2000). A novel regulatory role of glucose transporter of *Escherichia coli:* membrane sequestration of a global repressor Mlc. EMBO J., 19:5344-5352.

Tempest, D.W., and Neijssel, O.M. (1987). Growth yield and energy distribution. In: Neidhardt FC, editor. *Escherichia coli* and Salmonella typhimurium. ASM Press, Washington, DC, p.797-806.

Unden, G., Achebach, S., Holighaus, G., Tran, H.G., Wackwitz, B., and Zeuner, Y. (2002). Control of FNR function of *Escherichia coli* by O_2 and reducing conditions. J Mol Microbiol Biotechnol., 4(3);263-8.

Vemuri, G.N., Eiteman, M.A., and Altman, E. (2002). Effects of growth mode and pyruvate carboxylase on succinic acid production by metabolically engineered strains of *Escherichia coli*. Appl Environ Microbiol., 68:1715-1727.

Vemuri, G.N., Altman, E., Sangurdekar, D.P., Khodursky, A.B., and Eiteman, M.A. (2006). Overflow metabolism in *Escherichia coli* during steady-state growth: transcriptional regulation and effect of the redox. Appl Environ Microbiol., 72(5):3653-3661.

Zhang, X., Jantama, K., Moore, J.C., Jarboe, L.R., Shanmugam, K.T., and Ingram, L.O. (2010). Metabolic evolution of energy-conserving pathways for succinate production in *Escherichia coli*. Proc Natl Acad Sci USA., 106(48):20180-20185.