RICE UNIVERSITY

Metabolic Network Design and Engineering in *Escherichia coli*

by

Henry Lin

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APPROVED, THESIS COMMITTEE:

Ka-Yiu San, E.D. Butcher Professor
Bioengineering and Chemical Engineering

Kyriacos Zygalakis, A.J. Hartsook Professor, Chair
Bioengineering and Chemical Engineering

George N. Bennett, Professor, Chair
Biochemistry and Cell Biology

HOUSTON, TEXAS

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ABSTRACT

Metabolic Network Design and Engineering in *Escherichia coli*

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This thesis is a study of metabolic engineering to design and engineer novel metabolic networks with improved metabolic processes that can increase product yield and enhance cellular properties. The succinate synthesis pathways in *E. coli* were chosen as the model system for network design and optimization. The ultimate goal is to create succinate production systems in *E. coli* that not only achieve the maximum theoretical yield of succinate, but also are highly efficient and robust.

Different carbon sources with different oxidation states and transport systems, such as sorbitol and xylose, were used to address the requirements of cofactor NADH and precursor PEP in order to improve succinate synthesis.

Phosphoenolpyruvate carboxylase and pyruvate carboxylase were coexpressed to drive the carbon flux toward succinate. Competing pathways of succinate synthesis, the lactate and acetate pathways, were also inactivated to increase more carbon flux toward succinate. The intracellular acetyl-CoA pool was increased by overexpressing pantothenate kinase to enhance the activity of PEPC and PYC in order to improve succinate production.

Novel metabolic networks were designed and constructed to enable *E. coli* to produce succinate as a product under complete aerobic conditions. Since this is naturally not possible, extensive pathway manipulations had to be carried out.
The potential to produce succinate aerobically in *E. coli* would offer great advantages over anaerobic fermentation in terms of higher biomass generation, faster carbon throughput and product formation. After a series of pathway reconstructions, several aerobic succinate production systems were finally developed that could achieve the maximum theoretical succinate yield predicted by pathway modeling and simulation.

Fed batch reactor experiments were carried out for the most efficient succinate production system under aerobic conditions and the results demonstrated that it has a tremendously high capacity for succinate production. This system not only sustained fast productivity and maximal yield, it also produced succinate at a level never imagined feasible under aerobic conditions. Examination of the metabolite profiles, enzyme activities, and gene expression profiles showed that the metabolic processes of the most efficient aerobic succinate production system were more robust than the other systems.
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Chapter 1

1. Introduction

1.1 Metabolic Engineering

In recent years, there has been considerable interest in controlling metabolic fluxes in microorganisms in order to achieve desired effects. An example of this is to increase the production of a metabolite in a microorganism that normally produces it only in minute quantities. Increasing efforts in this direction have contributed to the development of the field of metabolic engineering. Metabolic engineering is defined as the “directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology” (Stephanopoulos, 1999). Metabolic engineering is multidisciplinary in nature. Its methodology encompasses a system of scientific techniques drawn from chemical engineering, computational sciences, biochemistry, molecular biology, and microbiology used to alter and control the metabolic behavior of microorganisms. Underlying principles for metabolic engineering include genetic engineering by recombinant DNA technology, bioreactor design, mathematical modeling, and bioinformatics.

Rapid advancements in genomic technology have helped fuel the field of metabolic engineering. Before the emergence of technology for DNA manipulation, random processes such as mutagenesis and screening were the only means of improving cellular properties. These often lead to creation of mutant microorganisms with unknown metabolic and genetic backgrounds. The availability of recombinant DNA technology
has now provided a more controlled approach for targeting modifications to metabolic pathways of microorganisms. Mutant microorganisms created in this fashion have well-defined genetic backgrounds. Directed manipulation of metabolic pathways and the study of the metabolic network as a whole rather individual reactions have paved way to new areas of research in metabolic engineering (Bailey, 1991; Stephanopoulos and Vallino, 1991; Stephanopoulos and Sinskey, 1993; Farmer and Liao, 1996; Jacobsen and Khosla, 1998; Stephanopoulos et al., 1998; Yang et al., 1998; Stephanopoulos, 1999; Nielsen, 2001).

Metabolic engineering has various applications in the chemical, food, and pharmaceutical industries. Microorganisms can be genetically engineered to serve as biocatalysts for the production of useful proteins and chemicals. Examples include enhancing ethanol production in Escherichia coli by overexpressing the Zymomonas mobilis PET operon genes (Ingram et al., 1987; Ingram and Conway, 1998; Ingram et al., 1999), production of 1,3-propanediol by recombinant E. coli carrying the dha regulon from Klebsiella pneumoniae (Cameron et al., 1998; Biebl et al., 1999), and production of 3-dehydroshikimic acid by recombinant E. coli with amplified expression of DAHP synthase (Li et al., 1999). Other microorganisms have also been engineered for enhanced amino acid and peptide production like using Lactococcus lactis for glutathione production (Li et al., 2004) and Corynebacterium glutamicum for L-lysine production (de Graaf et al., 2001).

Production of valuable chemicals from microorganisms for commercial use has instigated interest in metabolic engineering from the industry. The potential to improve process productivity and reduce the cost of production with biomass has numerous
companies positioning themselves in this rapidly growing field. Companies like DuPont, Cargill Dow, Genencor, and ADM are heavily exploiting biotechnology to produce “sustainable” products for human consumption. Examples include microbial production of indigo (Genencor), propylene glycol (DuPont), and ethanol and amino acids (ADM).

The use of biological processes for chemical production can serve as a viable alternative to traditional chemical processes. Petroleum-based chemical synthesis can be expensive and have tremendous hazardous impact on the environment. Instead, using biomass driven by renewable resources, such as agricultural waste, can reduce the cost of production and the impact on the environment. Such manufacturing process can be a sustainable source of the chemical, while being environmentally friendly through “green chemistry”.

1.2 Metabolic Network Design and Engineering

1.2.1 Research Objective

The research is to design and engineer novel metabolic networks in *E. coli* for improved metabolic function in order to maximize product yield. A systematic approach is taken throughout the process that allows understanding of the cellular response to precise pathway manipulations. Pathway modeling and simulation are performed to help direct the design of optimal metabolic networks. The model system focused in this research is the succinate synthesis pathways of *E. coli*. Succinate production is important because it is a valuable specialty chemical used as a precursor for the synthesis of a variety of commodity chemicals. Succinate is naturally produced by *E. coli* only in
minimal quantities under anaerobic conditions. For this reason, the metabolic network of
*E. coli* is extensively characterized and new networks are designed for dedicated
succinate production. The environmental variables for growth and production are also
considered when optimizing succinate production in *E. coli*. In the end, novel metabolic
networks in *E. coli* are created that are robust and efficient for succinate production.

1.2.2 Background

The valuable specialty chemical succinate and its derivatives have extensive
industrial applications. It can be used as an additive and flavoring agent in foods, a
supplement for pharmaceuticals, a surfactant, a detergent extender, a foaming agent, and
an ion chelator (Zeikus *et al.*, 1999). The domestic market for succinate and its
derivatives is more than $1.3 billion per year and is expected to expand 6 to 10 percent
per year. Currently, succinate is produced through petrochemical processes that can be
expensive and can lead to pollution problems. Much effort has shifted toward making
biocatalysts a viable and improved alternative for the production of succinate. Despite
much progress that has been made, producing succinate economically with biocatalysts is
still not feasible today. The success of microbial fermentation someday coupled with the
use of renewable carbohydrates will significantly improve the economics of the succinate
market (Schilling, 1995).

Succinate is a metabolite that is formed under both anaerobic and aerobic
conditions. Under anaerobic conditions, *E. coli* undergoes mixed-acid fermentation that
yields acetate, ethanol, formate, and lactate as its major fermentation products with
succinate a minor product (Figure 1-1). Succinate is formed under anaerobic conditions
Figure 1-1: Central anaerobic pathways of *E. coli*.
first through a carboxylation reaction and then a series of reductive reactions (Figure 1-1). In the first enzymatic step, the precursor phosphoenolpyruvate (PEP) is converted to oxaloacetate (OAA) through fixation of CO₂. This reaction is catalyzed by phosphoenolpyruvate carboxylase (PEPC). OAA is then sequentially reduced to malate, where it is eventually converted to succinate. During this process, two moles of NADH are oxidized to two moles of NAD⁺. Therefore, for every mole of succinate formed, one mole of CO₂ and two moles of NADH are required.

Under aerobic conditions, succinate is only an intermediate of the tricarboxylic acid (TCA) cycle (Figure 1-2). It is eventually oxidized to supply electrons for oxidative phosphorylation (ATP generation) and to regenerate OAA. Succinate is, therefore, not produced as a product under aerobic conditions in *E. coli*. PEPC is also active under aerobic conditions to help replenish OAA. The TCA cycle consists of a series of oxidative reactions that generate intermediates for biosynthesis of secondary metabolites. Within the TCA cycle exists a detour called the glyoxylate bypass that branches out from isocitrate. This bypass consists of two reactions. The first one catalyzed by isocitrate lyase (ICL) converts isocitrate to glyoxylate and succinate. The second reaction catalyzed by malate synthase (MS) condenses glyoxylate and acetyl-CoA to malate. The glyoxylate bypass is essential for growth on the two-carbon acetate or fatty acids because it prevents carbon loss as CO₂ in the TCA cycle (Kornberg, 1966). In other situations such as glucose as a carbon source, the glyoxylate bypass is inactive. When the glyoxylate bypass is predominantly active, isocitrate dehydrogenase (ICD) is minimally active, and *vice versa*. 
Figure 1-2: Central aerobic pathways of E. coli.
Figure 1-3: Model network of succinate synthesis pathways under anaerobic and aerobic conditions. The pyc pathway is not indigenous in E. coli, which would have to be expressed with a plasmid.
Based on the succinate synthesis pathways active under both anaerobic and aerobic conditions, a complete network of all the possible succinate synthesis pathways is assembled (Figure 1-3). This model provides the framework for the design of a new succinate production network.

1.2.3 Advances in Metabolic Engineering for Succinate Production

Microorganisms that have the innate capability for high yield succinate production have been studied. Examples include the high yield production of succinic acid from wood hydrolysate, whey, or glycerol by *Anaerobiospirillum succiniciproducens* through improvement of its fermentation conditions (Lee *et al.*, 2000, 2001, 2003b; Samuelov *et al.*, 1999). The obligate anaerobe *A. succiniciproducens*, however, is not practical for commercial fermentation because it is unstable due to its tendency to degenerate, and requires environments absolutely free of oxygen for cultivation (Nghiem *et al.*, 1999). *E. coli* has been extensively genetically engineered through the use of recombinant DNA technology in recent years to show promising potential for succinate production. *E. coli* naturally produces succinate as a minor fermentation product under anaerobic conditions (Clark, 1989). Under aerobic conditions, succinate is not produced as a product in *E. coli* and acetate is the main product. Numerous metabolic engineering strategies to enhance succinate production in *E. coli* have met with success. By amplifying enzymatic steps involved in the succinate pathway under anaerobic conditions, higher succinate production could be achieved. An example of this was shown when phosphoenolpyruvate carboxylase (*pepc*) from *E. coli* was overexpressed (Millard *et al.*, 1996). Conversion of fumarate to succinate was
improved by overexpressing native fumarate reductase (*frd*) in *E. coli* (Goldberg et al., 1983; Wang et al., 1998). Certain enzymes are not indigenous in *E. coli*, but can potentially help increase succinate production. By introducing pyruvate carboxylase (*pyc*) from *Rhizobium etli* into *E. coli*, succinate production was enhanced (Gokarn et al., 1998; Gokarn et al., 2000; Gokarn et al., 2001). Other metabolic engineering strategies also include inactivating competing pathways of succinate. When malic enzyme was overexpressed in the presence of inactivated pyruvate formate lyase (*pfl*) and lactate dehydrogenase (*ldh*), succinate became the major fermentation product (Stols and Donnelly, 1997; Hong and Lee, 2001). In this *pfl* and *ldh* mutant, there is large pyruvate accumulation. Overexpression of malic enzyme in this mutant increased succinate production driven by the high pyruvate pool toward the direction of malate formation, which was subsequently converted to succinate. An inactive glucose phosphotransferase system (*ptsG*) in the same mutant strain (*pfl*- and *ldh*-) had also been shown to yield higher succinate production in *E. coli* (Chatterjee et al., 2001).

Genetic engineering coupled with optimization of production conditions has shown promising results for large-scale production of succinate from *E. coli*. This makes succinate production in *E. coli* competitive with that of other organisms like *Anaerobiospirillum succiniciproducens*. A genetically improved *E. coli* mutant strain (AFP111/pTrc99A-*pyc*) grown anaerobically in optimized fed batch conditions was shown to achieve succinate production of 99.2 g/L with yield of 110% and productivity of 1.3 g/L h (Vemuri et al., 2002a, 2002b). Because of anaerobic fermentation process disadvantages that include poor biomass generation, slow carbon throughput, and, therefore, slow product formation, *E. coli* was also genetically engineered to produce
succinate under aerobic conditions (Lin et al., 2005a b). Aerobic fed batch culture of a genetically modified E. coli mutant strain (HL27659k(pKK313)) was shown to produce 58.3 g/L of succinate with yield of 94% and productivity of 1.1 g/L h (Lin et al., 2005c).

Currently, industrial (Arkenol and Applied CarboChemicals) and governmental (Argonne National Laboratory and Oak Ridge National Laboratory) research efforts are underway to commercially produce succinate using biomass as the biocatalyst. If succinate can be produced from biomass, the lower cost will allow it to compete with more chemicals currently produced from petroleum-based feedstocks. The competively-priced succinate can then be used directly or as a precursor for many industrial chemicals. The result will be a significant reduction in the use of petroleum resources. In addition to the energy savings that accrue by substituting biomass for imported petroleum, carbon dioxide is also “fixed” during the fermentation process, providing the potential to reduce greenhouse gas emissions.

1.3 Thesis Outline

Chapter 2 describes the materials and methods that were used in the research. Chapter 3 describes manipulation of culture conditions with different carbon sources to improve succinate production in E. coli. Chapter 4 explores genetic manipulation of the succinate synthesis pathways in E. coli to improve succinate production. In this chapter, PEPC and PYC pathways were coexpressed in mutant strains of E. coli to show their effect on succinate production. Chapter 5 introduces the strategy of cofactor manipulation to enhance activity of pathways and thus product formation. This chapter describes increasing the intracellular acetyl-CoA pool to enhance the activities of the
PEPC and PYC pathways. As a result of this, succinate production in *E. coli* was improved. Chapter 6 describes the genetic reconstruction of *E. coli*’s aerobic metabolism to enable succinate production under aerobic conditions. This chapter is significant in that it is the first time that succinate has been shown to be produced by *E. coli* under aerobic conditions. Previous efforts to improve succinate production have all been done under anaerobic conditions, since *E. coli* cannot produce succinate aerobicly. Chapter 7 describes further work to improve the aerobic succinate production platform developed in Chapter 6. In this chapter, novel aerobic succinate production systems were developed that could achieve the maximum theoretical succinate yield of 1.0 mol/mol glucose under aerobic conditions. This maximum theoretical yield was obtained by mathematical simulation and prediction. Chapter 8 describes a large-scale production study of the most efficient succinate production system developed in Chapter 7 using fed batch reactor. The results in Chapter 8 show that high level succinate production can be achieved under aerobic conditions. The aerobic succinate production system developed holds great potential for industrial-scale succinate production. Chapter 9 describes the characterization of the metabolic network of various aerobic succinate production systems in chemostat cultures. The metabolic function of genetically engineered networks can be better understood by examining the connections between genes, proteins, and metabolites. This was done by studying the metabolite profiles, enzyme activities, and gene expression profiles of various *E. coli* mutant strains constructed. Chapter 10 includes recommendations for future directions on the research presented in this thesis.
Chapter 2

2. Materials and Methods

This section contains the materials and methods that were used to perform this research in general. A more detailed description, specific to each section of the work, is included in each chapter.

2.1 Bacterial Strains and Plasmids

Strains and plasmids used are listed in Tables 2-1 and 2-2. The laboratory standard strain is GJT001, a spontaneous cadR mutant of MC4100, Δlac strain (arg-lac) U169 rspL150 relAI, (Tolentino et al., 1992). All pathway deletions were performed in GJT001 using P1 phage transduction and the one-step inactivation based on λ red recombinase (Datsenko and Wanner, 2000). All gene expressions via plasmids were also performed in GJT001. The construction of plasmids and mutant E. coli strains is described in detail in the chapters that present the research where they were used.

2.2 Cultivation Media

2.2.1 LB Medium

Luria-Bertani broth (LB) with an adjusted pH of 7.5 was used. The composition of LB consists of 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl. The medium was prepared using distilled water. Carbon sources such as glucose, sorbitol, and xylose were added to the LB medium in concentrations specific to the experiment. The medium is autoclaved at 121 °C for 20-45 min depending on the load, without
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GJT001</td>
<td>Spontaneous cadR mutant of MC4100(ATC35695) (\Delta lac(arg-lac)U169p8sL150relA1ptsF Sm^R)</td>
<td>Tolentino et al., 1992</td>
</tr>
<tr>
<td>YBS121</td>
<td>GJT001Δ(ackA-pta), Cm^R</td>
<td>Yang et al., 1999b</td>
</tr>
<tr>
<td>YBS131</td>
<td>GJT001Δ(idhA), Te^R</td>
<td>Yang et al., 1999b</td>
</tr>
<tr>
<td>YBS132</td>
<td>GJT001(Δ(ackA-pta), ΔidhA), Te^R/Km^R</td>
<td>Yang et al., 1999b</td>
</tr>
<tr>
<td>SB202</td>
<td>GJT001Δ(idhA-pta), Te^R/Cm^R</td>
<td>Berrios-Rivera, 2002</td>
</tr>
<tr>
<td>GJT001(pKK313)</td>
<td>GJT001 overexpressing S8D mutant Sorgum sordum Ap^R</td>
<td>Lin et al., 2005d</td>
</tr>
<tr>
<td>YBS121(pKK313)</td>
<td>YBS121 overexpressing S8D mutant Sorgum sordum Ap^R</td>
<td>Lin et al., 2005d</td>
</tr>
<tr>
<td>YBS132(pKK313)</td>
<td>YBS132 overexpressing S8D mutant Sorgum sordum Ap^R</td>
<td>Lin et al., 2005d</td>
</tr>
<tr>
<td>GJT(pDHK29, pTrc99A)</td>
<td>GJT001 control strain</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>GJT(pHL333, pTrc99A)</td>
<td>GJT001 overexpressing PEPC</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>GJT(pHL333, pHL413)</td>
<td>GJT001 overexpressing PEPC and PYC</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>YBS121(pDHK29, pTrc99A)</td>
<td>YSB121 control strain</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>YBS121(pHL333, pTrc99A)</td>
<td>YSB121 overexpressing PEPC</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>YBS121(pHL333, pHL413)</td>
<td>YSB121 overexpressing PEPC and PYC</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>YBS131(pDHK29, pTrc99A)</td>
<td>YBS131 control strain</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>YBS131(pHL333, pTrc99A)</td>
<td>YBS131 overexpressing PEPC</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>YBS131(pHL333, pHL413)</td>
<td>YBS131 overexpressing PEPC and PYC</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>YBS132(pDHK29, pTrc99A)</td>
<td>YBS132 control strain</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>YBS132(pHL333, pTrc99A)</td>
<td>YBS132 overexpressing PEPC</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>YBS132(pHL333, pHL413)</td>
<td>YBS132 overexpressing PEPC and PYC</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>SB202(pDHK29, pTrc99A)</td>
<td>SB202 control strain</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>SB202(pHL333, pTrc99A)</td>
<td>SB202 overexpressing PEPC</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>SB202(pHL333, pHL413)</td>
<td>SB202 overexpressing PEPC and PYC</td>
<td>Lin et al., 2004b</td>
</tr>
<tr>
<td>GJT(pDHK29, pUC19)</td>
<td>GJT001 control strain</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>GJT(pHL333, pRV380)</td>
<td>GJT001 overexpressing PANK</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>GJT(pHL333, pUC19)</td>
<td>GJT001 overexpressing PEPC</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>GJT(pHL333, pRV380)</td>
<td>GJT001 overexpressing PEPC and PANK</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>GJT(pTrc99, pRV480)</td>
<td>GJT001 overexpressing PANK</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>GJT(pHL413, pDHK29)</td>
<td>GJT001 overexpressing PYC</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>GJT(pHL413, pRV480)</td>
<td>GJT001 overexpressing PYC and PANK</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>HL2k</td>
<td>GJT001(sdhAB::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL26k</td>
<td>GJT001(sdhAB, poxB::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL267k</td>
<td>GJT001(sdhAB, poxB, (ackA-pta)::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL2671k</td>
<td>GJT001(sdhAB, poxB, (ackA-pta), icsd::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL26715k</td>
<td>GJT001(sdhAB, poxB, (ackA-pta), ics, icslR::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL27615k</td>
<td>GJT001(sdhAB, (ackA-pta), poxB, ics, icslR::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL26765k</td>
<td>GJT001(ics, sdxAB, poxB, (ackA-pta), icslR::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL51267k</td>
<td>GJT001(icslR, ics, sdxAB, poxB, (ackA-pta)::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL51267k</td>
<td>GJT001(icslR, ics, sdxAB, (ackA-pta), ics, icslR::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL51267k</td>
<td>GJT001(ics, sdxAB, ics, ics, icslR::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL51276k</td>
<td>GJT001(ics, sdxAB, (ackA-pta), ics, icslR::Km^R)</td>
<td>Lin et al., 2005a</td>
</tr>
<tr>
<td>HL51276k</td>
<td>GJT001(pKK313) overexpressing S8D mutant Sorgum sordum Ap^R</td>
<td>Lin et al., 2005b</td>
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<tr>
<td>HS12769k(pKK313)</td>
<td>HS12769k overexpressing S8D mutant Sorgum sordum Ap^R</td>
<td>Lin et al., 2005b</td>
</tr>
<tr>
<td>HS12769k(pKK313)</td>
<td>HS12769k overexpressing S8D mutant Sorgum sordum Ap^R</td>
<td>Lin et al., 2005b</td>
</tr>
<tr>
<td>HS12765k(pKK313)</td>
<td>HS12765k overexpressing S8D mutant Sorgum sordum Ap^R</td>
<td>Lin et al., 2005b</td>
</tr>
</tbody>
</table>
Table 2-2: List of plasmids used.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK311</td>
<td>Wildtype <em>Sorghum pepe</em> Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Wang et al., 1992</td>
</tr>
<tr>
<td>pKK313</td>
<td>S8D mutant <em>Sorghum pepe</em> Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Wang et al., 1992</td>
</tr>
<tr>
<td>pCPYC1</td>
<td><em>L. lactis</em> pyc Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Wang et al., 2000</td>
</tr>
<tr>
<td>pGC1002</td>
<td><em>E. coli</em> frdABCD Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Cecchini et al., 1984</td>
</tr>
<tr>
<td>pICL11</td>
<td><em>R. equi</em> aceA-fadB Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Kelly et al., 2002</td>
</tr>
<tr>
<td>pLOI3514</td>
<td><em>B. subtilis</em> gltA Ap&lt;sup&gt;R&lt;/sup&gt; : Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Underwood et al., 2002</td>
</tr>
<tr>
<td>pSM1</td>
<td><em>S. clavuligerus</em> aceB Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Chan and Sim, 1998</td>
</tr>
<tr>
<td>pUK1</td>
<td><em>S. xylosus</em> gtcU-gltA Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Fiegler et al., 1999</td>
</tr>
<tr>
<td>pUK1C</td>
<td>Control vector of pUK1 Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Not published</td>
</tr>
<tr>
<td>pHL323</td>
<td>Wildtype <em>Sorghum pepe</em> in pHDC29 Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Not published</td>
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<tr>
<td>pHL333</td>
<td>S8D mutant <em>Sorghum pepe</em> in pHDK29 Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>pHL413</td>
<td><em>L. lactis</em> pyc in pTrc99A Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>pTrc99A</td>
<td>Cloning vector Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pHDC29</td>
<td>Cloning vector Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Phillips et al., 2000</td>
</tr>
<tr>
<td>pHDK29</td>
<td>Cloning vector Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Phillips et al., 2000</td>
</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Yanisch-Perron et al., 1985</td>
</tr>
<tr>
<td>pRV380</td>
<td><em>E. coli</em> panK in pUC19 Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Vadali et al., 2004a</td>
</tr>
<tr>
<td>pRV480</td>
<td><em>E. coli</em> panK in pHDK29 Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Vadali et al., 2004b</td>
</tr>
<tr>
<td>pH513</td>
<td><em>B. subtilis</em> gltA in pTrc99A Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Not published</td>
</tr>
<tr>
<td>pH531</td>
<td><em>B. subtilis</em> gltA in pHDK29 Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Not published</td>
</tr>
</tbody>
</table>

Table 2-3: List of antibiotics and their concentrations.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stock Concentration</th>
<th>Working Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (Ap)</td>
<td>25 mg/ml in H₂O</td>
<td>100-200 mg/l</td>
</tr>
<tr>
<td>Oxacillin (Ox)</td>
<td>25 mg/ml in H₂O</td>
<td>100-200 mg/l</td>
</tr>
<tr>
<td>Carbenicillin (Cn)</td>
<td>25 mg/ml in H₂O</td>
<td>100-200 mg/l</td>
</tr>
<tr>
<td>Chloramphenicol (Cm)</td>
<td>34 mg/ml in ethanol</td>
<td>34 mg/l</td>
</tr>
<tr>
<td>Kanamycin (Km)</td>
<td>50 mg/ml in H₂O</td>
<td>100 mg/l</td>
</tr>
<tr>
<td>Streptomycin (Sm)</td>
<td>20 mg/ml in H₂O</td>
<td>50 mg/l</td>
</tr>
<tr>
<td>Tetracycline (Tc)</td>
<td>12.5 mg/ml in 1:1 H₂O-ethanol</td>
<td>50 mg/l</td>
</tr>
<tr>
<td>Nalidixic Acid (Nal)</td>
<td>5 mg/ml in H₂O</td>
<td>15 mg/l</td>
</tr>
</tbody>
</table>

glucose, sorbitol, or xylose. Afterwards, glucose, sorbitol, or xylose is then aseptically added to the medium.

One millimolar of isopropyl-β-D-thiogalactoside (IPTG) was added to the medium when necessary for induction of gene expression from plasmids. Antibiotics were added to maintain a selection pressure for plasmid containing cells and for chromosomal markers in mutant strains. Antibiotic solutions used are listed in Table 2-3. All antibiotics are stored at -20 °C. Stock solutions of the antibiotics are filter-sterilized through a 0.22-micron sterile membrane filter. The research in the following chapters will describe in detail the medium composition used including the carbon source and antibiotics.

**2.2.2 Production**

Medium used for succinate production was modified to improve the growth and production conditions. In experiments requiring the use of anaerobic tubes for succinate production (anaerobic), 5 g of MgCO₃ was added to 18 ml of medium. In experiments requiring the use of flasks for succinate production (microaerobic), 3 g of MgCO₃ was added to 10 ml of medium. MgCO₃ is added to the tubes and flasks prior to autoclaving, and then the medium is added to these tubes and flasks during experimental setup. The
use of MgCO$_3$ improves the medium because it buffers the pH of the medium during fermentation, and it also indirectly provides CO$_2$, which is necessarily for the succinate synthesis pathway. Medium used for succinate production was also supplemented with 2 g/l of NaHCO$_3$, which serves the same purpose as MgCO$_3$ except it is soluble in the medium. MgCO$_3$ is not soluble in the medium when using the concentrations stated above.

2.2.3 Storage Medium

2.2.3.1 Glycerol Stocks

Glycerol stocks (50% glycerol) were made for long-term storage of strains. These stocks were made with 0.5 ml of 100% autoclaved glycerol and 0.5 ml of cell culture. The cell culture was grown in LB medium for 12 to 18 hours from an individual colony. The mixture was then vortexed and left in room temperature for 1 to 2 hours to allow the cells to acclimate to the glycerol environment. The glycerol stocks were then stored in -80°C.

2.2.3.2 Agar Plates

Agar plates were made from LB media and 15 g/l of technical agar. The agar solution was autoclaved before it was used for making the plates. The solution was then cooled to approximately 55°C before adding the appropriate antibiotics, as well as the X-gal and IPTG if needed. Colonies maintained on plates with antibiotics are fresh for up to two weeks.
2.2.3.3 Strain Propagation

Strains stored for a long period of time were propagated to maintain viability. Liquid cultures were grown from old glycerol stocks overnight at 37°C and 250 rpm in appropriate antibiotics. The cells from the culture were then streaked on agar plates containing the right antibiotics and incubated at 37°C overnight. A single colony from the plate was then used to inoculate 5 ml of LB with antibiotics. This culture was used for preparation of new glycerol stocks of the strains.

2.3 Cell Cultivation

Initial cultures used in gene cloning work or fermentation experiments were grown overnight for 12 to 18 hours in 5 ml of LB with the appropriate antibiotics at 37°C and 250 rpm. The cultures were washed twice with fresh LB when used for experiments.

2.3.1 Anaerobic Tube

For general experiments under anaerobic conditions, 45-ml capped tubes with PTFE/silicone rubber septa on open top caps were used. Each capped tube contained 40 ml of medium. The gas in the headspace of each sealed tube was removed with needle and syringe. A seed inoculum of 100 µl from an overnight 5 ml culture was used for each tube.

Anaerobic experiments for succinate production were performed in the same type of capped tubes as above. Each tube was filled with 18 ml of medium, instead. The air in the headspace was replaced with CO₂ at 1 atm by purging CO₂ into the tube at a flow
rate of 1 l/min for 8 sec. A seed inoculum of 45 μl from an overnight 5 ml culture was used for each tube.

2.3.2 Aerobic Shake Flask

For general experiments under aerobic conditions, 250-ml flasks containing 50 ml of medium were used. The flasks were plugged with foam tube plugs (Identi-Plugs, Jaece Industries, Inc., North Tonawanda, NY) to allow gas exchange with the environment. A seed inoculum of 200 μl from an overnight 5 ml culture was used for each flask.

For succinate production under microaerobic conditions, 250-ml capped flasks containing 10 ml of medium were used. The large air-to-liquid ratio was used to provide sufficient O₂ in the headspace for aerobic respiration. Capping the flask conserves the CO₂ and provides the microaerobic environment. A seed inoculum of 40 μl from an overnight 5 ml culture was used for each flask.

2.3.3 Bioreactor – Batch

2.3.3.1 Inoculum Preparation

Cultures from frozen glycerol stocks were used to streak for fresh colonies on LB agar plates containing the appropriate antibiotics. A single colony was then selected and grown in 5 ml of LB with the appropriate antibiotics. This culture was grown at 37°C and 250 rpm in a rotary shaker for 12 to 18 hours. Then the 5 ml culture was washed twice with fresh LB to remove undesired proteins, such as β-lactamase, which degrades
ampicillin. The whole washed culture was then used to inoculate the bioreactor. This inoculum constituted less than 1% of the liquid volume in the bioreactor.

2.3.3.2 Medium

The medium used was LB with 2 g/L NaHCO₃. This medium was autoclaved with the bioreactor. Glucose was added at concentrations required for the experiment. The appropriate antibiotics and their concentrations were also added. IPTG was added for cultures of strains that harbor plasmids that could be induced by IPTG.

2.3.3.3 Batch Conditions

The initial medium volume was 600 ml in a 1.0-L New Brunswick Scientific Bioflo 110 fermenter (Figure 2-1). The pH was measured using a glass electrode and controlled at 7.0 using 1.5N HNO₃ and 2N Na₂CO₃. The temperature was maintained at 37°C, and the agitation speed was constant at 800 rpm. The inlet airflow used was 1.5 L/min. The dissolved oxygen was monitored using a polarographic oxygen electrode (New Brunswick Scientific) and was maintained above 80% saturation throughout the experiment.

2.3.4 Bioreactor – Fed Batch

2.3.4.1 Inoculum Preparaion

Cultures from frozen glycerol stocks were used to streak for fresh colonies on LB agar plates containing the appropriate antibiotics. A single colony was then selected and
grown in 5 ml of LB with the appropriate antibiotics. This culture was grown at 37°C and 250 rpm in a rotary shaker for 12 to 18 hours. Then the 5 ml culture was washed twice with fresh LB. A 1% (v/v) inoculum was used to inoculate the bioreactor.

2.3.4.2 Medium

Fed batch reactor medium used is SB (Ausubel et al., 1988) with 2 g/L NaHCO₃. SB consists of 5 g/l NaCl, 20 g/l yeast extract, 32 g/l tryptone, 7 g/l Na₂HPO₄, 3 g/l KH₂PO₄, and 1 g/l NH₄Cl. NaHCO₃ was added to the culture medium because it yielded better cell growth and succinate production due to its pH-buffering capacity and its ability to supply CO₂. Appropriate antibiotics were added to the bioreactor. For ampicillin, carbenicillin, and oxacillin, they were added to the medium at a concentration of 200 mg/l each. The use of ampicillin, carbenicillin, and oxacillin in combination at such concentration was to enforce that the plasmids were retained throughout aerobic fermentation. IPTG was added at 1.0 mM to the medium to induce expression of genes from plasmids.

2.3.4.3 Fed Batch Conditions

Fed batch reactor experiments were conducted under aerobic conditions (Figure 2-2). The initial medium volume is 400 ml in a 1.0-L New Brunswick Scientific Bioflo 110 fermenter. The pH was measured using a glass electrode and controlled at 7.0 using 1.5N HNO₃ and 2N Na₂CO₃. The temperature was maintained at 37°C, and the agitation speed was constant at 800 rpm. The inlet airflow used was 2.0 l/min. The dissolved oxygen was monitored using a polarographic oxygen electrode (New Brunswick
Scientific, Edison, New Jersey) and was maintained above 50% saturation throughout the experiment. Glucose was fed exponentially according to the specific growth rate of the strain studied, obtained from batch experiment results. The program used for glucose feeding was BioCommand Plus BioProcessing Software from New Brunswick Scientific. After inoculation, the culture in the bioreactor was grown in batch mode for up to 14 hrs before the glucose pump was turned on to start the fed batch.

2.3.5 Bioreactor – Chemostat

2.3.5.1 Inoculum Preparation

Cultures from frozen glycerol stocks were used to streak for fresh colonies on LB agar plates containing the appropriate antibiotics. A single colony was then selected and grown in 5 ml of LB with the appropriate antibiotics. This culture was grown at 37°C and 250 rpm in a rotary shaker for 12 to 18 hours. Then the 5 ml culture was washed twice with fresh LB. A 1% (v/v) inoculum was used to inoculate the bioreactor.

2.3.5.2 Medium

The medium used was Luria-Bertani broth (LB), 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl (Sambrook et al., 1989), added with 2 g/l NaHCO₃ and 20 g/l of glucose. NaHCO₃ was added to the culture medium because it promoted cell growth and succinate production due to its pH-buffering capacity and its ability to supply CO₂. Appropriate antibiotics were added depending on the strain. For cultures of mutant
strains with kanamycin marker in their chromosome, 50 mg/l of kanamycin was added to the medium.

2.3.5.3 Chemostat Conditions

Chemostat experiments were performed under aerobic conditions at a dilution rate of 0.1 hr\(^{-1}\) (Figure 2-3). This dilution rate was selected based on specific growth rates of the five mutant strains, obtained from log phase growth data of previous batch culture studies. The working volume was maintained at 600 ml in a 1.0-L New Brunswick Scientific Bioflo 110 fermenter. The pH was measured using a glass electrode and controlled at 7.0 using 1.5 N HNO\(_3\) and 2 N Na\(_2\)CO\(_3\). The temperature was maintained at 37\(^\circ\)C, and the agitation speed was constant at 500 rpm. The inlet airflow used was 0.6 l/min. The dissolved oxygen was monitored using a polarographic oxygen electrode (New Brunswick Scientific) and was maintained above 50% saturation throughout the experiment. After inoculation, the culture was allowed to grow in batch mode for 12 to 14 hours before the feed pump and waste pump were turned on to start the chemostat. The continuous culture reached steady state after 5 residence times. Optical density and metabolites were measured from samples at 5 and 6 residence times and then compared to ensure that steady state had been established. Actual samples used for obtaining the results presented were taken after 6 residence times.
**Figure 2-1**: Batch reactor setup.
Figure 2-2: Fed batch reactor setup.
Figure 2-3: Chemostat reactor setup.
2.4 Analytical Techniques

2.4.1 Cell Density

Optical density (OD) was measured at 600 nm with a spectrophotometer (Bausch & Lomb Spectronic 1001); the culture was diluted to the linear range with 0.15 M NaCl and the dilution factor was accounted in the calculation of OD. The measured OD was correlated with dry cell weight if necessary. Cell dry weight was determined by collection of 100 ml of culture in an ice batch. The samples were centrifuged at 4,000g and 4°C for 10 minutes, washed with 0.15M sodium chloride solution, and dried in an oven at 55°C until constant weight. The final weight of the dried samples was corrected for the weight of NaCl in the washing solution.

2.4.1 Metabolite Analysis – HPLC

For analyzing the culture medium broth, 1 ml of culture was centrifuged at 8000g for 5 min and the supernatant was then filtered through a 0.2 μm PVDF membrane syringe filter and stored frozen at -20°C until analysis.

The HPLC system (Shimadzu-10A Systems, Shimadzu, Columbia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A) and a differential refractive index (RI) detector (Waters 2410, Waters, Milford, MA). A 0.6 ml/min mobile phase using 2.5 mM H₂SO₄ solution was applied to the column. The column was operated at 55 °C. Standards were prepared for both the RI detector and UV detector, and calibration curves were created. Glucose, xylose, arabinose, sorbitol, gluconate, succinate, lactate, formate, acetate, ethanol and
glyoxylate were all measured by the RI detector and pyruvate was measured by the UV detector at 210 nm.

2.4.1 Enzyme Assays

Crude extracts for all enzyme assays were prepared by taking 20 OD units of culture (OD_{660nm} * vol (ml) = 20) and centrifuging the appropriate volume at 5000g and 4°C for 20 min. The cell pellet was then washed once in 15 ml of the appropriate buffer for each type of enzyme assay. The pellet was then centrifuged again and resuspended in 10 ml of that buffer. The cells were then subjected to sonication for 10 min in an ice bath. The sonicated cells were centrifuged at 5000g and 4°C for 60 min to remove cell debris. The supernatant was then used for the enzyme assay. Total protein concentration of the crude extract was measured by Lowry’s method (Sigma Lowry Reagent, Modified) using bovine serum albumin as standard. All enzyme activities were expressed as U/mg, μmol of substrate converted to product per min per mg protein. The specific enzyme activity was calculated by the following equation: specific activity (U/mg protein) = [1000 * (total volume in cuvette) * (difference in rate of absorbance between control and sample)] / [(molar extinction coefficient) * (volume of cell extract) * (dilution factor of cell extract) * (protein concentration)].

2.4.3.1 PEPC Assay

Phosphoenolpyruvate carboxylase activity was measured by a modified method of Terada et al., 1991. This assay is a coupled enzyme assay with malate dehydrogenase. Phosphoenolpyruvate carboxylase converts phosphoenolpyruvate to oxaloacetate through
a carboxylation reaction. Malate dehydrogenase then reduces oxaloacetate to malate with the oxidation of NADH.

The following solutions were required: 50 mM Tris-HCl/MgCl₂ buffer (pH = 8.0) (adjusted with 20% NaOH to pH of 8.0 and added enough MgCl₂ for a final concentration of 1mM), 0.15 M Tris-HCl buffer (pH = 8.5) (adjusted with 20% NaOH to a pH of 8.5), 0.3 M MgCl₂, 0.6 M NaHCO₃ (prepared fresh), 3.0 mM NADH (prepared fresh), 0.1 M phosphoenolpyruvate (prepared fresh), 15 mM acetyl-CoA (prepared fresh), and malate dehydrogenase (10,000 U/ml). Cold Tris/MgCl₂ (pH = 8.0) was used for cell extract preparation. UV grade cuvettes were used. For the control cuvette, add the following to a total volume of 1350 ul: 190 ul DI H₂O, 1000 ul Tris (pH = 8.5), 25 ul NaHCO₃, 25 ul MgCl₂, 50 ul NADH, 50 ul acetyl-CoA, 10 ul malate dehydrogenase, and no PEP. For the sample cuvette, add the following to a total volume of 1350 ul: 140 ul DI H₂O, 1000 ul Tris (pH = 8.5), 25 ul NaHCO₃, 25 ul MgCl₂, 50 ul NADH, 50 ul acetyl-CoA, 10 ul malate dehydrogenase, and 50 ul PEP. The cuvette was blanked in the spectrophotometer and then 150 ul of cell extract were then added. The solution was quickly mixed by pipetting or inversion and the reading was started. The reaction was measured at 340 nm and 37°C, and the molar extinction coefficient used was 6.22 mM⁻¹ cm⁻¹. The rate of decrease in absorbance was used to calculate phosphoenolpyruvate carboxylase activity.

Another method that could be used to measure PEPC activity was based on the citrate synthase-coupled assay by the method of Payne and Morris, 1969. The oxaloacetate produced by PEPC is reacted with acetyl-CoA by citrate synthase to yield CoA. The CoA then reacts with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) to produce a
product that can be measured spectrophotometrically at 412 nm and 37°C. Reactions were initiated by adding the crude extract. Phosphoenolpyruvate is the substrate required for PEPC. The DTNB extinction coefficient used was \(1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}\). The rate of increase in absorbance was used to calculate PEPC activity.

### 2.4.3.2 PYC Assay

Pyruvate carboxylase activity was measured by a modified method of Payne and Morris, 1969. Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate using ATP and carbonate as co-substrates. The oxaloacetate produced by PYC is condensed with acetyl-CoA by citrate synthase to yield citrate and CoA. The CoA then reacts with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) that can be measured spectrophotometrically.

The following solutions were required: PBS buffer (NaCl (1.17 g/l), KCl (0.20 g/l), \(\text{Na}_2\text{HPO}_4\cdot7\text{H}_2\text{O}\) (2.72 g/l) and \(\text{K}_2\text{HPO}_4\) (0.24 g/l) adjusted to pH 7.8 with HCl and sterilized and stored at 4°C), 0.5 M NaHCO\(_3\), 0.1 M MgCl\(_2\), 1.0 mM acetyl-CoA (prepared fresh), 0.1 M pyruvate (stored at -20°C), 0.1 M ATP (prepared fresh), 0.039 g DTNB in 10 ml 100% ethanol (prepared fresh), and citrate synthase (1000 U/ml). Cold PBS buffer was used for cell extract preparation. UV grade cuvettes were used. For the control cuvette, add the following to a total volume of 950 ul: 480 ul DI \(\text{H}_2\text{O}\), 140 ul PBS, 100 ul NaHCO\(_3\), 50 ul MgCl\(_2\), 100 ul acetyl-CoA, 25 ul DTNB, 5 ul citrate synthase, 50 ul ATP and no pyruvate. For the sample cuvette, add the following to a total volume of 950 ul: 430 ul DI \(\text{H}_2\text{O}\), 140 ul PBS, 100 ul NaHCO\(_3\), 50 ul MgCl\(_2\), 100 ul acetyl-CoA, 25 ul DTNB, 5 ul citrate synthase, 50 ul ATP and 50 ul pyruvate. The
The cuvette was blanked in the spectrophotometer and then 50 ul of cell extract were then added. The solution was quickly mixed by pipetting or inversion and the reading was started. The reaction was measured at 412 nm and 30°C, and the molar extinction coefficient used was $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The rate of increase in absorbance was used to calculate pyruvate carboxylase activity.

### 2.4.3.3 CS Assay

Citrate synthase activity was measured by a modified method of Aoshima et al., 2003. Citrate synthase produces citrate and CoA from the condensation of acetyl-CoA and oxaloacetate. DTNB, 5,5-dithiobis(2-nitrobenzoic acid), was added to react with the sulfhydryl group of the CoA, which could be spectrophotometrically.

The following solutions were required: 40 mM Tris-HCl buffer (adjusted to pH 8.1 with 20% KOH), 5.0 mM acetyl-CoA (prepared fresh), 100 mM oxaloacetate (stored at -20°C), 10.0 mg DTNB in 10 ml 100% ethanol (prepared fresh), and 100 mM KCl. Cold Tris-HCl buffer was used for cell extract preparation. UV grade cuvettes were used. For the control cuvette, add the following to a total volume of 950 ul: 150 ul DI H₂O, 500 ul Tris-HCl, 100 ul DTNB, 100 ul acetyl-CoA, 100 ul KCl and no oxaloacetate. For the sample cuvette, add the following to a total volume of 950 ul: 50 ul DI H₂O, 500 ul Tris-HCl, 100 ul DTNB, 100 ul acetyl-CoA, 100 ul KCl and 100 ul oxaloacetate. The cuvette was blanked in the spectrophotometer and then 50 ul of cell extract were then added. The solution was quickly mixed by pipetting or inversion and the reading was started. The reaction was measured at 412 nm and 37°C, and the molar extinction coefficient used was
13.6 mM\(^{-1}\) cm\(^{-1}\). The rate of increase in absorbance was used to calculate citrate synthase activity.

2.4.3.4 ICD Assay

Isocitrate dehydrogenase activity was measured by a modified method of Soundar et al., 1996. Isocitrate dehydrogenase oxidizes isocitrate to 2-ketoglutarate with a concomitant release of CO\(_2\) and reduction of NADP\(^+\) to NADPH.

The following solutions were required: 100 mM triethanolamine chloride buffer (pH 7.4) (adjusted with 3 N NaOH), 100 mM isocitrate (stored at -20\(^\circ\)C), 50 mM MnSO\(_4\) and 2 mM NADP\(^+\) (prepared fresh). Cold triethanolamine chloride buffer was used for cell extract preparation. UV grade cuvettes were used. For the control cuvette, add the following to a total volume of 900 ul: 510 ul DI H\(_2\)O, 300 ul triethanolamine chloride buffer, 40 ul MnSO\(_4\), and 50 ul NADP\(^+\) and no isocitrate. For the sample cuvette, add the following to a total volume of 900 ul: 470 ul DI H\(_2\)O, 300 ul triethanolamine chloride buffer, 40 ul MnSO\(_4\), 50 ul NADP\(^+\) and 40 ul isocitrate. The cuvette was blanked in the spectrophotometer and then 50 ul of cell extract were then added. The solution was quickly mixed by pipetting or inversion and the reading was started. The reaction was measured at 340 nm and 25\(^\circ\)C and the molar extinction coefficient used was 6.22 mM\(^{-1}\) cm\(^{-1}\). The rate of increase in absorbance was used to calculate isocitrate dehydrogenase activity.

2.4.3.5 ICL Assay
Isocitrate lyase activity was measured by a modified method of Dixon and Kornberg, 1959. Isocitrate lyase catalyzes the hydrolysis of isocitrate into glyoxylate and succinate. The glyoxylate formed in the presence of phenylhydrazine was measured spectrophotometrically as glyoxylic acid phenylhydrazone.

The following solutions were required: 250 mM potassium phosphate buffer pH 7.0 (mix 36 ml of 250 mM KH₂PO₄ and 45 ml of 250 mM K₂HPO₄), 0.1 M MgCl₂, 0.1 M cysteine HCl (prepared fresh), 0.1 M phenylhydrazine HCl (prepared fresh), and 0.1 M isocitrate (stored at -20°C). Cold potassium phosphate buffer was used for cell extract preparation. UV grade cuvettes were used. For the control cuvette, add the following to a total volume of 990 ul: 370 ul DI H₂O, 400 ul potassium phosphate buffer, 60 ul MgCl₂, 40 ul phenylhydrazine, 120 ul cysteine and no isocitrate. For the sample cuvette, add the following to a total volume of 990 ul: 290 ul DI H₂O, 400 ul potassium phosphate buffer, 60 ul MgCl₂, 40 ul phenylhydrazine, 120 ul cysteine and 80 ul isocitrate. The cuvette was blanked in the spectrophotometer and then 10 ul of cell extract were then added. The solution was quickly mixed by pipetting or inversion and the reading was started. The reaction was measured at 324 nm and 37°C. The molar extinction coefficient used was 17 mM⁻¹ cm⁻¹. The rate of increase in absorbance in the linear range was used to calculate isocitrate lyase activity.

2.4.3.6 MS Assay

Malate synthase activity was measured by the method of de Jong-Gubbels et al., 1995. The assay is a coupled enzyme assay in which the malate formed by malate
synthase is oxidized to oxaloacetate by malate dehydrogenase with the concomitant reduction of NAD\(^+\) to NADH.

The following solutions were required: 250 mM Tris-HCl pH 8.0, 100 mM MgCl\(_2\), 5.0 mM acetyl-CoA (prepared fresh), 40 mM NAD\(^+\) (prepared fresh), 5 mM sodium glyoxylate (stored at -20°C), citrate synthase (1008 U/ml) and malate dehydrogenase (8568 U/ml). Cold Tris-HCl buffer was used for cell extract preparation. UV grade cuvettes were used. For the control cuvette, add the following to a total volume of 950 ul: 356.6 ul DI H\(_2\)O, 400 ul Tris-HCl, 80 ul acetyl-CoA, 50 ul MgCl\(_2\), 10 ul NAD\(^+\), 2 ul citrate synthase, 1.4 ul malate dehydrogenase and no sodium glyoxylate. For the sample cuvette, add the following to a total volume of 950 ul: 306.6 ul DI H\(_2\)O, 400 ul Tris-HCl, 80 ul acetyl-CoA, 50 ul MgCl\(_2\), 10 ul NAD\(^+\), 2 ul citrate synthase, 1.4 ul malate dehydrogenase and 50 ul sodium glyoxylate. The cuvette was blanked in the spectrophotometer and then 50 ul of cell extract were then added. The solution was quickly mixed by pipetting or inversion and the reading was started. The reaction was measured at 340 nm and 25°C and the molar extinction coefficient used was 6.22 mM\(^{-1}\) cm\(^{-1}\). The rate of increase in absorbance was used to calculate malate synthase activity.

2.4.3.7 MDH Assay

Malate dehydrogenase activity was measured by a modified method of Zeikus \textit{et al.}, 1977. Malate dehydrogenase reduces oxaloacetate to malate through the oxidation of NADH to NAD\(^+\).

The following solutions were required: 200 mM tricine pH 8.1 (adjusted with 20% KOH), 2 mM NADH (prepared fresh) and 40 mM oxaloacetate (prepared fresh).
Cold tricine was used for cell extract preparation. UV grade cuvettes were used. For the control cuvette, add the following to a total volume of 950 ul: 350 ul DI H₂O, 500 ul tricine, 100 ul NADH and no oxaloacetate. For the sample cuvette, add the following to a total volume of 950 ul: 300 ul DI H₂O, 500 ul tricine, 100 ul NADH and 50 ul oxaloacetate. The cuvette was blanked in the spectrophotometer and then 1 ul of cell extract were then added. The solution was quickly mixed by pipetting or inversion and the reading was started. This reaction was measured at 340 nm and 37°C and the molar extinction coefficient used was 6.22 mM⁻¹ cm⁻¹. The rate of decrease in absorbance was used to calculate malate dehydrogenase activity. Normally, cell extracts have very high levels of malate dehydrogenase activity.

2.4.3.8 PDH Assay

Pyruvate dehydrogenase activity was measured by a modified method of Szutowicz et al., 1981. Pyruvate dehydrogenase catalyzes the conversion of pyruvate to acetyl-CoA and CO₂ using NAD⁺ as the co-substrate.

The following solutions were required: 0.25 M Tris-HCl pH 8.0, 0.2 M sodium pyruvate (stored at -20°C), 4 mM sodium CoA (prepared fresh), 40 mM NAD⁺ (prepared fresh), 40 mM thiamine pyrophosphate (TPP) (prepared fresh), 10 mM MgCl₂, 200 mM dithiothreitol (DTT) (prepared fresh), 25 mM oxaloacetate (stored at -20°C), 0.05 g DTNB in 10 ml 100% ethanol (prepared fresh), and citrate synthase (250 U/ml). Cold Tris-HCl was used for cell extract preparation. For the control, add the following in a microcentrifuge tube to a total volume of 825 ul: 325 ul DI H₂O, 200 ul Tris-HCl, 50 ul sodium pyruvate, 50 ul NAD⁺, 50 ul TPP, 100 ul MgCl₂, 50 ul DTT, and no sodium CoA.
For the sample, add the following in a microcentrifuge tube to a total volume of 825 ul: 275 ul DI H$_2$O, 200 ul Tris-HCl, 50 ul sodium pyruvate, 50 ul NAD$^+$, 50 ul TPP, 100 ul MgCl$_2$, 50 ul DTT, and 50 ul sodium CoA. To each microcentrifuge tube, 100 ul of cell extract was added and then incubated at 37°C in a waterbath for 15 min. The solutions in the microcentrifuge tubes were transferred to UV grade cuvettes. Then 50 ul of OAA and 25 ul of DTNB were added to each cuvette and mixed by vortex. The cuvettes were then blanked in the spectrophotometer and 5 U of citrate synthase was then added to each cuvette. The cuvettes were mixed again by pipetting or inversion. The reading was started immediately. The reaction was measured at 412 nm and 30°C. The molar extinction coefficient used was 13.6 mM$^{-1}$ cm$^{-1}$. The rate of increase in absorbance was used to calculate pyruvate dehydrogenase activity.

2.4.3.9 PYK Assay

Pyruvate kinase activity was measured by a modified method of Malcovati and Valentini, 1982. Pyruvate kinase dephosphorylates phosphoenolpyruvate into pyruvate using ADP as the phosphate group acceptor.

The following solutions were required: 50 mM HEPES (N-$\text{-2-hydroxyethylpiperazine-N'$\text{-2-ethane sulfonic acid}$}$ pH 7.5, 100 mM MgCl$_2$, 500 mM KCl, 40 mM ADP (prepared fresh), 100 mM PEP (prepared fresh), 10 mM NADH (prepared fresh), and lactate dehydrogenase. Cold HEPES was used for cell extract preparation. UV grade cuvettes were used. For the control cuvette, add the following to a total volume of 950 ul: 200 ul HEPES, 428 ul DI H$_2$O, 100 ul MgCl$_2$, 100 ul KCl, 50 ul ADP, 50 ul NADH, 22 ul lactate dehydrogenase and no PEP. For the sample cuvette, add
the following to a total volume of 950 ul: 200 ul HEPES, 328 ul DI H$_2$O, 100 ul MgCl$_2$, 100 ul KCl, 50 ul ADP, 50 ul NADH, 22 ul lactate dehydrogenase and 100 ul PEP. The cuvette was blanked in the spectrophotometer and then 50 ul of cell extract were then added. The solution was quickly mixed by pipetting or inversion and the reading was started. This reaction was measured at 340 nm and 25°C and the molar extinction coefficient used was 6.22 mM$^{-1}$ cm$^{-1}$. The rate of decrease in absorbance was used to calculate pyruvate kinase activity.

2.4.3.10 Intracellular Metabolites

For analyzing intracellular metabolites and acetyl-CoA, 40 OD units (OD$_{660nm}$ * vol (mL) = 40) of cell culture were taken into a precooled centrifuge tube, immediately chilled on ice, and centrifuged at 5000g at 4°C for 10 min. The pellet was cold washed and resuspended in 1 mL of cold 6% perchloric acid to lyse the cells and then placed on an ice bath for 10 min. Then 0.3 mL of cold 3 M potassium carbonate was added while vortexing to neutralize the acid. The solution was then centrifuged again at 4°C for 10 min and the supernatant was filtered through a 0.2 µm PVDF membrane syringe filter. The filtered supernatant was then stored at -80°C until analysis. Approximate intracellular concentrations relative to the cell volume were calculated based on the cell volume constant of 2.7 µL/mg dry weight (Winkler and Wilson, 1966).

The quantification of acetyl-CoA was based on a modified protocol of Boynton (Boynton et al., 1994) and used previously by others (Vadali et al., 2004; Lin et al., 2004). Acetyl-CoA was analyzed by HPLC (Thermofinnigan, San Jose, CA) using a UV detector set at 254 nm. The column used was a 5 µm octyldecyl silane column (Cell
Technologies, Inc., Houston, TX) preceded by an Allsphere ODS-2 (C18) guard column (Alltech, Deerfield, IL). The column was operated at room temperature. Two mobile phases of buffer were used at a flow rate of 1 mL/min. One buffer was 0.2 M sodium phosphate (pH 5.0) and the other buffer was 800 mL of 0.25 M sodium phosphate (pH 5.0) mixed with 200 mL of 100% acetonitrile. The run profile was adopted from Boynton (Boynton et al., 1994).

Citrate, isocitrate, and malate were measured by enzyme assay kits obtained from R-Biopharm. Metabolites such as glucose, succinate, lactate, formate, acetate, ethanol and glyoxylate were measured by the RI detector using HPLC. Pyruvate was measured by the UV detector at 210 nm using HPLC.

2.4.4 Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed in a two-step process. The first step is the cDNA synthesis, and the second step is the quantitative real-time PCR using the cDNA synthesized.

2.4.4.1 RNA Preparation

Total RNA was isolated with the Promega SV Total RNA Isolation System (Promega Corporation, Madison, WI) according to the manufacturer’s protocol. The isolated RNA was then treated again with DNase (Promega) and RNase Inhibitor (Promega). The reaction was incubated at 37°C for 30 min. The RNA was then extracted with phenol once, phenol/chloroform (50:50) once, and then chloroform twice. The RNA was then precipitated with ethanol and resuspended in RNase-free H2O (DEPC treated).
The RNase-free H₂O (DEPC treated) was prepared by adding DEPC (1µL/mL) to distilled water, then storing it overnight at 37°C, and then autoclaving it. The concentration of RNA was quantified by measuring the absorbance at 260 nm and applying the formula, concentration (µg/mL) = A₂₆₀ * 40 * dilution factor. The purity of the RNA was determined by reading the absorbance at 260 nm and 280 nm. RNA samples used were ensured to have the A₂₆₀/A₂₈₀ ratio between 1.8-2.1 for clean RNA.

2.4.4.2 cDNA Synthesis by Reverse Transcription

   cDNA was synthesized by reverse transcription using the RNA prepared as template. This was performed by using the Promega Reverse Transcription System (Promega, Madison, WI) and the reaction was carried out in a RoboCycler Gradient 96 (Stratagene, La Jolla, CA). The cDNA was synthesized in a total reaction mixture volume of 60 µL containing 1 µg of RNA template. The reaction mixture was incubated for 10 min at room temperature for primer extension, 30 min at 50°C for reverse transcription, and then 5 min at 95°C and 10 min at 6°C for inactivation of the reverse transcriptase. Control samples of the cDNA were also prepared following the same protocol, except reverse transcriptase was not added to the reaction mixture. The single-stranded cDNA sample was then diluted into the working range with nuclease free H₂O and stored at -20°C until further use.

2.4.4.3 Quantitative Real-Time PCR

   Quantitative real-time PCR was performed in a ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR Green PCR
Master Mix (Applied Biosystems, Foster City, CA). Direct detection of PCR product is monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA (SYBR Green PCR Master Mix and RT-PCR Protocol, Applied Biosystems). Forward and reverse primers were designed for each gene studied. The list of genes studied and primers designed is shown in Table 3. Reactions were carried out in a 96-well plate using the cDNA prepared as template. Each well contains a reaction mixture consisting of cDNA, forward and reverse primers, SYBR Green PCR Master Mix, and nuclease free water. PCR reaction was performed three times under identical reaction conditions for every gene using a particular strain’s cDNA in order to calculate a standard deviation; reaction using the control cDNA sample of that strain was also performed concurrently to ensure that there was no contamination.

Following the manufacturer’s protocol, the comparative $C_T$ method for relative quantification of gene expression was used (ABI Prism 7700 Sequence Detection System User Bulletin #2, Applied Biosystems). The threshold cycle, $C_T$, was the data obtained. The *rrsA* gene encoding rRNA 16S ribosomal RNA was used as an endogenous control in order to standardize the amount of sample DNA added to a reaction. This gene is not subjected to variable expression because its expression is abundant and relatively constant in most cells. In addition, since ribosome level varies with cell growth rate, chemostat cultures should maintain a steady level of this expression between strains. The difference between the $C_T$ of the studied gene and the *rrsA* gene was calculated ($\Delta C_T$) and a sensitivity test to confirm that the $\Delta C_T$ was independent of the RNA concentration was performed for each gene. The $\Delta \Delta C_T$ was then calculated by taking the difference between the $\Delta C_T$ of a gene in one strain and the $\Delta C_T$ of the same gene in the control
strain. The relative expression of a particular gene between a strain and its control strain is given by the formula $2^{-\Delta\Delta C_T}$. Using this method, it is important to emphasize that the expression levels of different genes cannot be compared. Expression levels can only be compared between different strains for the same gene, since the primers in those reactions would be the same.

2.5 Genetic Manipulations

2.5.1 Plasmid Isolation and DNA Purification

Plasmid isolation (miniprep) was performed using the GenElute Plasmid Mini-Prep Kit from Sigma (St. Louis, MO) or the QIAprep Spin Miniprep Kit from QIAGEN (Valencia, CA) by following the protocol suggested by the manufacturer. The method involves chemical lysis of the cells, binding the plasmid DNA to a spin column, then washing the column with ethanol-based solution to remove protein contaminants and finally eluting the plasmid using alkaline solution such Tris-HCl.

PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) by following the manufacturer’s protocol. DNA products such as plasmids and PCR products in agarose gels were purified using the QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA) following the manufacturer’s protocol. Chromosomal DNA from *Escherichia coli* and *Saccharomyces cerevisiae* used for genomic PCR were extracted and purified using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) by following the manufacturer’s protocol.
2.5.2 DNA Digestion and Ligation

Plasmid and DNA digestion was performed by using restriction enzymes purchased from New England Biolabs (Beverly, MA), Promega (Madison, WI), and Fisher (Pittsburgh, PA) following the protocol for each specific restriction enzyme. Ligation of DNA fragments for the construction of plasmids was performed using the DNA Ligation Kit from Sigma (St. Louis, MO) by following the manufacturer’s protocol.

2.5.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed to analyze and confirm the sizes of DNA fragments from either PCR or plasmid. A 1% agarose gel was made with TAE buffer (40 mM Tris-acetate, 2 mM EDTA at pH 8) and ethidium bromide. After making and solidifying the gel, the gel was placed in an apparatus containing TAE buffer. The DNA samples and DNA markers were then loaded into the wells of the gel. The DNA fragments were separated based on size after applying 100 volts across the gel for 60 minutes. The negative charge of DNA allows separation of different sizes across the negative and positive ends of the potential field. DNA fragments bound with ethidium bromide were visualized under UV light due to the fluorescence emitted by the ethidium bromide that behaves as an intercalating dye.

2.5.4 PCR

Primers were designed for PCR following general primer design methodology. Primer lengths vary between 18 to 30 nucleotides, including sites for restriction endonucleases. In general, the following guidelines in primer design are to avoid
stretches of A's, T's, G's, and C's, anchor the 3' end of primers with G's or C's, create primers with close to 50% G's and C's, match the T_m's of the forward and reverse primers, and be aware of primers forming dimers and hairpin loops.

PCR was conducted using the RoboCycler Gradient 96 (Stratagene, La Jolla, CA). Primers were designed and purchased from Integrated DNA Technologies, Inc (Coralville, IA). The rTth DNA Polymerase Kit from Applied Biosystems (Foster City, CA) and the MasterTaq Kit from Eppendorf (Hamburg, Germany) were used for cloning of genes following the manufacturer's protocol. PCR reaction mixtures in general contain the manufacturer's supplied PCR buffer, MgCl_2, dNTP, DNA template (plasmid or chromosomal DNA), forward and reverse primers, DNA polymerase, and Sigma H_2O. PCR conditions used depend on the characteristics of the primers such as melting temperature (T_m). Generally, different temperatures with different number of cycles are used for denaturing DNA, annealing primers, and extending primers. Denaturation is normally performed at 94°C, annealing/extension at 50°C to 65°C, and extension at 72°C.

2.5.5 Cell Transformation

One transformation method is chemical transformation. Chemical competent cells were prepared using the following protocol. The culture was grown with 5 ml of LB overnight at 37°C. The overnight culture was used to inoculate another 5 ml of LB and was grown for approximately 5 hrs to reach the early log phase (OD_{600nm} = 0.3 to 0.6). The culture was then centrifuged at 1000 g for 10 min and then resuspended in cold sterile TSB solution (93 ml LB, 10 g PEG, 5 ml DMSO, 1 ml 1.0 M MgCl_2, and 1 ml 1.0
M MgSO₄). The solution was frozen with dry ice submersed in isopropanol and stored in -80°C for storage.

For chemical transformation, 0.1 ug of plasmid DNA or 5 ul of miniprep was added to 100 ul of competent cells. The mixture was placed on ice for 5 to 30 min. Then 0.9 ml of TSB with 20 mM glucose was added to the mixture. The mixture was incubated at 37°C for 60 min with shaking at 250 rpm. A 100 ul aliquot from the mixture was then plated on appropriate selection agar plates. The agar plates were then placed in a 37°C incubator overnight and transformants were selected the following day.

Another transformation method is by electroporation. Electrocompetent cells were prepared using the following protocol. The culture was grown with 5 ml of LB overnight at 37°C. The overnight culture was used to inoculate another 5 ml of LB and was grown for approximately 5 hrs to reach the early log phase (OD₆₀₀nm = 0.3 to 0.6). The culture was then chilled on ice for 15 min. The culture was then kept on ice for all subsequent steps. The culture was washed twice with 5 ml of cold 1.0 mM HEPES solution (pH 7.0). The culture was then washed twice with 5 ml of cold 10% glycerol. The 5 ml culture was finally resuspended in 2 ml of 10% glycerol. This 2 ml culture solution contains electrocompetent cells ready for electroporation. The solution was frozen with dry ice submersed in isopropanol and stored in -80°C for storage.

Electroporation was performed using a Bio-Rad Gene Pulser (Hercules, CA). The electroporator was set at 2.5 kV and 25 μF, and the Pulse Controller (Bio-Rad, Hercules, CA) was set at 200 ohms. One to two microliters of plasmid DNA was added to 100 ul of electrocompetent cells and mixed by tapping of the microcentrifuge tube. The mixture was then transferred to a 0.1 cm Gene Pulser Cuvette (Bio-Rad, Hercules, CA) and the
cuvette was chilled on ice for 5 min. The conducting surface of the cuvette was then wiped dried. The volume of DNA added must be kept small because large concentration of DNA can decrease the transformation efficiency. Since the resistance (ohms) of sample should be high, make sure DNA added to the cells does not increase the total salt concentration in the cuvette by more than 1 mM. The cuvette was then finally placed into the sample chamber of the electroporator. The pulse was then applied to the cuvette. The cuvette was immediately removed after pulsing and added with 1 ml of SOC medium (autoclaved solution containing 20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl; then 10 ml of filter sterilized 1.0 M MgCl₂ and 10 ml of filter sterilized 1.0 M MgSO₄ per liter were added; then 1 ml of 2 M sterile glucose was added to 100 ml of this filter sterilized solution) to the transformation mixture. The solution was transferred back to a microcentrifuge tube and incubated for 1 hr at 37°C and 250 rpm for the cells to grow. An aliquot of 100 ul was then used to spread on an agar antibiotic selection plate, and the plate was incubated overnight at 37°C. Transformants were selected from the plate the following day for propagation.

2.5.6 Gene Cloning and Expression

Genes can be cloned with PCR and then expressed in cells using plasmids. This requires the construction of plasmids. Examples of plasmid construction are shown in Figures 2-4, 2-5, 2-6, and 2-7. The sequence maps of the gene and plasmid are required to facilitate construction. In general, restriction enzymes are used to digest plasmids and PCR products for ligation. The DNA fragment and the plasmid will need to have same flanking restriction sites in order for the ligation to occur. The gene fragment is ligated to
the plasmid after digestion. The ligation mixture is then transformed into appropriate host cells, and selection for colonies containing possible correct plasmid constructs is then performed on appropriate selection plates, such as Xgal and antibiotic containing plates. The plasmids of the cells from cultures grown with the selected colonies are then extracted by miniprep. Appropriate restriction enzymes are then used to digest the extracted plasmids, and the digested plasmids are electrophoresed on an agarose gel to check for the right construct. The correct plasmid construct will contain the gene of interest under the control of a promoter. That plasmid can then be transformed into a host strain for expression of that particular gene.

2.5.7 Gene Inactivation

2.5.7.1 P1-Mediated Phage Transduction

P1-mediated phage transduction was used to create mutations in the genes of \textit{E. coli}. Transduction is composed of two stages. The first stage involves the preparation of P1 lysate from the donor strain. The donor strain contains the mutation that is to be transferred to the chromosome of the recipient strain. Donor cells were grown overnight in LB and infected with appropriate amount of P1 lysate using a plating method (Miller, 1972). The P1 lysate from the donor containing DNA fragments that carry the gene mutation and a selection marker were stored at 4°C. In the second step, the P1 lysate of the donor was used to import the gene mutation into the recipient strain. Recipient cells grown overnight on LB were infected with appropriate amounts of P1 lysate from the donor. The infection occurred in sterile centrifuge tubes incubated at 37°C and 250 rpm
for 20 min. The excess media containing the P1 phage was removed by centrifugation and the pellet resuspended in minimal medium (10.5 g/l K$_2$HPO$_4$, 4.5 g/l KH$_2$PO$_4$, 1 g/l (NH$_4$)$_2$SO$_4$, and 0.5 g/l sodium citrate). The transductants were spread on the appropriate selection plates and incubated at 37°C.

2.5.7.2 λ Red Recombinase

Inactivation of genes can also be performed using the one-step inactivation method of Datsenko and Wanner (Datsenko and Wanner, 2000). This method first requires the construction of the single mutations using the phage λ Red recombinase. P1 phage transduction was then used to combine various mutations into one strain. Each mutation had to be added to the strain one at a time before the introduction of the next mutation because the kanamycin cassette had to be removed at each stage to enable selection of the next mutation. PCR products of the kanamycin cassette gene flanked by FRT (FLP recognition target) sites and homologous sequences to the gene of interest were made using pKD4 (Datsenko and Wanner, 2000) as the template. These PCR products were then transformed into the cells by electroporation (Gene Pulser, Bio-Rad, Valencia, CA) for insertional inactivation of the gene of interest. These transformed cells carry the plasmid pKD46 (Datsenko and Wanner, 2000) that expresses the λ Red system (γ, β, exo) for recombination of the PCR product into the chromosome. Once the kanamycin cassette is inserted, it can be removed using the helper plasmid, pCP20 (Datsenko and Wanner, 2000), that expresses FLP. The removal of the FRT-flanked kanamycin cassette leaves behind an 84 base pair insertion cassette. All disrupted genes
were verified with genomic PCR after construction to ensure that the gene of interest had been disrupted.

**Figure 2-4:** Cloning of *pepc* into pCR2.1-TOPO.
Figure 2-5: Cloning of pepe into pDHC29.
Figure 2-6: Cloning of pepc into pDHK29.
Figure 2-7: Cloning of \textit{pyc} into pTrc99A.
Chapter 3

3. Effect of Carbon Sources Differing in Oxidation State and Transport Route on Succinate Production in Metabolically Engineered

*Escherichia coli*

* This chapter has been accepted for publication in the *Journal of Industrial Microbiology & Biotechnology* (Lin et al., 2005d).

3.1 Abstract

In mixed-acid fermentation, succinate synthesis requires one mole of phosphoenolpyruvate (PEP), one mole of CO₂, and two moles of NADH for every mole of succinate to be formed. Different carbon sources with different properties were used to address these requirements. Sorbitol generates one more mole of NADH than glucose. Fermentation of sorbitol was shown to produce significantly more succinate than fermentation of glucose due to increased NADH availability. Xylose fermentation conserves the intracellular PEP pool, since its transport does not require the phosphotransferase system normally used for glucose transport. The extra PEP can then be assimilated in the succinate pathway to improve production. Fermentation of xylose did yield higher succinate production than glucose fermentation as a result. Subsequent inactivation of the acetate and lactate pathways was performed to study metabolite redistribution and the effect on succinate production. With the acetate pathway inactivated, significant carbon flux shifted toward lactate rather than succinate. When both acetate and lactate pathways were inactivated, succinate yield ultimately increased with a concomitant increase in ethanol yield.
3.2 Introduction

Succinate is a C_4-dicarboxylic acid with broad industrial value. It acts as a potential precursor for many commercial chemicals, such as 1,4-butanediol, with applications in the pharmaceutical, agricultural, fine chemical, and polymer industries (Zeikus et al., 1999). Traditionally, succinate is produced through petrochemical processes that are expensive and can lead to pollution problems. Recently, efforts have shifted toward microbial fermentation as a viable alternative for the production of succinate. The success of fermentation processes with renewable feedstocks can potentially supplant energy intensive processes that use nonrenewable sources (Nghiem et al., 1999).

The use of recombinant DNA technology to alter pathways in E. coli has been successful in increasing succinate production. By amplifying enzymatic steps involved in the succinate pathway, higher succinate production could be achieved. An example of this was demonstrated when native E. coli phosphoenolpyruvate carboxylase (PEPC) was overexpressed in E. coli (Millard et al., 1996), which increased succinate production. Conversion of fumarate to succinate was enhanced by overexpressing fumarate reductase (FRD) in E. coli (Goldberg et al., 1983; Wang et al., 1998). Certain pathways are not indigenous in E. coli, but can potentially improve succinate production. By introducing pyruvate carboxylase (PYC) from Rhizobium etli into E. coli, succinate production was enhanced (Gokarn et al., 1998). Other metabolic engineering strategies include inactivating competing pathways of the desired product. Inactivating pyruvate formate lyase (PFL) and lactate dehydrogenase (LDH) while overexpressing malic enzyme significantly increased succinate production (Stols and Donnelly, 1997). An inactive
glucose phosphotransferase system (PTSG) had also been shown to yield higher succinate production and better cell growth in the same mutant *E. coli* (Chatterjee *et al.*, 2001).

Under anaerobic conditions, *E. coli* undergoes mixed-acid fermentation to produce acetate, ethanol, formate, and lactate with succinate as a minor product. The succinate synthesis pathway branches from the glycolytic backbone at the phosphoenolpyruvate (PEP) node (Figure 3-1). PEP is converted to oxaloacetate (OAA) by PEPC through a carboxylation reaction. For every mole of succinate to be formed, one mole of CO$_2$ and two moles of NADH are required (Figure 3-1). PEPC has been shown to be important in enhancing succinate production (Millard *et al.*, 1996). Various PEPC enzymes from different species have been characterized (Annette *et al.*, 1980; Luinenberg and Coleman, 1992; Naide *et al.*, 1979; Nakamura *et al.*, 1995; Svensson *et al.*, 1997; Takai *et al.*, 1998; Terada *et al.*, 1991). In particular, PEPCs from plant species have drawn much attention due to their natural involvement in CO$_2$ fixation. For this reason, a mutant PEPC from a plant species (*Sorghum*) is overexpressed in all the strains used in the study. This mutant PEPC is particularly insensitive to malate feedback inhibition (Wang *et al.*, 1992). Therefore, the mutation is advantageous and favorable for improving succinate production.

Different carbon sources are used to examine the pathway requirements of succinate. Sorbitol, with an oxidation state of -1, generates one mole of NADH more than glucose per mole consumed. Therefore, the use of sorbitol in fermentation will
Figure 3-1: Mixed-acid fermentation pathways in E. coli.
increase the NADH availability. This, in turn, should increase succinate production compared to glucose. PEP is the first precursor in the succinate pathway. This substrate can be limiting since it is also assimilated in glycolysis and by the PTSG (Figure 3-1). Xylose can be used as a carbon source to conserve the PEP pool for the succinate pathway. The transport of xylose does not require the phosphotransferase system unlike glucose. It is transported by high-affinity permease driven by ATP (Li and Frost, 1999). With PTSG, a mole of PEP is converted to a mole of pyruvate while a mole of glucose is transported and phosphorylated. For this reason, xylose fermentation should yield higher succinate production than glucose fermentation. The acetate (ackA-pta) and lactate (ldhA) pathways are also inactivated in appropriate strains to study the effect on metabolite redistribution and the response to different carbon sources. Inactivating these pathways could eliminate competition for carbon flux with the succinate pathway, therefore improving succinate production.

3.3 Materials and Methods

3.3.1 Strains and Plasmids

Our laboratory wild type GJT001, a spontaneous S-(2-aminoethyl)-L-cysteine-resistant mutant of MC4100, Δlac strain (arg-lac) U169 rspL150 relA1, (Tolentino et al., 1992) was used for all the genetic manipulations. YBS121 is GJT001Δ(ackA-pta) (Yang, 1999) with the acetate pathway inactivated. YBS132 is GJT001Δ(ackA-pta, ldhA) (Yang et al., 1999b) with both the acetate and lactate pathways inactivated.
Plasmid pKK313, carrying the S8D mutation in *Sorghum PEPC* (Wang et al., 1992), was used to overexpress PEPC in GJT001, YBS121, and YBS132. Plasmid pKK313 confers ampicillin resistance. The altered *Sorghum* PEPC has a site-directed mutation that advantageously relieves malate feedback inhibition from the enzyme (Wang et al., 1992). This plasmid, pKK313, was transformed into GJT001, YSB121, and YSB132. GJT(pKK313) is used as the basic host for all the experiments. To study pathway inactivation, GJT(pKK313) is used as the control for YBS121(pKK313) and YBS132(pKK313).

### 3.3.2 Medium

The medium used was Luria-Bertani broth (LB) adjusted to a pH of 7.4. It was supplemented with 40 g/l of carbon source (glucose, sorbitol, or xylose) depending on the experiment. An IPTG concentration of 0.1 mM was added to the medium to induce the expression of the *Sorghum* PEPC under the control of the *trc* promoter. An ampicillin concentration of 50 mg/l was used for selection of the plasmid.

### 3.3.3 Analytical Techniques

Cell density was measured at 600 nm in a Spectronic 1001 spectrophotometer and optical density (OD) was recorded as absorbance. Samples were centrifuged at 6000g and 4°C for 10 min. The supernatant was then filtered through a 0.45-μm syringe filter and stored in HPLC vials at -20°C. The carbon source and extracellular metabolites (succinate, lactate, formate, acetate, and ethanol) were quantified using an HPLC system (Thermo-Separation Products) equipped with a cation-exchange column (HPX-87H,
BioRad Lbs, Hercules, CA) and a differential refractive index detector (Waters). A 0.6 ml/min mobile phase using 2.5 mM H₂SO₄ solution was applied to the column. The 2.5 mM H₂SO₄ solution was prepared with Milli-Q water (Milli-Q Water System, Millipore, Bedford, MA) and filtered through a 0.45-μm membrane filter. The column was operated at 55°C.

3.3.4 Experimental Setup A

For experimental setup A, 45-ml capped tubes containing 18 ml of medium were used. The air in the headspace was replaced with CO₂ at 1 atm by purging CO₂ into the tube at a flow rate of 1 l/min for 8 sec. A seed inoculum of 45 μl from an overnight 5 ml culture was used for each tube. An MgCO₃ concentration of 27.78 g/l was also supplied in the medium to buffer the pH during growth and act as an indirect means of supplying CO₂. All experiments were performed in triplicates. Cultures were grown in an orbital shaker at 250 rpm and 37°C. Results were used only when all the carbon source had been consumed.

3.3.5 Experimental Setup B

For experimental setup B, 250-ml capped flasks containing 10 ml of medium were used. The headspace was not replaced with CO₂. This setup was used when studying mutant E. coli strains since it allowed the strains to grow faster in the presence of oxygen. A seed inoculum of 40 μl from an overnight 5 ml culture was used for each flask. MgCO₃ concentration of 27.78 g/L was also supplied to the medium. All experiments were performed in triplicates. Cultures were grown in 37°C at 250 rpm.
Results were examined only when all the carbon source had been consumed. The CO₂ and biomass contributions were not determined.

3.4 Results

3.4.1 Effect of Carbon Sources with Different Oxidation States and Transport Routes on Succinate Production

Satisfying the cofactor requirement of metabolic pathways is essential in facilitating production formation. One of the requirements for succinate formation is the cofactor NADH. Two moles of NADH are needed for the production of one mole of succinate from PEP (Figure 3-1). The maximum theoretical succinate yield under anaerobic conditions is two moles of succinate formed for every mole of glucose consumed if excess CO₂ is available. This theoretical yield for succinate is not achievable, though, under natural conditions since NADH is limiting when metabolizing glucose. For every mole of glucose metabolized via glycolysis, only two moles of NADH are formed. This, therefore, will only allow one mole of succinate to be produced. To study the effect of increased NADH availability on succinate production, sorbitol is used as a carbon source for comparison to glucose. Studies have shown the importance of sorbitol’s reducing power in enhancing succinate production (Hong and Lee, 2002). This is related to NADH formation since the catabolism of sorbitol produces more NADH than glucose (San et al., 2002). Sorbitol has an oxidation state of -1 whereas glucose has an oxidation state of 0 (San et al., 2002). For every mole of sorbitol consumed, three moles of NADH are formed compared to only two moles of NADH formed for every mole of glucose consumed.
The glucose phosphotransferase system (PTSG) is utilized to transport glucose into the cytoplasm of the cell. The transport process is mediated at the expense of one mole of phosphoenolpyruvate (PEP) converted to one mole of pyruvate per mole of glucose transported (Figure 3-1). During this process, glucose is phosphorylated with the phosphate group from PEP. Since PEP is the first precursor in the succinate synthesis pathway, the depletion of PEP by the PTSG should hamper succinate productivity. To study the importance of the PEP requirement in succinate production, a carbon source that does not use the PTS system for transport was used and compared to glucose. Xylose is a five carbon sugar with the same oxidation state, 0, as glucose. Its uptake into the cell uses a non-PTS system. In this regard, using xylose as a carbon source would be expected to conserve more PEP for succinate synthesis, therefore yielding higher succinate production than glucose. Sorbitol like glucose is transported into the cell by the PTS system. Sorbitol and xylose are compared to glucose to examine the effect of different oxidation states and transport routes on succinate production.

Sorbitol was a more effective carbon source in succinate production than glucose with experimental setup A when the strain GJT(pKK313) was examined. Succinate production and yield with sorbitol were 96% and 81% higher than glucose, respectively (Table 3-1 and Table 3-2). This effect was also observed in studies by others (Hong and Lee, 2002). The results show the importance of NADH as a rate-limiting factor in the succinate synthesis pathway. Succinate productivity increases when more NADH is available. Ethanol production also increased substantially with sorbitol as the carbon source compared to glucose. Production and yield of ethanol with sorbitol increased by 75% and 62% over glucose, respectively (Table 3-1 and Table 3-2). Since formation of
one mole of ethanol also requires two moles of NADH, the increased NADH pool generated by the use of sorbitol would be expected to enhance ethanol production. Interestingly, lactate production and yield with sorbitol decreased substantially compared

**Table 3-1:** Comparison of carbon source on metabolite production using strain GJ7(pKK313). Experiment setup A was used with 40 g/l of carbon source supplemented. Experiments were performed in triplicates.

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Succinate (mM)</th>
<th>Lactate (mM)</th>
<th>Acetate (mM)</th>
<th>Ethanol (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>32.74 ± 0.14</td>
<td>45.66 ± 3.2</td>
<td>128.14 ± 2.38</td>
<td>150.35 ± 1.40</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>64.11 ± 0.66</td>
<td>6.270 ± 0.3</td>
<td>66.850 ± 0.99</td>
<td>263.08 ± 4.17</td>
</tr>
<tr>
<td>Xylose</td>
<td>49.54 ± 1.10</td>
<td>6.680 ± 0.6</td>
<td>163.30 ± 3.16</td>
<td>172.83 ± 3.28</td>
</tr>
</tbody>
</table>

**Table 3-2:** Comparison of carbon source on metabolite molar yield using strain GJ7(pKK313). Experiment setup A was used with 40 g/l of carbon source supplemented. Results are from the experiment in Table 3-1. Molar yield is mole of product formed per mole of carbon source consumed.

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Succinate yield</th>
<th>Lactate yield</th>
<th>Acetate yield</th>
<th>Ethanol yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.172 ± 0.001</td>
<td>0.240 ± 0.017</td>
<td>0.675 ± 0.013</td>
<td>0.792 ± 0.007</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0.312 ± 0.003</td>
<td>0.031 ± 0.002</td>
<td>0.325 ± 0.001</td>
<td>1.280 ± 0.009</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.201 ± 0.004</td>
<td>0.027 ± 0.002</td>
<td>0.663 ± 0.013</td>
<td>0.701 ± 0.013</td>
</tr>
</tbody>
</table>

to glucose (Table 3-1 and Table 3-2). Formation of lactate also requires NADH. The succinate and ethanol pathways could possibly be preferred routes for establishing redox balance under reduced environments when sorbitol is being consumed since both pathways oxidize two moles of NADH for every mole of product formed. This could explain why lactate production decreased with sorbitol since more carbon flux was channeled toward succinate and ethanol. Production and yield for acetate also decreased substantially with sorbitol compared to glucose (Table 3-1 and Table 3-2). The partition of carbon flux at the acetyl-CoA node was imbalanced under sorbitol with a molar ethanol to acetate ratio of approximately 4 to 1 (Table 3-1). This molar ratio under glucose is approximately 1 to 1 showing a more balanced partition between acetate and
ethanol at the acetyl-CoA node (Table 3-1). These results show the significant effect of sorbitol in rendering reduced environments through the formation of extra NADH during its catabolism.

The use of xylose as a carbon source by strain GJT(pKK313) in experimental setup A resulted in higher succinate production than the use of glucose. Succinate production and yield with xylose increased by 51% and 17%, respectively (Table 3-1 and Table 3-2). Lactate production and yield with xylose decreased substantially compared to glucose (Table 3-1 and Table 3-2). This decrease could be in part attributed to more carbon flux being directed toward the succinate pathway at the PEP node. Therefore, less carbon flux was being channeled toward pyruvate and the lactate pathway. Importantly, the ethanol to acetate ratio under xylose was approximately 1 to 1 similar to glucose (Table 3-1). This is the expected result since the oxidation state of xylose and glucose is 0, which does not alter the redox state of the intracellular environment.

3.4.2 Effect of Acetate and Lactate Pathway Inactivation on Metabolite Production under Glucose, Sorbitol, and Xylose Fermentation

Inactivating competing pathways of succinate can channel more carbon flux toward the succinate synthesis pathway, therefore resulting in higher succinate production. An example is an engineered E. coli strain where its lactate dehydrogenase (LDH) and pyruvate formate-lyase (PFL) have both been inactivated (Bunch et al., 1997). This inactivation in the central metabolic pathway, as a result, caused a net accumulation of pyruvate. The strategy is direct, but the effect is sometimes haphazard since the cellular response to perturbation is often stochastic. The effect of inactivating
the acetate pathway on metabolite production is characterized under glucose, sorbitol, and xylose fermentation. Subsequently, the lactate pathway is inactivated in combination with the acetate pathway to characterize further the effect on metabolite production.

Strain GJT(pKK313) is used in the study as the control strain for the pathway mutant strains. Strain YBS121(pKK313) is GJT(pKK313) with the acetate pathway inactivated (ΔackA-pta). YSB132(pKK313) is GJT(pKK313) with both the acetate and lactate pathways inactivated (ΔackA-pta, ldhA). Experimental setup B (in Material and Methods) was employed for studying these strains in order to promote faster cell growth. Succinate, lactate, acetate, and ethanol production and yield were examined.

The results of YBS121(pKK313) showed that inactivating the acetate pathway had a significant effect on metabolite redistribution. Under all three carbon sources (glucose, sorbitol, and xylose) lactate production and yield increased substantially. For glucose, lactate production and yield both increased by 15 fold (Table 3-3 and Table 3-4). For sorbitol, there was no lactate production in GJT(pKK313). Once the acetate pathway was inactivated, substantial lactate production was observed (Table 3-3). For xylose, lactate production and yield also increased substantially by over 5 fold (Table 3-3 and Table 3-4). Acetate production did decrease substantially in strain YSB121(pKK313) with all three carbon sources. Ethanol production also decreased as a result of the acetate pathway inactivation. The cell response to acetate pathway inactivation, apparently, is to channel significant excess carbon flux toward the lactate pathway. With glucose and sorbitol, succinate yield decreased in strain YBS121(pKK313) compared to the control strain GJT(pKK313) (Table 4). With xylose, the succinate yield of YSB121(pKK313) was relatively unchanged compared to GJT(pKK313) (Table 4). The most significant
metabolite redistribution observed with acetate pathway inactivation was changes in lactate production.

Table 3-3: Effect of pathway inactivation on metabolite production with various carbon sources. Experiment setup B was used with 40 g/l of carbon source supplemented. Experiments were performed in triplicates.

<table>
<thead>
<tr>
<th></th>
<th>Succinate (mM)</th>
<th>Lactate (mM)</th>
<th>Acetate (mM)</th>
<th>Ethanol (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GJT(pKK313)</td>
<td>80.02 ± 2.18</td>
<td>11.80 ± 5.25</td>
<td>220.6 ± 7.88</td>
<td>13.99 ± 4.40</td>
</tr>
<tr>
<td>YBS121(pKK313)</td>
<td>34.51 ± 2.74</td>
<td>190.5 ± 9.54</td>
<td>40.62 ± 3.83</td>
<td>3.140 ± 0.43</td>
</tr>
<tr>
<td>YSB132(pKK313)</td>
<td>49.68 ± 2.69</td>
<td>6.720 ± 0.70</td>
<td>13.82 ± 1.39</td>
<td>46.42 ± 1.67</td>
</tr>
<tr>
<td>Sorbitol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GJT(pKK313)</td>
<td>102.3 ± 0.90</td>
<td>0</td>
<td>143.0 ± 4.44</td>
<td>87.57 ± 6.32</td>
</tr>
<tr>
<td>YBS121(pKK313)</td>
<td>105.1 ± 2.92</td>
<td>93.47 ± 5.00</td>
<td>26.51 ± 3.99</td>
<td>57.91 ± 9.00</td>
</tr>
<tr>
<td>YSB132(pKK313)</td>
<td>102.4 ± 9.75</td>
<td>8.990 ± 1.00</td>
<td>8.790 ± 0.73</td>
<td>101.9 ± 21.2</td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GJT(pKK313)</td>
<td>83.21 ± 1.79</td>
<td>28.95 ± 5.28</td>
<td>239.8 ± 8.38</td>
<td>10.62 ± 1.65</td>
</tr>
<tr>
<td>YBS121(pKK313)</td>
<td>83.30 ± 4.98</td>
<td>182.6 ± 11.3</td>
<td>42.85 ± 5.93</td>
<td>3.920 ± 0.51</td>
</tr>
<tr>
<td>YSB132(pKK313)</td>
<td>72.77 ± 4.95</td>
<td>6.650 ± 0.18</td>
<td>15.97 ± 1.13</td>
<td>47.42 ± 2.42</td>
</tr>
</tbody>
</table>

Table 3-4: Effect of pathway inactivation on metabolite molar yield with various carbon sources. Experiment setup B was used with 40 g/l of carbon source supplemented. Results are from the experiment in Table 3-3. Molar yield is mole of product formed per mole of carbon source consumed.

<table>
<thead>
<tr>
<th></th>
<th>Succinate yield</th>
<th>Lactate yield</th>
<th>Acetate yield</th>
<th>Ethanol yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GJT(pKK313)</td>
<td>0.424 ± 0.012</td>
<td>0.063 ± 0.028</td>
<td>1.169 ± 0.042</td>
<td>0.074 ± 0.023</td>
</tr>
<tr>
<td>YBS121(pKK313)</td>
<td>0.183 ± 0.015</td>
<td>1.009 ± 0.051</td>
<td>0.215 ± 0.020</td>
<td>0.017 ± 0.002</td>
</tr>
<tr>
<td>YSB132(pKK313)</td>
<td>0.307 ± 0.016</td>
<td>0.042 ± 0.004</td>
<td>0.085 ± 0.009</td>
<td>0.287 ± 0.011</td>
</tr>
<tr>
<td>Sorbitol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GJT(pKK313)</td>
<td>0.565 ± 0.004</td>
<td>0</td>
<td>0.790 ± 0.023</td>
<td>0.483 ± 0.030</td>
</tr>
<tr>
<td>YBS121(pKK313)</td>
<td>0.515 ± 0.014</td>
<td>0.458 ± 0.025</td>
<td>0.130 ± 0.020</td>
<td>0.284 ± 0.044</td>
</tr>
<tr>
<td>YSB132(pKK313)</td>
<td>0.661 ± 0.007</td>
<td>0.058 ± 0.006</td>
<td>0.057 ± 0.004</td>
<td>0.657 ± 0.120</td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GJT(pKK313)</td>
<td>0.342 ± 0.008</td>
<td>0.119 ± 0.022</td>
<td>0.986 ± 0.035</td>
<td>0.044 ± 0.007</td>
</tr>
<tr>
<td>YBS121(pKK313)</td>
<td>0.342 ± 0.002</td>
<td>0.751 ± 0.047</td>
<td>0.176 ± 0.024</td>
<td>0.016 ± 0.002</td>
</tr>
<tr>
<td>YSB132(pKK313)</td>
<td>0.433 ± 0.018</td>
<td>0.040 ± 0.002</td>
<td>0.095 ± 0.003</td>
<td>0.282 ± 0.008</td>
</tr>
</tbody>
</table>

Since lactate production increased substantially upon acetate pathway inactivation, the lactate pathway was also inactivated alone with the acetate pathway. Mutant strain YBS132(pKK313) has both the acetate and lactate pathways inactivated. The metabolite production of this strain was compared to YBS121(pKK313) and GJT(pKK313). In YBS132(pKK313), lactate production and yield decreased
substantially compared to YSB121(pKK313) (Table 3-3 and Table 3-4). Acetate production and yield of YBS132(pKK313) further decreased compared to YSB121(pKK313).

The most significant shift in metabolite distribution upon lactate and acetate pathway inactivation is toward ethanol. The ethanol yield of YSB132(pKK313) increased substantially compared to YSB121(pKK313) and GJT(pKK313) under all three carbon sources (Table 4). The succinate yield was also high in YBS132(pKK313). With glucose, the succinate yield of YSB132(pKK313) was higher than YSB121(pKK313), but not higher than GJT(pKK313) (Table 4). With sorbitol and xylose, the succinate yield of YSB132(pKK313) was higher than either YSB121(pKK313) or GJT(pKK313) (Table 4). Ethanol production had the largest increase after the lactate and acetate pathways were inactivated; indicating that the ethanol pathway was the cell’s preferred pathway for relieving excess carbon flux and maintaining redox balance.

Among the three carbon sources, sorbitol still retained the highest succinate and ethanol production in YSB121(pKK313) and YSB132(pKK313) compared to glucose and xylose (Table 3-3). Lactate production in YSB121(pKK313) with sorbitol fermentation was the lowest compared to glucose and xylose fermentation (Table 3-3). With xylose, succinate production in YSB121(pKK313) and YSB132(pKK313) was consistently higher than glucose. These results showed that the effect of sorbitol’s oxidation state and xylose’s transport property on succinate production remained the same despite inactivation of the lactate and acetate pathways.

3.5 Discussion
The use of sorbitol as a carbon source increased succinate production substantially in *E. coli* compared to glucose. This result showed that the cofactor NADH is a rate-limiting factor in the succinate synthesis pathway. Ethanol also increased since its formation also requires two moles of NADH, same as succinate. The effect of the reducing power of sorbitol can be observed by the molar ethanol to acetate ratio increasing substantially above 1. With glucose, this ratio is approximately 1 to 1. This altered ethanol to acetate ratio is a result of imbalanced carbon flux partitioning at the acetyl-CoA node with more carbon flux directed toward the ethanol pathway. This is caused by the recycling of excess NADH generated from sorbitol catabolism.

PEP is an essential limiting precursor for driving succinate synthesis. This was observed by the use of xylose as a carbon source, which increased succinate production compared to glucose. The transport of xylose does not require the PTS system unlike glucose. As a result, more intracellular PEP is conserved, which can then be assimilated through the succinate pathway to increase production.

Attempts to redirect carbon flux toward a desired pathway by means of pathway inactivation cannot always be premeditated. Inactivating the acetate pathway only resulted in a substantial increase in lactate production rather than succinate production. Succinate production did finally increase when both the acetate and lactate pathways were inactivated. The effect of inactivating these two pathways, though, also caused ethanol production to increase substantially. The dynamic response of the cell to reach equilibrium due to perturbation in its metabolic network is sometimes difficult to foresee. Sequential pathway deletion in this study exemplifies the intricate nature of the metabolic network.
3.6 Acknowledgements

The authors would like to thank Dr. Jean Vidal for providing the *Sorghum pepc* plasmid (pKK313). This work was supported by grants from the National Science Foundation (BES-0222691 and BES-0000303). Henry Lin was supported by a training grant from the National Science Foundation (DGE0114264).
Chapter 4

4. Effect of *Sorghum vulgare* Phosphoenolpyruvate Carboxylase and *Lactococcus lactis* Pyruvate Carboxylase Coexpression on Succinate Production in Mutant Strains of *Escherichia coli*

* This chapter has been accepted for publication in *Applied Microbiology and Biotechnology* (Lin et al., 2004b).

4.1 Abstract

*Sorghum vulgare* phosphoenolpyruvate carboxylase (PEPC) and *L. lactis* pyruvate carboxylase (PYC) were overexpressed in *E. coli* concurrently to improve production of succinate, a valuable industrial specialty chemical. This coexpression system was also applied to *E. coli* mutant strains strategically designed by inactivating competing pathways of succinate formation. The highest level of succinate production was observed in *E. coli* strains coexpressing both PEPC and PYC when compared to *E. coli* strains individually overexpressing either PEPC or PYC. Lactate production was also significantly reduced with PEPC and PYC coexpression. Lactate and acetate pathways were inactivated to eliminate competing pathways of succinate formation. Results showed that inactivation of both the lactate and acetate pathways with the coexpression of PEPC and PYC was the most effective in improving succinate production. Inactivating the lactate or acetate pathway alone only caused a majority of the carbon flux to shift to other metabolites rather than succinate. Coexpression of PEPC and PYC was also applied to an *ldh* and *pfl* *E. coli* mutant strain that accumulates a substantial amount of the intermediate metabolite pyruvate during growth. The results showed that PEPC
and PYC coexpression was effective in depleting the pyruvate accumulation and increasing the production of metabolites.

### 4.2 Introduction

Succinate, a C$_4$-dicarboxylic acid, is produced only as a minor product in mixed-acid fermentation of glucose by *E. coli* (Blackwood *et al.*, 1956). Acetate, formate, lactate, and ethanol are the major products of *E. coli* fermentation. Succinate is a useful specialty chemical serving as a precursor to many commodity chemicals such as 1,4-butanediol, tetrahydrofuran, and γ-butyrolactone. These chemicals are applied in areas such as polymers, solvents, and additives (Zeikus *et al.*, 1999). The capability to produce succinate commercially using a biocatalyst with renewable carbohydrate feedstocks could serve as an alternative to traditional petrochemical processes that are expensive and prone to cause pollution. A useful bioprocess could improve the economics of the succinate market (Schilling, 1995).

In the succinate formation pathway, the first enzymatic step is the carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (OAA) through the fixation of CO$_2$ (Figure 4-1). This reaction is catalyzed by phosphoenolpyruvate carboxylase (PEPC). OAA is then sequentially reduced to malate, where it is eventually converted to succinate. During this process, two moles of NADH are oxidized to two moles of NAD$^+$ for every mole of succinate formed. By overexpressing native PEPC in *E. coli*, others have already shown increased production of succinate (Millard *et al.*, 1996). Overexpression of a mutant *Sorghum vulgare* PEPC that is resistant to malate feedback inhibition in *E. coli* has also been shown to increase succinate production (Lin *et al.*
2004a). OAA is an important precursor of the succinate pathway. Means of increasing the OAA pool could translate to increased succinate production. Pyruvate carboxylase (PYC) converts pyruvate to OAA through fixation of CO$_2$ and hydrolysis of ATP. However, PYC is not indigenous to *E. coli*. By introducing pyruvate carboxylase (PYC) from *Rhizobium etli* into *E. coli*, succinate production was significantly enhanced (Gokarn *et al.*, 1998, 2000, 2001). The glucose phosphotransferase system (PTSG) is responsible for transporting glucose across the cell membrane. During this process, glucose is phosphorylated to glucose-6-P with the concomitant dephosphorylation of PEP to pyruvate. Therefore, for every mole of glucose transported, one mole of PEP is converted to pyruvate (Figure 4-1). *E. coli* strains with a mutation in PTSG have been shown to increase succinate production (Chatterjee *et al.*, 2001). Inactive PTSG in wildtype *E. coli* causes slower glucose uptake, but enhanced succinate production. This is likely due to an increased PEP pool that could be converted to OAA for succinate formation.

In this study, we applied a PEPC and PYC coexpression system in *E. coli* to examine its effect on succinate productivity. Coexpression of PEPC and PYC is also applied in mutant strains of *E. coli* strategically designed to have the competing pathways of succinate inactivated. The origins of PEPC and PYC used in this study are from *Sorghum vulgare* and *Lactococcus lactis*, respectively. Overexpressing PYC concurrently with PEPC can recapture the pyruvate produced from PTSG and direct it back to OAA. Together with PEPC, the enhanced OAA pool can subsequently increase succinate production. This coexpression strategy can improve productivity without inactivating PTSG which would impede glucose uptake and cell growth. Overexpressing
Figure 4-1: Mixed-acid fermentation pathways in *E. coli*. The dashed line of pyruvate carboxylase (*pye*) is to indicate that it is not indigenous in *E. coli*. PEP is phosphoenolpyruvate, OAA is oxaloacetate, *pepc* is PEP carboxylase, *ldh* is lactate dehydrogenase, *ack* is acetate kinase, *pta* is phosphotransacetylase, *ptsG* is glucose phosphotransferase system, and *frd* is fumarate reductase.
PEPC and PYC concurrently would provide two routes for diverting the glycolytic flux toward OAA.

The lactate (ldh) and acetate (ackA-pta) pathways compete with the succinate pathway for carbon flux at the pyruvate node (Figure 4-1). These pathways were inactivated in the presence of overexpressed PEPC and PYC in E. coli. The effect of inactivation of the lactate and acetate pathways on metabolite production was examined. Coexpression of PEPC and PYC was also applied in a mutant strain deficient in lactate dehydrogenase (LDH) and pyruvate:formate lyase (PFL). This ldh− and pfl− mutant strain accumulates a significant amount of the intermediate, pyruvate and is characterized by poor carbon-throughput. If the accumulated pyruvate can be redirected to OAA, this could potentially increase succinate production in this mutant strain. A similar mutant strain, NZN111, had also been shown to exhibit slow growth, but high succinate production (Donnelly et al., 1998). NZN111 also accumulates a substantial amount of pyruvate and is unable to ferment glucose to completion. The coexpression of PEPC and PYC was, therefore, applied to the ldh− and pfl− mutant strain to examine its efficacy in reducing pyruvate accumulation.

### 4.3 Materials and Methods

#### 4.3.1 Strains and Plasmids

Our laboratory parental E. coli wildtype strain is GJT001, which is a spontaneous cadR mutant of E. coli strain MC4100 (Table 4-1). All pathway inactivations were performed in GJT001 using P1 phage transduction. E. coli mutant strain YBS121 is
Table 4-1: List of *E. coli* strains and plasmids used in the study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GJT001</td>
<td>Spontaneous cadR mutant of MC4100(ATCC35695), Δlac(arg-lac)U169rpsL150relA1ptsF Sm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Tolentino et al., 1992</td>
</tr>
<tr>
<td>YBS121</td>
<td>GJT001Δ(ackA-pta), Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Yang et al., 1999b</td>
</tr>
<tr>
<td>YBS131</td>
<td>GJT001Δ(ldhA), Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Yang et al., 1999b</td>
</tr>
<tr>
<td>YBS132</td>
<td>GJT001Δ(ackA-pta, ldhA), Tc&lt;sup&gt;R&lt;/sup&gt;/Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Yang et al., 1999b</td>
</tr>
<tr>
<td>SB202</td>
<td>GJT001Δ(ldhA, pfl), Tc&lt;sup&gt;R&lt;/sup&gt;/Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Berrios-Rivera, 2002</td>
</tr>
<tr>
<td>GJT(pDHK29, pTrc99A)</td>
<td>GJT001 control strain</td>
<td>This study</td>
</tr>
<tr>
<td>GJT(pHL333, pTrc99A)</td>
<td>GJT001 overexpressing PEPC</td>
<td>This study</td>
</tr>
<tr>
<td>GJT(pHL333, pHL413)</td>
<td>GJT001 overexpressing PEPC and PYC</td>
<td>This study</td>
</tr>
<tr>
<td>YBS121(pDHK29, pTrc99A)</td>
<td>YSB121 control strain</td>
<td>This study</td>
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<td>YSB121 overexpressing PEPC and PYC</td>
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<td>This study</td>
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<tr>
<td>YBS131(pHL333, pHL413)</td>
<td>YSB131 overexpressing PEPC and PYC</td>
<td>This study</td>
</tr>
<tr>
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<td>YSB132 control strain</td>
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</tr>
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<tr>
<td>SB202(pDHK29, pTrc99A)</td>
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<tr>
<td>SB202 (pHL333, pHL413)</td>
<td>SB202 overexpressing PEPC and PYC</td>
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<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHL333</td>
<td>S8D mutant <em>Sorghum vulgare pepc</em> in pDHK29, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>pHL413</td>
<td><em>L. lactis pyc</em> in pTrc99A, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lin et al., 2004a</td>
</tr>
<tr>
<td>pTrc99A</td>
<td>Cloning vector, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pDHK29</td>
<td>Cloning vector, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Phillips et al., 2000</td>
</tr>
</tbody>
</table>
GJT001Δ(ackA-pta) with the acetate pathway inactivated (Yang, 1999). *E. coli* mutant strain YBS131 is GJT001ΔldhA with the lactate pathway inactivated (Yang, 1999). *E. coli* mutant strain YBS132 is GJT001(Δ(ackA-pta), ΔldhA) with both the acetate and lactate pathways inactivated (Yang et al., 1999b). *E. coli* mutant strain SB202 is YBS131Δpfl with both the lactate and pyruvate:formate lyase pathways inactivated (Berrios-Rivera, 2002).

The malate feedback inhibition resistant *Sorghum vulgare pepc* gene was cloned into the pDHK29 vector and designated pHL333 (Lin et al., 2004a). *L. lactis pyc* was cloned into the pTrc99A vector and designated pHL413 (Lin et al., 2004a). The origins of replication of pHL333 and pHL413 are compatible for plasmid stability. Both pHL333 and pHL413 are high-copy plasmids with the trc promoter controlling gene expression. Plasmid pHL333 confers kanamycin resistance and pHL413 confers ampicillin resistance. The four plasmids, pDHK29, pHL333, pTrc99A, and pHL413, were transformed in pairs in different combinations into the *E. coli* strains GJT001, YBS121, YBS131, YSB132, and SB202 to study the coexpression of PEPC and PYC. A complete list of *E. coli* strains created with two plasmids is shown in Table 4-1.

### 4.3.2 Medium and Experimental Setup

Luria-Bertani broth (LB) (Sambrook et al., 1989) with an adjusted pH of 7.5 was used. Glucose was supplemented at 20 g/l. Since pHL413 contains the *lacI* repressor gene, 1mM of isopropyl-β-D-thiogalactoside (IPTG) was added to the medium for induction. An ampicillin concentration of 100 mg/l was supplemented for studying strains carrying only one plasmid. An ampicillin concentration of 200 mg/l was
supplemented for studying strains carrying two plasmids. A kanamycin concentration of 100 mg/l was supplemented in all cases.

All experiments were performed using 250-mL flasks sealed with rubber plugs. Each flask contained 0.3g of MgCO$_3$ prior to autoclaving. The MgCO$_3$ serves to buffer the pH during cell growth. A volume of 10 ml of medium was then added to each flask for cultivation. All cultures in the experiments were started at an initial optical density of 0.5. The cultures were grown in an orbital shaker at 250 rpm and 37 °C for 24 hrs. All experiments were performed in triplicates. Optical density of cultures was not measured due to interference from MgCO$_3$.

4.3.3 Analytical Techniques

Samples for analysis were centrifuged at 6000xg and 4°C for 10 min. The supernatant was then filtered through a 0.45-μm syringe filter and stored in vials at 20°C. The carbon source and extracellular metabolites (succinate, lactate, formate, acetate, and ethanol) were quantified using high-throughput liquid chromatography (HPLC) from Thermo-Separation Products (West Palm Beach, FL). It is equipped with a cation-exchange column (HPX-87H, BioRad Lbs, Hercules, CA) and a differential refractive index detector (Waters, Milford, MA). A 0.6 ml/min mobile phase using 2.5 mM H$_2$SO$_4$ solution was applied to the column. The 2.5 mM H$_2$SO$_4$ solution was prepared with Milli-Q water (Milli-Q Water System, Millipore, Bedford, MA) and filtered through a 0.45-μm membrane filter. The column was operated at 55°C.

4.3.4 Enzyme Assays
To prepare crude extracts for enzyme assays, mid-exponential phase cultures were taken and washed twice with cold 100 mM Tris-HCl buffer at pH 8.0. Centrifugation was done at 4000xg and 4°C for 20 min for these washing steps. The cells were then resuspended in the same solution and subjected to sonication for 6 min in an ice bath. The sonicated cells were centrifuged at 1500xg and 4°C for 60 min to remove cell debris. PEPC and PYC activities were measured based on the citrate synthase-coupled assay by the method of Payne and Morris (Payne and Morris, 1969). The oxaloacetate produced by PYC or PEPC is reacted with acetyl-CoA by citrate synthase to yield CoA. The CoA then reacts with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) to produce a yellow colored product that can be measured spectrophotometrically at 412 nm. All reactions were performed in duplicates and carried out at 37°C. Reactions were initiated by adding the crude extract. The PYC assay differed from the PEPC assay in that pyruvate and ATP were added for the PYC assay. For PEPC, phosphoenolpyruvate was added instead of pyruvate and ATP. The specific activity of the enzymes was measured in μmole product/mg protein/hr (DTNB extinction coefficient used is $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). Total protein concentration of the crude extract was measured by Lowry’s method (Sigma Lowry Reagent, Modified) using bovine serum albumin as standard.

### 4.4 Results

#### 4.4.1 Effect of PEPC and PYC Coexpression

The effect of *L. lactis* pyc overexpression on succinate production in *E. coli* was first examined before it was coexpressed with *Sorghum vulgare pepc*. Plasmid pHL413 bearing the *L. lactis* pyc was transformed into the parental wildtype strain GJT001 and
three other mutant strains, YBS121, YBS131, and YBS132 (Table 4-1). The backbone plasmid pTrc99A was also transformed into these four strains as a control. Succinate production increased with overexpression of *L. lactis* PYC in *E. coli*. Results showed that PYC from *L. lactis* was effective in increasing succinate production and molar yield (mole succinate formed/mole glucose consumed) by 145% (data not shown). Others have also shown that by overexpressing *Rhizobium etli* PYC in *E. coli*, succinate production also increased significantly (Gokarn *et al.*, 1998, 2000, 2001). A similar effect on succinate production by *L. lactis* PYC overexpression was observed in the three mutant strains YBS121, YBS 131, and YBS132 (data not shown).

Coexpression of mutant *Sorghum vulgare pepc* and *L. lactis pyc* was carried out using two high-copy plasmids with compatible origins of replication. Plasmid pHL333 overexpresses PEPC and pHL413 overexpresses PYC. The control vector plasmids for pHL333 and pHL413 are pDHK29 and pTrc99A, respectively. Parental strains carrying two plasmids were constructed for the systematic study of the effect of PEPC and PYC coexpression on metabolite production. The strain GJT(pDHK29, pTrc99A) is the control (Table 4-1). Strain GJT(pHL333, pTrc99A) overexpresses only the *Sorghum vulgare* PEPC. Strain GJT(pHL333, pHL413) overexpresses both the *Sorghum vulgare* PEPC and *L. lactis* PYC. The combined effect of PEPC and PYC overexpression is examined by comparing with the control strain with the vector plasmids and the strain overexpressing only *Sorghum vulgare* PEPC.

The results showed that by overexpressing only the *Sorghum vulgare* PEPC (GJT(pHL333, pTrc99A)), succinate production and yield both increased by over 2-fold compared to the control GJT(pDHK29, pTrc99A) (Figure 4-2). Previous studies have
Figure 4-2: Succinate and lactate production with PEPC and PYC coexpression in the parental wildtype *E. coli* strain GJT001. Control represents the strain GJT(pDHK29, pTrc99A) without PEPC or PYC overexpression; PEPC represents the strain GJT(pHL333, pTrc99A) with only PEPC overexpression; PEPC+PYC represents the strain GJT(pHL333, pHL413) with both PEPC and PYC overexpression. (1a) Succinate production; (1b) Succinate yield (mole succinate formed/mole glucose consumed); (2a) Lactate production; (2b) Lactate yield (mole lactate formed/mole glucose consumed). No significant trend was observed for acetate or ethanol production in these three strains. Mean and standard deviations were calculated based on triplicate experiments. Cultures were grown for 24 hours.
shown that overexpression of PEPC increases succinate production in *E. coli* (Millard *et al.*, 1996). Coexpression of PYC with PEPC (GJT(pHL333, pHL413)) increased succinate production and yield by another 26 % compared to GJT(pHL333, pTrc99A). The coexpression of PYC and PEPC increased overall succinate production and yield by 3-fold compared to the control GJT(pDHK29, pTrc99A) (*t*=2.776; *p*<0.05). The results showed the substantial effect of PYC coexpressed with PEPC on succinate production. PYC, not indigenous in *E. coli*, provides an alternative route for salvaging the pyruvate that is generated from glycolysis and the glucose phosphotransferase system (PTSG). Overexpression of PYC increases the oxaloacetate (OAA) pool. Presumably, the increased OAA pool would result in higher succinate production, since OAA is a major precursor in the succinate synthesis pathway. Therefore, with the coexpression of PYC and PEPC, the intracellular OAA pool increased substantially. As a result, this produced the highest increase in succinate production and yield compared to the control and overexpression of PEPC alone.

The effect of PYC could also be seen on lactate production. Lactate is formed directly from pyruvate in a reduction reaction catalyzed by lactate dehydrogenase (LDH) (Figure 4-1). Results showed that with only PEPC overexpression, lactate production and yield decreased by 23 % (Figure 4-2) in strain GJT(pHL333, pTrc99A) compared to the control strain GJT(pDHK29, pTrc99A) (*t*=2.776; *p*<0.05). When PYC was coexpressed with PEPC, lactate production and yield decreased significantly by 82 % in strain GJT(pHL333, pHL413) compared to strain GJT(pHL333, pTrc99A) with only PEPC overexpressed (*t*=2.776; *p*<0.05). With PYC and PEPC coexpression, lactate decreased overall by 86 % compared to the control strain GJT(pDHK29, pTrc99A) (*t*=2.776;
$p<0.05$). The significant decrease in lactate when PYC was introduced into the *E. coli* metabolism demonstrated that PYC was competitive with LDH at the pyruvate node. PEPC alone did not substantially reduce the lactate production, since carbon flux was still being channeled from pyruvate to lactate. With both PEPC and PYC overexpressed, a substantial amount of the carbon flux was directed from the PEP and pyruvate nodes toward the succinate synthesis pathway resulting in a substantial decrease in lactate production.

Acetate production and ethanol production did not change significantly with the coexpression of PEPC and PYC (data not shown). Because the acetate and ethanol pathways are competing pathways of succinate, the PEPC and PYC coexpression system can be further applied in mutant strains where these competing pathways of succinate production have been inactivated. By inactivating competing pathways, the coexpression of PEPC and PYC could become even more efficient in redirecting the carbon flux toward the succinate pathway.

### 4.4.2 Effect of Competing Pathway Inactivation upon PEPC and PYC Coexpression

The effect of coexpression of PEPC and PYC on succinate production was further studied with the inactivation of competing pathways of succinate. Pyruvate is the main precursor of both the acetate and lactate pathways (Figure 4-1). Inactivating these two pathways would reduce the competition at the pyruvate node thereby enhancing the carbon flux toward the succinate pathway. Inactivation of the acetate and lactate pathway was carried out in the parental wildtype strain GJT001 (Table 4-1). YBS121 is GJT001 with only the acetate pathway (*ackA-pta*) inactivated. YBS131 is GJT001 with only the
lactate pathway (*ldh*) inactivated. YBS132 is GJT001 with both the acetate and lactate pathways (*ackA-pta, ldh*) inactivated. All four strains were transformed with the appropriate plasmids, so strains overexpressing PEPC and PYC would be examined. The effect of these pathway mutations on succinate, acetate, lactate and ethanol production was analyzed. GJT001, YBS121, YBS131, and YBS132 were transformed with both pHL333 and pHL413, which overexpress PEPC and PYC, respectively. Simultaneous PEPC and PYC overexpression was shown to be effective in increasing succinate production for the three mutant strains, YBS121, YBS131, and YBS132, just as in the parental wildtype strain GJT001 (data not shown).

Inactivating competing pathways has a significant effect on succinate production with the coexpression of PEPC and PYC. Succinate production in strains YBS121(pHL333, pHL414), YBS131(pHL333, pHL413) and YBS132(pHL333, pHL413) was higher than the control strain GJT(pHL333, pHL413) in each case (Figure 4-3). Strain YBS132(pHL333, pHL413) had the highest succinate production and yield of all four strains (Figure 4-3). Succinate production and yield were the second highest in strain YSB131(pHL333, pHL413) and then followed by strain YBS121(pHL333, pHL413). The results showed that inactivating the acetate pathway was less effective in increasing succinate production than inactivating the lactate pathway. Inactivating both the acetate and lactate pathways was the most effective in increasing succinate production. Strain GJT(pHL333, pHL413), used as the control, showed the lowest succinate production.

Inactivating pathways caused shifts in carbon flux to different metabolites. When the acetate pathway (*ackA-pta*) was inactivated (YBS121(pHL333, pHL413)), acetate
Figure 4-3: Metabolite production in mutant strains with PEPC and PYC coexpression. GJT(PEPC+PYC) is the parental wildtype strain GJT(pHL333, pHL413); YBS121(PEPC+PYC) is strain GJT(pHL333, pHL413) with the acetate pathway (ackA-pta) inactivated; YBS131(PEPC+PYC) is strain GJT(pHL333, pHL413) with the lactate pathway (ldhA) inactivated; YBS132(PEPC+PYC) is strain GJT(pHL333, pHL413) with both the acetate and lactate pathways inactivated. (1a) Succinate production; (1b) Succinate yield (mole succinate formed/mole glucose consumed); (2a) Lactate production; (2b) Lactate yield (mole lactate formed/mole glucose consumed); (3a) Acetate production; (3b) Acetate yield (mole acetate formed/mole glucose consumed); (4a) Ethanol production; (4b) Ethanol yield (mole ethanol formed/mole glucose consumed). Mean and standard deviations were calculated based on triplicate experiments. Cultures were grown for 24 hours.
production decreased by 79% (Figure 4-3) \((t=2.776; p<0.05)\). This led to a substantial increase in lactate production of about 24-fold. Succinate production, however, only increased by 20% \((t=2.776; p<0.05)\). Ethanol production decreased by 25% \((t=2.776; p<0.05)\). The disproportionate shift in carbon flux toward lactate significantly reduced the effect of acetate pathway inactivation on increasing succinate production. In the presence of an inactivated acetate pathway, the lactate pathway was more competitive than the succinate pathway for the excess carbon flux even when PEPC and PYC were overexpressed.

When the lactate pathway \((ldhA)\) was inactivated instead \((YBS131(pHL333, pHL413))\), succinate production increased by 46% over the control GJT(pHL333, pHL413) \((t=2.776; p<0.05)\). This increase showed that inactivating the lactate pathway was more effective than inactivating the acetate pathway in enhancing succinate production. With the lactate pathway inactivated, there was no detectable lactate production. Acetate production increased by 19% \((t=2.776; p<0.05)\) although the yield was relatively the same compared to the control strain GJT(pHL333, pHL413). Interestingly, a significant amount of the carbon flux was channeled instead toward ethanol production. Ethanol production increased by 150% whereas the yield increased by 110% when the lactate pathway was inactivated \((t=2.776; p<0.05)\). With a significant portion of the carbon flux partitioned toward ethanol, not a substantial amount could be channeled toward succinate production. However, inactivating the lactate pathway is still more effective than inactivating the acetate pathway to increase succinate production.

When both the acetate \((ackA-pta)\) and lactate pathways \((ldhA)\) were inactivated \((YBS132(pHL333, pHL413))\), succinate production and yield increased by 67% and
76%, respectively, compared to the control strain GJT(pHL333, pHL413) (Figure 4-3) 
($t=2.776; p<0.05$). No trace of lactate production was detected. Acetate production 
decreased by 76% ($t=2.776; p<0.05$). Ethanol production increased by only 15% 
($t=2.776; p<0.05$). This increase in ethanol was significantly less than the increase in 
ethanol when only the lactate pathway was inactivated. This suggests that more carbon 
flux has been drawn toward the succinate pathway with the inactivation of both the 
acetate and lactate pathways. As a result, succinate production and yield were the highest 
in strain YBS132(pHL333, pHL413) compared to strains GJT(pHL333, pHL413), 
YBS121(pHL333, pHL413), and YBS132(pHL333, pHL413).

4.4.3 Reduction of Pyruvate Accumulation with Coexpression of *pepc* and *pyc*

Coexpression of PEPC and PYC has been shown to be effective in enhancing 
succinate production in the parental wildtype strain GJT001 and the three mutant strains 
YBS121, YBS131, and YBS132. Overexpression of PEPC and PYC appears to increase 
the oxaloacetate pool by drawing carbon flux from the PEP and pyruvate nodes, 
respectively (Figure 4-1). Addition of PYC to the central metabolic pathway was also 
competitive with LDH at the pyruvate node since lactate decreased significantly.

The PEPC and PYC coexpression system was used as a tool to study the potential 
to reduce pyruvate accumulation in a particular *E. coli* mutant strain designed for 
succinate production. SB202, constructed from YBS131, has both the LDH and 
pyruvate:formate lyase (PFL) inactivated (Berrios-Rivera, 2002). This strain as a result is 
characterized by slow growth and inability to ferment glucose to completion in anaerobic 
conditions. Inactive LDH and PFL cause the carbon flux to bottle up at the pyruvate
node causing pyruvate to accumulate as a major product. SB202, therefore, accumulates a substantial amount of pyruvate that is secreted into the medium. A similar strain, NZN111, with the same pathway deletions as SB202 also accumulates pyruvate and is inefficient in fermenting glucose (Bunch et al., 1997). NZN111 has been extensively studied. AFP111, a spontaneous mutant of NZN111, with mutation in the PTSG (Chatterjee et al., 2001) had an increased growth rate and was shown to produce 11.3 g/l of succinate with no accumulation of pyruvate (Donnelly et al., 1998).

Coexpression of PEPC and PYC is applied in the strain SB202 to promote dissimilation of accumulated pyruvate. With this approach, succinate productivity in SB202 may be improved. Three different strains derived from SB202 were created by transformation of different pairs of plasmids (Table 4-1). SB202(pDHK29, pTrc99A) is the control strain, SB202(pHL333, pTrc99A) overexpresses only PEPC, and SB202(pHL333, pHL413) overexpresses both PEPC and PYC. The effect of PEPC and PYC coexpression in SB202 on succinate, pyruvate, lactate, acetate, and ethanol production was examined.

In the control strain SB202(pDHK29, pTrc99A), 32mM of pyruvate was secreted into the medium (Figure 4-4). When PEPC was overexpressed in SB202, pyruvate decreased by 40% to 19mM ($t=2.776; p<0.05$). Overexpressing PYC alone had similar effect on pyruvate production as overexpressing PEPC alone (data not shown). When PYC and PEPC were both overexpressed in SB202, there was no trace of pyruvate detected in the medium. The results showed that coexpression of PEPC and PYC eliminated the pyruvate accumulation in SB202. PEPC and PYC coexpression would convert the excess pyruvate to OAA. The increase in the OAA pool could then be
Figure 4-4: Metabolite production in the \textit{ldh} and \textit{pfl} mutant strain SB202 with PEPC and PYC coexpression. Control represents the strain SB202(pDHK29, pTrc99A) without PEPC or PYC overexpression; PEPC represents the strain SB202(pHL333, pTrc99A) with only PEPC overexpression; PEPC+PYC represents the strain SB202(pHL333, pHL413) with both PEPC and PYC overexpression. (1a) Succinate production; (1b) Succinate yield (mole succinate formed/mole glucose consumed); (2a) Pyruvate production; (2b) Pyruvate yield (mole pyruvate formed/mole glucose consumed); (3a) Acetate production; (3b) Acetate yield (mole acetate formed/mole glucose consumed). No lactate production was detected in these three strains. No significant trend was observed for ethanol production in these three strains. Mean and standard deviations were calculated based on triplicate experiments. Cultures were grown for 24 hours.
channeled to the formation of other products. PEPC and PYC coexpression provided an efficient detour for relieving the carbon flux congestion at the pyruvate node in SB202.

Succinate production by SB202(pDHK29, pTrec99A) was 12 mM (Figure 4-4). This amount is higher than parental wildtype strain GJT(pDHK29, pTrec99A) due to competing pathway inactivation of both LDH and PFL. When PEPC was overexpressed in SB202(pHL333, pTrec99A), succinate increased by 59% over the control strain SB202(pDHK29, pTrec99A) \((t=2.776; p<0.05)\). With the coexpression of both PEPC and PYC in SB202(pHL333, pH413), succinate increased by 65% compared to the control strain SB202(pDHK29, pTrec99A) \((t=2.776; p<0.05)\). Between SB202(pHL333, pTrec99A) and SB202(pHL333, pH413), there was no significant difference in succinate production, although pyruvate was completely dissimilated when both PEPC and PYC were overexpressed. In SB202(pHL333, pTrec99A), acetate production also increased by 35% when PEPC was overexpressed compared to the control strain (Figure 4-4) \((t=2.776; p<0.05)\). Acetate production increased significantly by 81% in SB202(pHL333, pH413) when both PEPC and PYC were overexpressed compared to the control \((t=2.776; p<0.05)\). SB202(pHL333, pH413) had a 34% higher acetate production than SB202(pHL333, pTrec99A) \((t=2.776; p<0.05)\).

4.5 Discussion

In this study, we have shown that coexpression of PEPC and PYC enhances succinate production by simultaneously drawing carbon flux from the PEP and pyruvate nodes of the glycolytic pathway. PYC, not indigenous in *E. coli*, provides an alternative route for salvaging the pyruvate that is generated from glycolysis and the glucose
phosphotransferase system (PTSG). PEPC and PYC work in tandem to convert PEP and pyruvate to OAA. As a result, the OAA pool is increased. Presumably, the increased OAA pool results in higher succinate production because OAA is a major precursor in the succinate synthesis pathway. Coexpression of PEPC and PYC results in higher succinate production than when only PEPC is overexpressed. *L. lactis* PYC is also competitive with LDH at the pyruvate node resulting in reduced lactate production.

Competing pathways of succinate were inactivated to improve the efficiency of PEPC and PYC coexpression in enhancing succinate production. The results showed that inactivating competing pathways effectively increased succinate production, but the carbon flux was still being channeled to other metabolites. When the acetate pathway was inactivated, a significant portion of the carbon flux was channeled toward lactate production rather than succinate production. When the lactate pathway was inactivated instead, more carbon flux shifted toward ethanol production than acetate and succinate production. Inactivating the lactate pathway was observed to be more effective than inactivating the acetate pathway in increasing succinate production. This is because the lactate pathway is one of the major pathways used by *E. coli* in oxidation-reduction for NADH recycling to NAD$^+$; the acetate pathway is not involved in this process. By inactivating the lactate pathway, the redox state of the cell becomes imbalanced forcing the cell to utilize the ethanol pathway and to a lesser extent, the succinate pathway for maintaining redox balance. When the acetate pathway was inactivated, the cell preferably utilized the lactate pathway to displace congestion of carbon flux at the pyruvate node. Based on the results, the ethanol and lactate pathways are committed routes for maintaining redox balance and metabolic equilibrium. Inactivating both the
acetate and lactate pathways was the most effective in enhancing succinate production with coexpression of PEPC and PYC. Interestingly, acetate and lactate pathway inactivation induced the cell to utilize the succinate pathway for NADH recycling more than the ethanol pathway. As a result, succinate production increased significantly, while the increase in ethanol production was relatively insignificant.

A mutant *E. coli* strain deficient in both LDH and PFL, a strain similar to NZN111 that produces high level of succinate (Donnelly *et al.*, 1998), has been studied and improved for its potential to produce large amount of succinate. This strain has also been shown to accumulate a high level of pyruvate impeding its growth and productivity. Results showed that coexpression of PEPC and PYC in this type of mutant strain could completely alleviate the pyruvate accumulation under the conditions used. With PEPC and PYC coexpression, no trace of pyruvate was detected in the medium.

4.6 Acknowledgements

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Chapter 5

5. Increasing the Acetyl-CoA Pool in the Presence of Overexpressed Phosphoenolpyruvate Carboxylase or Pyruvate Carboxylase Enhances Succinate Production in Escherichia coli

* This chapter has been published in Biotechnology Progress (Lin et al., 2004a).

5.1 Abstract

An in vivo strategy to apply the activation effect of acetyl-CoA on phosphoenolpyruvate carboxylase (PEPC) and pyruvate carboxylase (PYC) to increase succinate production in Escherichia coli was studied. This approach relies on the increased intracellular acetyl-CoA and CoA levels by overexpressing E. coli pantothenate kinase (PANK). The results showed that coexpression of PANK and PEPC, and PANK and PYC did improve succinate production compared to the individual expression of PEPC and PYC, respectively. The intracellular acetyl-CoA and CoA levels were also measured, and each showed a significant increase when the PANK was overexpressed. Another effect observed was a decrease in lactate production. The least amount of lactate was produced when PANK and PEPC, and PANK and PYC were coexpressed. This result showed increased competitiveness of the succinate pathway at the phosphoenolpyruvate and pyruvate nodes for the carbon flux; as a result reducing the carbon flux toward the lactate pathway. The study also demonstrates a feasible method for metabolic engineering to modulate enzyme activity in vivo through specific activators and inhibitors.
5.2 Introduction

One common limitation in overexpressing enzymes for metabolic engineering purposes is the inability to modulate their \textit{in vivo} activities. One method of manipulating enzymatic activities is by utilizing the enzymes’ specific activators or inhibitors. Activators can significantly enhance the activities of the corresponding enzymes. Acetyl-CoA has been shown to be a powerful allosteric activator of phosphoenolpyruvate carboxylase (PEPC) from a range of species of organisms. PEPC from \textit{Escherichia coli} has been shown to be strongly activated by acetyl-CoA (Izui \textit{et al.}, 1981). PEPC from \textit{Corynebacterium glutamicum} exhibited sixfold higher activity in the presence of acetyl-CoA when expressed in transgenic plant \textit{Solanum tuberosum} (Gehlen \textit{et al.}, 1996).

Acetyl-CoA, in general, is known to be a better activator of bacterial PEPCs than plant PEPCs (Chen \textit{et al.}, 2002). In spite of this, individual cases still need to be examined for PEPCs from different species. For example, acetyl-CoA showed no effect on the activity of the PEPC from the thermophilic cyanobacterium, \textit{Synechococcus vulcanus} (Chen \textit{et al.}, 2002). PEPC branches from glycolysis by converting phosphoenolpyruvate (PEP) to oxaloacetate (OAA) through a carboxylation reaction. Pyruvate carboxylase (PYC) activity has also been shown to be largely dependent on acetyl-CoA. For example, PYC from the photosynthetic bacterium \textit{Rhodobacter capsulatus} was shown to be absolutely dependent on acetyl-CoA (Modak and Kelly, 1995). Acetyl-CoA also effectively protected the \textit{R. capsulatus} PYC from thermal denaturation (Modak and Kelly, 1995). PYC converts pyruvate to OAA also through fixation of CO$_2$. In glycolysis, PEP is converted to pyruvate in a one-step reaction by pyruvate kinase or during uptake of sugars by the phosphotransferase system (PTS).
Studies of activation of PEPC or PYC by acetyl-CoA or other activators have involved in vitro methods through enzyme extraction and activity measurement (Chen et al., 2002; Dong et al., 1998). The activation effect of acetyl-CoA on PEPC and PYC has never been accessed and applied in vivo by any means. Manipulation of intracellular enzymatic activity has been an ongoing interest and effort in metabolic engineering. The ability to modulate a particular enzyme’s activity in vivo means the possibility to successfully control the cellular system for achieving a desired purpose, such as enhancing the production of a certain product.

In this study, we seek to provide and corroborate a new means of exploiting the acetyl-CoA activation effect on PEPC and PYC entirely in vivo. In the context of metabolic engineering, this approach will be applied to enhance the production of succinate. PEPC and PYC are the first enzymatic steps in the succinate pathway. Overexpression of E. coli PEPC or Rhizobium etli PYC in E. coli has been shown to enhance succinate production (Millard et al., 1996; Gokarn et al., 1998). Interest in succinate production has been fueled by its practicality as a specialty industrial chemical and by an ever increasing effort to replace chemical processes with biocatalysts for production. To increase the intracellular acetyl-CoA pool, E. coli pantothenate kinase (PANK) is overexpressed in E. coli. This strategy has been shown to significantly enhance both the acetyl-CoA and CoA pools (Vadali et al., 2004a). Strains bearing the PANK plasmid will be used to coexpress a malate feedback inhibition resistant Sorghum PEPC and also separately, a PYC from Lactococcus lactis. PYC is not indigenous in E. coli (Gokarn et al., 1998), therefore the L. lactis PYC is used as the model PYC to be studied in the E. coli host system. Both the L. lactis PYC and Sorghum PEPC have been
shown in our lab to increase succinate production in *E. coli*. The increased intracellular acetyl-CoA pool will be used to study its effect on the native *E. coli* PEPC, *Sorghum* PEPC, and *Lactococcus lactis* PYC through the subsequent effect on succinate production. By simultaneously overexpressing PANK and PEPC, or PANK and PYC, succinate production was found to increase as compared to individual overexpression of PEPC or PYC.

**5.3 Materials and Methods**

**5.3.1 Plasmids**

The laboratory wildtype *E. coli* is GJT001, a spontaneous *cadR* mutant of MC4100 (Tolentino *et al.*, 1992). All plasmid transformation and gene expression studies were performed in GJT001. A malate feedback inhibition resistant *Sorghum pepc* was cloned from pKK313 (Wang *et al.*, 1992) into the high-copy number plasmid pDHK29 (Phillips *et al.*, 2000) to yield pHL333. The *trc* promoter and transcription terminators of the *Sorghum pepc* on pKK313 were also included in the PCR product. The *rTh* DNA polymerase was used (Applied Biosystems), and pKK313 served as the DNA template. The forward primer is 5'-CAGCTCGAGCGTAAATCAGTCGATAATTCCG-3', and the reverse primer is 5'-CACCATGGGTTATCAGGGTTATTGTCTCATGAGC-3'. The PCR product of *Sorghum pepc* was first ligated into the intermediate plasmid pCR2.1-TOPO (Invitrogen). The 3.8-kb *Sorghum pepc* fragment was then digested from the pCR2.1-TOPO vector.
using SpeI and EcoRI, and then ligated into pDHK29. The *Sorghum pepe* cloned in pDHK29 is designated pH333, which confers kanamycin (Km) resistance.

The *L. lactis* pyc was digested from pCPYC1 (Wang et al., 2000) with SmaI and PstI and then ligated into pTrc99A (Pharmacia). The final vector is designated pH413 and confers ampicillin (Ap) resistance.

The *E. coli* panK was cloned from pSJ380 (Calder et al., 1999) into both pDHK29 and pUC19 (Yanisch-Perron et al., 1985) to yield pRV480 (Vadali et al., 2004b) and pRV380 (Vadali et al., 2004a), respectively. The origin of replication of pDHK29 is compatible with the ColE1 origin of pTrc99A and pUC19. This feature allows concurrent overexpression of two genes in the same system. The antibiotic markers are also compatible since pDHK29 has kanamycin resistance, and pTrc99A and pUC19 express ampicillin resistance.

### 5.3.2 Medium and Cultivation

The medium used throughout the study was Luria-Bertani broth (LB) adjusted to pH of 7.5. A 20 g/L concentration of glucose was supplemented. Kanamycin concentration used was 100 mg/L and ampicillin concentration used was 1 g/L to maintain plasmid stability. MgCO₃ concentration of 20 g/L was also added to the LB. MgCO₃ serves to buffer the pH during fermentation and provides an indirect means of supplying CO₂ required in succinate synthesis. For induction of gene expression, 1 mM of isopropyl-β-D-thiogalactoside (IPTG) was used in the medium. Pantothenic acid was added to the culture medium at a concentration of 5 mM. This is a required substrate for the pantothenate kinase.
The experiments were performed in 250-mL flasks sealed with rubber plugs containing 15 ml of media. The results from this experimental setup were demonstrated to be reproducible through repetition of the experiments. All experiments were done in triplicates. The fermentation runs were for 24 hours. The flask cultures were grown at 37 ºC at 250 rpm in an orbital shaker.

5.3.3 Analytical Techniques

For analyzing the extracellular metabolites such as succinate and lactate, 1 mL of culture was centrifuged and the supernatant was then filtered through a 0.45-µm syringe filter for HPLC analysis. The HPLC system (Shimadzu-10A Systems, Shimadzu, Columbia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A) and a differential refractive index detector (Waters 2410, Waters, Milford, MA). A 0.6 mL/min mobile phase using 2.5 mM H₂SO₄ solution was applied to the column. The column was operated at 55 ºC.

To quantify intracellular acetyl-CoA and CoA compounds, 40 optical density (OD) units of cell culture was taken (OD₆₆₀nm * vol (mL) = 40) and centrifuged at 5000g at 4 ºC for 10 min. The pellet was then resuspended in 1 mL of 6% perchloric acid to lyse the cells. Then 0.3 mL of 3 M potassium carbonate was added while vortexing to neutralize the acid. The solution is then centrifuged and the supernatant filtered through a 0.2 µm PVDF membrane syringe filter.

Acetyl-CoA and CoA were analyzed on HPLC (Thermofinnigan, San Jose, CA) using a UV detector and a 5 µm octyldecyl silane column (Cell Technologies, Inc., Houston, TX). Two mobile phases of buffer were used at a flow rate of 1 mL/min. One
buffer is 0.2 M sodium phosphate (pH 5.0) and the other buffer is 800 mL of 0.25 M sodium phosphate (pH 5.0) mixed with 200 mL of 100% acetonitrile).

5.3.4 Enzyme Assays

To prepare crude extracts for enzyme assays, mid-exponential phase cultures were taken and washed twice with cold 100 mM Tris-HCl buffer at pH 8.0. Centrifugation was done at 4000 g and 4°C for 20 min for these washing steps. The cells were then resuspended in the same solution and subjected to sonication for 6 min in an ice bath. The sonicated cells were centrifuged at 1500 g and 4°C for 60 min to remove cell debris. PEPC and PYC activities were measured based on the citrate synthase-coupled assay by the method of Payne and Morris (Payne and Morris, 1969). The oxaloacetate produced by PYC or PEPC is reacted with acetyl-CoA by citrate synthase to yield CoA. The CoA then reacts with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) to produce a yellow colored product that can be measured spectrophotometrically at 412 nm. All reactions were performed in duplicates and carried out at 37°C. Reactions were initiated by adding the crude extract. The PYC assay differed from the PEPC assay in that pyruvate and ATP were added for the PYC assay. For PEPC, phosphoenolpyruvate was added instead of pyruvate and ATP. The specific activity of the enzymes was measured in μmole product/mg protein/hr (DTNB extinction coefficient used is $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). Total protein concentration of crude extract was measured by Lowry’s method (Sigma Lowry Reagent, Modified) using bovine serum albumin as standard.

5.4 Results and Discussion
The intracellular acetyl-CoA level is increased by overexpressing *E. coli* PANK and adding pantothenic acid to the medium, a necessary substrate for PANK (Vadali *et al.*, 2004a). The effect of modulating intracellular PEPC or PYC activity is therefore studied with coexpression of PEPC and PANK, and coexpression of PYC and PANK. The subsequent effect on succinate production and other metabolites is then examined.

To perform the coexpression effectively with two plasmids without the difficulty of imbalanced plasmid replication, two origin-compatible plasmids were used. For PEPC and PANK coexpression, PEPC was cloned into pDHK29 to form pHL333, and PANK was cloned into pUC19 to form pRV380. For PYC and PANK coexpression, PYC was cloned into pTrc99A to form pHL413, and PANK was cloned into pDHK29 to form pRV480. The vector, pTrc99A, is also compatible with pDHK29. Plasmids pRV380 and pRV480 have both been shown to be similar in significantly increasing the acetyl-CoA and CoA pools (Vadali *et al.*, 2004a, 2004b).

### 5.4.1 Coexpression of PEPC and PANK

To systematically study the effect of the individual enzymes and then the combined effect, four strains cotransformed with two plasmids were created. The strains are described in Table 5-1 for PEPC and PANK coexpression. The strain, GJT(pDHK29,pUC19), is the control strain without PEPC or PANK overexpression. The strain, GJT(pDHK29,pRV380), overexpresses PANK only. The strain, GJT(pHL333,pUC19), overexpresses *Sorghum* PEPC only. The strain, GJT(pHL333,pRV380), overexpresses *Sorghum* PEPC and PANK simultaneously. Experiments with these four strains were performed simultaneously, and succinate,
lactate, and intracellular acetyl-CoA and CoA levels were measured. All four strains consumed the same amount of glucose at the end of the experiment and no pyruvate accumulation was detected. The acetate production was similar for all four strains.

### Table 5-1: List of strains and plasmids used in the study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GJT001</td>
<td>Laboratory wildtype</td>
<td>Tolentino <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>GJT(pDHK29, pUC19)</td>
<td>Control for PEPC and PANK study</td>
<td>This study</td>
</tr>
<tr>
<td>GJT(pDHK29, pRV380)</td>
<td>Overexpression of PANK only</td>
<td>This study</td>
</tr>
<tr>
<td>GJT(pHL333, pUC19)</td>
<td>Overexpression of PEPC only</td>
<td>This study</td>
</tr>
<tr>
<td>GJT(pHL333, pRV380)</td>
<td>Overexpression of PEPC and PANK</td>
<td>This study</td>
</tr>
<tr>
<td>GJT(pTrc99, pDHK29)</td>
<td>Control for PYC and PANK study</td>
<td>This study</td>
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<tr>
<td>GJT(pTrc99, pRV480)</td>
<td>Overexpression of PANK only</td>
<td>This study</td>
</tr>
<tr>
<td>GJT(pHL413, pDHK29)</td>
<td>Overexpression of PYC only</td>
<td>This study</td>
</tr>
<tr>
<td>GJT(pHL413, pRV480)</td>
<td>Overexpression of PYC and PANK</td>
<td>This study</td>
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<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
<td>pUC19</td>
<td>ColE1 origin control vector Ap(^R)</td>
<td>Yanisch-Perron <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>pTrc99A</td>
<td>ColE1 origin control vector Ap(^R)</td>
<td>Pharmacia</td>
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<tr>
<td>pDHK29</td>
<td>ColE1 compatible control vector Km(^R)</td>
<td>Phillips <em>et al.</em>, 2000</td>
</tr>
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<td><em>Sorghum peps</em> in pDHK29 Km(^R)</td>
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</tr>
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<td><em>L. lactis pyc</em> in pTrc99A Ap(^R)</td>
<td>This study</td>
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<tr>
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<td><em>E. coli panK</em> in pUC19 Ap(^R)</td>
<td>Vadali <em>et al.</em>, 2004a</td>
</tr>
<tr>
<td>pRV480</td>
<td><em>E. coli panK</em> in pDHK29 Km(^R)</td>
<td>Vadali <em>et al.</em>, 2004b</td>
</tr>
<tr>
<td>pKK313</td>
<td>S8D mutant <em>Sorghum peps</em> Ap(^R)</td>
<td>Wang <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>pCPYC1</td>
<td><em>L. lactis pyc</em> Cm(^R)</td>
<td>Wang <em>et al.</em>, 2000</td>
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</tbody>
</table>

To show that the increase in succinate was due to the increased acetyl-CoA pool enhancing the activation of PEPC rather than differences in enzyme activity levels, enzyme assays were performed on the four strains GJT(pDHK29,pUC19), GJT(pDHK29,pRV380), GJT(pHL333,pUC19), and GJT(pHL333,pRV380). For the enzyme assays, pantothenic acid was not added during cultivation, since it could be subsequently converted to acetyl-CoA by PANK. This could prevent differences in
acetyl-CoA levels among the strains during the assay, which could have affected the activity of PEPC. For comparison, the basal chromosomal PEPC activities of GJT(pDHK29, pUC19) and GJT(pDHK29, pRV380) were observed to be the same (these two strains have no PEPC overexpressed) (data not shown). The basal PEPC activities of GJT(pHL333, pUC19) and GJT(pHL333, pRV380) were also observed to be the same (both strains overexpress the *Sorghum* PEPC) (data not shown). Therefore, the increase in succinate production by the two strains (GJT(pDHK29, pRV380) and GJT(pHL333, pRV380)) overexpressing PANK could only be attributed to the effect of increased acetyl-CoA pool on the native and *Sorghum* PEPC.

The results showed that the strain overexpressing PEPC and PANK concurrently resulted in the highest succinate final concentration at the 24 hr endpoint (Figure 5-1a). This strain had an intracellular acetyl-CoA concentration of 8.05 μM as compared to its control (PEPC and no PANK), which had only 0.24 μM, at the 24 hr endpoint (statistically significantly different at the 95% confidence level). Previous studies have shown that overexpressing PANK consistently yield higher acetyl-CoA levels throughout exponential and stationary phases (Vadali et al., 2004a). This significantly increased acetyl-CoA pool may have enhanced the activation of the abundant intracellular *Sorghum* PEPC throughout the cell culture causing an increase in succinate production. Individual overexpression of PANK or PEPC also increased succinate production compared to the control with no PEPC or PANK overexpression (GJT(pDHK29, pUC19)). The increase in succinate production caused by overexpression of PANK alone was likely a result of the effect of elevated acetyl-CoA level on the native *E. coli* PEPC. Acetyl-CoA has been shown to be an effective activator of *E. coli* PEPC (Izui et al., 1981). This increased
Figure 5-1: a) Effect on succinate production by PEPC and PANK coexpression. b) Effect on lactate production by PEPC and PANK coexpression. c) Effect of PANK on acetyl-CoA and CoA levels. GJTpDHK29pUC19 is control.
GJTpDHK29pRV380 overexpresses PANK only. GJTpHL333pUC19 overexpresses PEPC only. GJTpHL333pRV380 overexpresses both PEPC and PANK. Total glucose consumed is the same for all four strains. No pyruvate accumulation was detected.

activation of the native PEPC, therefore, would cause a subsequent increase in succinate production.

Lactate production decreased significantly when both PANK and PEPC were overexpressed compared to the control without PANK or PEPC overexpression (statistically significantly different at the 95% confidence level) (Figure 5-1b). When the individual genes were overexpressed, lactate also decreased substantially as compared to the control with no PEPC and PANK overexpression, but not as substantially as when both genes were coexpressed. Lactate is formed from pyruvate by lactate dehydrogenase (LDH). As a result, succinate increased and lactate decreased. The decline in lactate was caused by the PEPC being more competitive than the LDH for the carbon flux at the PEP-to-pyruvate node. The elevated *in vivo* pool of acetyl-CoA likely enhanced the activity of PEPC resulting in more succinate production and less lactate production. This effect became more prominent when both PEPC and PANK were overexpressed simultaneously. No pyruvate accumulation was detected at the end of fermentation. Acetate production was similar in all four strains studied (data not shown). Apparently, overexpression of PEPC and PANK did not have substantial effect on the acetate level. In addition, formate and ethanol production were observed to be higher in the strains overexpressing PANK compared to the same strains not overexpressing PANK (data not shown).
Figure 5-1c shows the intracellular acetyl-CoA and CoA levels of the four strains at the end of the experiment. The two strains carrying pRV380 overexpressing PANK showed significantly higher acetyl-CoA and CoA levels than their respective controls carrying pUC19 (statistically significantly different at the 95% confidence level). Especially the acetyl-CoA levels in the two strains with pRV380 were many fold higher than their respective controls. The acetyl-CoA and CoA levels have also been previously shown to be consistently higher throughout the growth phase when overexpressing the PANK in a batch bioreactor setting (Vadali et al., 2004a).

5.4.2 Coexpression of PYC and PANK

Coexpression of PYC and PANK was studied in the same manner as coexpression of PEPC and PANK. Four strains were created each carrying different combinations of two plasmids. These strains are described in Table 5-1 for PYC and PANK coexpression. The first strain, GJT(pTrc99A,pDHK29), is the control without overexpression of both PYC and PANK. The strain, GJT(pTrc99A,pRV480), overexpresses only PANK, and the strain, GJT(pHL413,pDHK29), overexpresses only PYC. The fourth strain, GJT(pHL413,pRV480), overexpresses both PYC and PANK simultaneously. All four strains consumed the same amount of glucose at the end of the experiment and no pyruvate accumulation was detected. The acetate production was the same for all four strains.

PYC assays were performed on the four strains GJT(pTrc99A,pDHK29), GJT(pTrc99A,pRV480), GJT(pHL413,pDHK29), and GJT(pHL413,pRV480) to show that the increase in succinate was due to the increased acetyl-CoA pool enhancing the
activation of PYC rather than differences in PYC level. Again, no pantothenic acid was added during cultivation to prevent any differences in acetyl-CoA levels in the extract affecting the measured activity. There was very minimal activity detected in the strains GJT(pTrc99A,pDHK29) and GJT(pTrc99A,pRV480) when the PYC assay was performed (data not shown). This is expected, since E. coli does not possess a native PYC. The increase in succinate concentration produced by GJT(pTrc99A,pRV480), therefore, could only be the effect of increased acetyl-CoA on the native chromosomal PEPC. The basal PYC activities of GJT(pHL413,pDHK29) and GJT(pHL413,pRV480) were observed to be the same (data not shown). Therefore, succinate increase by GJT(pHL413,pRV480) overexpressing PANK during experiment could only be attributed to the effect of increased acetyl-CoA pool on the L. lactis PYC.

The results of PYC and PANK coexpression showed similar trends as coexpression of PEPC and PANK for succinate, lactate, acetyl-CoA, and CoA. These similar trends are expected since acetyl-CoA is a potential allosteric activator for these two types of carboxylases. The enhanced PYC activity would increase conversion of pyruvate to OAA resulting in subsequent increase of succinate. Therefore, when PYC and PANK were both overexpressed, the succinate production was the highest after 24 hours compared to the other three strains (Figure 5-2a). The succinate production was also higher in the strain, GJT(pTrc99A,pRV480) overexpressing only PANK, than in the control, GJT(pTrc99A,pDHK29) with no overexpression of PYC or PANK. This increase in succinate production was caused by the enhanced activation of the native E. coli PEPC, since PYC is absent in E. coli. Although the strain, GJT(pHL413,pRV480), overexpressing both PYC and PANK showed the highest succinate production, it was not
Figure 5-2: a) Effect on succinate production by PYC and PANK coexpression. b) Effect on lactate production by PYC and PANK coexpression. c) Effect of PANK on acetyl-CoA and CoA levels. GJTpTrc99ApDHK29 is control. GJTpTrc99ApRV480 overexpresses PANK only. GJTpHL413pDHK29
overexpresses PYC only. GJTpHL413pRV480 overexpresses both PYC and PANK. Total glucose consumed is the same for all four strains. No pyruvate accumulation was detected.

substantially higher than the strain, GJT(pHL413,pDHK29), overexpressing PYC alone (Figure 5-2a). This may be that this particular PYC from *L. lactis* is not too sensitive to acetyl-CoA activation.

Lactate decreased significantly when both PYC and PANK were overexpressed compared to the control without PYC or PANK overexpressed (statistically significantly different at the 95% confidence level) (Figure 5-2b). Overexpression of PYC or PANK separately exhibited a lesser decrease in lactate. The formation of lactate for the strain overexpressing both PYC and PANK was not substantially less, though, than the strain overexpressing PYC alone. This may also be attributed to the low sensitivity of *L. lactis* PYC to acetyl-CoA. Nonetheless, the decrease in lactate can be similarly explained for PYC as with PEPC. At the pyruvate junction, the enhanced PYC activity by the increased acetyl-CoA level became more competitive for the carbon flux. As a result, the carbon flux toward the lactate pathway was reduced and channeled more toward the succinate pathway. No pyruvate accumulation was detected in all the four strains. Acetate production was similar in all four strains studied (data not shown). Overexpressing PYC and PANK did not have substantial effect on the acetate level. Formate and ethanol production were also observed to increase in the strains overexpressing PANK compared to the same strains not overexpressing PANK (data not shown). Figure 5-2c shows that the intracellular acetyl-CoA and CoA levels were significantly higher at the 24hr endpoint for the strains carrying pRV480 than the strains
carrying the control plasmid pDHK29 (statistically significantly different at the 95% confidence level.

5.5 Conclusion

The capability of regulating in vivo enzymatic activity in cellular systems still stands a tremendous challenge in metabolic engineering. This study shows an example of a method to modulate the activity of two specific enzymes, PEPC and PYC, through one of their activators, acetyl-CoA. The increased intracellular acetyl-CoA pool does effectively increase succinate production even without the coexpression of Sorghum PEPC or L. lactis PYC possibly by enhancing the activity of the native E. coli PEPC. Coexpression of Sorghum PEPC with E. coli PANK, and L. lactis PYC with E. coli PANK further enhances succinate production. This acetyl-CoA and CoA manipulation strategy, therefore, demonstrates the feasibility of modulating in vivo enzymatic activity. PEPC and PYC from other species can also be tested in the future using this technique to understand the effect of acetyl-CoA on their in vivo activities, and the subsequent effect on the flux to the succinate pathway.

5.6 Acknowledgements

The authors would like to thank Dr. R. Chollet at the University of Nebraska-Lincoln for providing the Sorghum pepc plasmid (pKK313) and Dr. L. L. McKay at the University of Minnesota for providing the L. lactis pyc plasmid (pCPYC1). This work was supported in part by grants from the U.S. Department of Agriculture (2002-35505-11638) and the National Science Foundation (BES-0118815 and BES-0000303). Henry
Lin was supported by a training grant from the National Science Foundation (DGE0114264).
6. Genetic Reconstruction of the Aerobic Central Metabolism in

*Escherichia coli* for the Absolute Aerobic Production of Succinate

*This chapter has been published in* Biotechnology and Bioengineering (Lin et al., 2005a).

6.1 Abstract

Most reported efforts to enhance production of the industrially valuable specialty
chemical succinate have been done under anaerobic conditions, where *E. coli* undergoes
mixed-acid fermentation. These efforts have often been hampered by the limitations of
NADH availability, poor cell growth and slow production. An aerobic succinate
production system has been strategically designed that allows *E. coli* to produce and
accumulate succinate efficiently and substantially as a product under absolute aerobic
conditions. Mutations in the tricarboxylic acid cycle (*sdhAB, icd, iclR*) and acetate
pathways (*poxB, ackA-pta*) of *E. coli* were created to construct the glyoxylate cycle for
aerobic succinate production. Experiments in flask studies showed that 14.28 mM of
succinate could be produced aerobically with a yield of 0.344 mole/mole using 55 mM
glucose. In aerobic batch reactor studies, succinate production rate was faster reaching
0.5 mole/mole in 24 hours with a concentration of 22.12 mM; further cultivation showed
that succinate production reached 43 mM with a yield of 0.7. There was also substantial
pyruvate and TCA cycle C₆ intermediate accumulation in the mutant. The results suggest
that more metabolic engineering improvements can be made to this system to make
aerobic succinate production more efficient. Nevertheless, this aerobic succinate
production system provides the first platform for enhancing succinate production aerobically in *E. coli* based on the creation of a new aerobic central metabolic network.

### 6.2 Introduction

The valuable specialty chemical succinate and its derivatives have extensive industrial applications. It can be used as an additive and flavoring agent in foods, a supplement for pharmaceuticals, a surfactant, a detergent extender, a foaming agent, and an ion chelator (Zeikus *et al.*, 1999). Currently, succinate is produced through petrochemical processes that can be expensive and can lead to pollution problems. Much effort has shifted toward making biocatalysts a viable and improved alternative for the production of succinate. The success of microbial fermentation coupled with the use of renewable carbohydrates would significantly improve the economics of the succinate market (Schilling, 1995).

Various strains such as *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, and *Escherichia coli* have been intensively studied for their potential as biocatalysts in succinate fermentation. The obligate anaerobe *A. succiniciproducens* has shown high potential for industrial scale succinate production because of its high conversion yield and productivity when fermented with whey (Lee *et al.*, 2000). However, *A. succiniciproducens* is not practical for commercial fermentation because it is unstable due to its tendency to degenerate, and requires environments absolutely free of oxygen for cultivation (Nghiem *et al.*, 1999). *E. coli* has also been extensively genetically engineered through the use of recombinant DNA technology in recent years to show promising potential for succinate fermentation. *E. coli* naturally produces succinate
as a minor fermentation product under anaerobic conditions (Clark, 1989). Under aerobic conditions, succinate is not produced as a byproduct in *E. coli* and acetate is the main byproduct. Numerous metabolic engineering strategies to enhance succinate production in *E. coli* have met with success. By amplifying enzymatic steps involved in the succinate pathway under anaerobic conditions, higher succinate production could be achieved. An example of this was shown when phosphoenolpyruvate carboxylase (*pepC*) from *E. coli* was overexpressed (Millard *et al.*, 1996). Conversion of fumarate to succinate was improved by overexpressing native fumarate reductase (*frd*) in *E. coli* (Goldberg *et al.*, 1983; Wang *et al.*, 1998). Certain enzymes are not indigenous in *E. coli*, but can potentially help increase succinate production. By introducing pyruvate carboxylase (*pyc*) from *Rhizobium etli* into *E. coli*, succinate production was enhanced (Gokarn *et al.*, 1998, 2000, 2001). Other metabolic engineering strategies also include inactivating competing pathways of succinate. When malic enzyme was overexpressed in the presence of inactivated pyruvate formate lyase (*pfl*) and lactate dehydrogenase (*ldh*), succinate became the major fermentation product (Stols and Donnelly, 1997; Hong and Lee, 2001). In this *pfl* and *ldh* mutant, there is a large pyruvate accumulation. Overexpression of malic enzyme in this mutant increased succinate production driven by the high pyruvate pool toward the direction of malate formation, which was subsequently converted to succinate. An inactive glucose phosphotransferase system (*ptsG*) in the same mutant strain (*pfl* and *ldh*) had also been shown to yield higher succinate production in *E. coli* (Chatterjee *et al.*, 2001).

The various genetic improvements described above for succinate production have all been done under anaerobic conditions utilizing the mixed-acid fermentation pathways
of E. coli. Unfortunately, anaerobic fermentation has inherent disadvantages that are difficult to surmount. Anaerobic conditions often cause poor cell growth and slow carbon throughput, therefore low production rates. Succinate formation in mixed-acid fermentation is also hampered by the limitations of NADH availability, since 2 moles of NADH are required for every mole of succinate to be formed. Strategies to overcome the anaerobic barrier have included generating enough biomass under aerobic conditions, then switching to anaerobic conditions for succinate production. This was shown to be effective using a “dual-phase” fermentation system, in which initial aerobic growth phase was started then followed by an anaerobic production phase (Vemuri et al., 2002a, 2002b). Absolute aerobic production of succinate in E. coli until now has not been metabolically engineered to show feasibility. Saccharomyces cerevisiae has been shown to increase succinate production aerobically when succinate dehydrogenase (sdh) is disrupted to utilize the oxidative pathway of the TCA cycle for production (Arikawa et al., 1999). The capability to produce succinate under aerobic conditions would mean an active oxidative phosphorylation for generating energy with O₂ present as the electron acceptor. This would lead to higher biomass generation, faster carbon throughput and product formation. In this study, we seek to develop an aerobic succinate production platform in E. coli, thus creating a new metabolic network for improving succinate production under aerobic conditions.

To create a novel succinate production system that functions under absolute aerobic conditions, the aerobic central metabolism of E. coli is genetically reconstructed. Pathway modeling and simulation were performed on the glycolysis, TCA cycle, and glyoxylate bypass to create a design for the network that would allow succinate to be
produced as a byproduct aerobically with a substantial achievable yield. Based on the design, various mutations were created. The two major acetate pathways (pyruvate oxidase \((poxB)\), and acetate kinase-phosphotransacetylase \((ackA-pta)\)) were inactivated to channel more carbon flux toward the TCA cycle (Figure 6-1). In the TCA cycle, \(sdhAB\) and \(icd\) were inactivated to redirect the fluxes toward succinate (Figure 6-1). The glyoxylate operon \(aceBAK\) repressor \((iclR)\) was inactivated in order to activate the glyoxylate bypass in the TCA cycle as a detour for succinate production. Studies have shown that disruption of \(iclR\) dramatically induces expression of the \(aceBAK\) operon when grown on glucose (Gui et al., 1996). This is not the case when grown on acetate, since growth on acetate yields induction of the \(aceBAK\) anyway (Gui et al., 1996). There are also other transcriptional regulators of the \(aceBAK\) operon, which include FadR (Maloy and Nunn, 1995), FruR (Chin et al., 1989), and the ArcAB system (Iuchi et al., 1988, 1989). The glyoxylate bypass, consisting of two enzymatic steps, is essential for growth on the two-carbon acetate or fatty acids because it prevents carbon loss as \(CO_2\) in the TCA cycle (Kornberg, 1966). The first step is isocitrate lyase \((aceA)\), which converts isocitrate to succinate and glyoxylate. Then in the secondary step, malate synthase \((aceB)\) condenses the glyoxylate with acetyl-CoA to form malate. The malate is then converted back to oxaloacetate \((OAA)\). These steps bypass the two oxidative steps of the TCA cycle in which \(CO_2\) is evolved. With these genetic modifications, the glyoxylate cycle is created which produces succinate as a byproduct. This will form the basis of the platform for aerobic succinate production. Through this aerobic system, succinate is produced through pathways that do not require NADH for formation. Cell growth and product formation would also be more efficient than anaerobic conditions. More importantly, the
Figure 6-1: Genetic engineering of the glycolysis and TCA cycle in the development of an aerobic succinate production system. 1 is icd knockout, 2 is sdh knockout, 5 is iclR knockout, 6 is poxB knockout, and 7 is ackA-pta knockout.
platform demonstrates the feasibility of producing succinate aerobically as a major product in *E. coli*.

6.3 Materials and Methods

6.3.1 Strains

Mutations were created in the laboratory wildtype GJT001, a spontaneous cadR mutant of MC4100 (Tolentino *et al.*, 1992). A library of mutants was created in the end throughout the process of constructing the pentamutant. A list of the mutant strains that were studied is shown in Table 6-1. Knockouts were created in succinate dehydrogenase (*sdhAB*), pyruvate oxidase (*poxB*), acetate kinase-phosphotransacetylase (*ackA-pta*), isocitrate dehydrogenase (*icd*), and the *aceBAK* operon repressor (*iclR*). Strains HL2k, HL26k, HL267k, HL2671k, and HL26715k were used to characterize the effect of each sequential addition of a mutation to the previously studied host strain, GJT001. The final mutant, GJT001(*sdhAB, poxB, ackA-pta, icd, iclR::Km*), is the pentamutant that sets the basis for aerobic succinate production. Other pentamutant strains were also constructed using different orders of introduction of the mutations to confirm the phenotype of GJT001(*sdhAB, poxB, ackA-pta, icd, iclR::Km*) in addition to genomic PCR verification. The Km resistant marker was left purposely on all the strains used in experiments for preventing contamination during cultivation with the use of kanamycin.
Table 6-1: Table of mutant strains constructed and studied.

<table>
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<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<td>Spontaneous cadR mutant of MC4100(ATC35695) Δlac(arg-lac)U169prsl150relA1ptsF Sm^R</td>
<td>Tolentino et al., 1992</td>
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<td>GJT001 (sdhAB, poxB::Km^R)</td>
<td>This study</td>
</tr>
<tr>
<td>HL267k</td>
<td>GJT001 (sdhAB, poxB, (ackA-pta)::Km^R)</td>
<td>This study</td>
</tr>
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</tr>
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<td>This study</td>
</tr>
<tr>
<td>HL27615k</td>
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<td>GJT001 (iclR, sdhAB, icd, (ackA-pta), poxB::Km^R)</td>
<td>This study</td>
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</table>

6.3.2 Mutant Construction

Mutations were created using the one-step inactivation method of Datsenko and Wanner (Datsenko and Wanner, 2000). This method first requires the construction of the single mutations using the phage λ Red recombinase. P1 phage transduction was then used to combine various mutations into one strain. Each mutation had to be added to the strain one at a time before the introduction of the next mutation because the kanamycin cassette had to be removed at each stage to enable selection of the next mutation. PCR products of the kanamycin cassette gene flanked by FRT (FLP recognition target) sites and homologous sequences to the gene of interest were made using pKD4 (Datsenko and Wanner, 2000) as the template. These PCR products were then transformed into the cells by electroporation (Bio-Rad Gene Pulser) for insertional inactivation of the gene of interest. These transformed cells carry the plasmid pKD46 (Datsenko and Wanner, 2000) that expresses the λ Red system (γ, β, exo) for recombination of the PCR product into the chromosome. Once the kanamycin cassette is inserted, it can be removed using the helper plasmid, pCP20 (Datsenko and Wanner, 2000), that expresses FLP. The removal of the FRT-flanked kanamycin cassette leaves behind an 84 base pair insertion cassette.
At each stage of mutation, experiments were performed to test the intermediate mutant for the effect on metabolite production. Throughout the process of constructing the aerobic succinate production system, a library of different mutants with varying types and numbers of mutations was created. All mutants were also verified with genomic PCR after construction to ensure that the gene of interest had been disrupted.

6.3.3 Flask Culture Medium and Conditions

The base medium used for studying the mutants is LB (10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, 10 g/L NaCl) with 2 g/L of NaHCO₃ and 55 mM of glucose. The medium used for inoculum preparation is also LB, except glucose was not added. The pH of the medium was adjusted to 7.5 with NaOH. NaHCO₃ was added to the culture medium because it helped yield better cell growth and succinate production due to its pH-buffering capacity and its ability to supply CO₂. The aerobic setup of the experiments utilized 250mL flasks with foam stoppers. Each flask contained 50 ml of the base medium and a 1% (v/v) inoculum was used, grown overnight from glycerol stocks kept at -85°C. Kanamycin was added to the flask at a concentration of 50 mg/L. Cultures were grown at 37°C and 250 rpm for 24 hours.

6.3.4 Bioreactor Culture Medium and Conditions

Aerobic batch reactor studies were conducted on the pentamutant HL27615k. The medium used is LB with 2 g/L NaHCO₃ and 63 mM of glucose. The initial medium volume is 600 ml in a 1.0-L New Brunswick Scientific Bioflo 110 fermenter. A 1% (v/v) inoculum was used from an overnight culture grown from a single colony for 12 hours.
The pH was measured using a glass electrode and controlled at 7.0 using 1.5N HNO₃ and 2N Na₂CO₃. The temperature was maintained at 37°C, and the agitation speed was constant at 800 rpm. The inlet airflow used was 1.5 L/min. The dissolved oxygen was monitored using a polarographic oxygen electrode and was maintained above 80% saturation throughout the experiment.

6.3.5 Analytical Techniques

Optical density was measured at 600nm with a spectrophotometer (Bausch & Lomb Spectronic 1001); the culture was diluted to the linear range with 0.15 M NaCl. For analyzing the extracellular metabolites, 1 ml of culture was centrifuged and the supernatant was then filtered through a 0.45-µm syringe filter for HPLC analysis. The HPLC system (Shimadzu-10A Systems, Shimadzu, Columbia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A) and a differential refractive index (RI) detector (Waters 2410, Waters, Milford, MA). A 0.6 mL/min mobile phase using 2.5 mM H₂SO₄ solution was applied to the column. The column was operated at 55 °C. Standards were prepared for glucose, succinate, acetate, and pyruvate for both the RI detector and UV detector, and calibration curves were created. Glucose, succinate, and acetate were measured by the RI detector and pyruvate was measured by the UV detector at 210nm.

6.4 Results and Discussion

6.4.1 Design and Construction of the Aerobic Succinate Production System
Succinate is naturally formed as a product under anaerobic conditions where *E. coli* undergoes mixed-acid fermentation. Under aerobic conditions, the production of succinate as a product is not naturally possible. In aerobic metabolism, succinate is only an intermediate of the TCA cycle. It is formed by succinyl-CoA synthetase, and then subsequently converted to fumarate by succinate dehydrogenase. Through the oxidation reaction by SDH, succinate also provides electrons to the electron transport chain for oxidative phosphorylation. Because of this recycling process, succinate is never detected in aerobic cultures of *E. coli*. Acetate is the only major byproduct of *E. coli* under aerobic conditions. To enable production of succinate as a byproduct under aerobic conditions, pathways in the central metabolism had to be redesigned by genetic manipulation. Pathways involved in the glycolysis, TCA cycle, and the glyoxylate bypass were engineered to create a platform for aerobic succinate production. First, to make succinate accumulation possible under aerobic conditions, succinate dehydrogenase (*sdh*) in the TCA cycle was inactivated (Figure 6-1). Once *sdh* was inactivated, succinate started accumulating during aerobic growth. Next, to increase the carbon flux toward the TCA cycle for succinate production, two competing acetate pathways were inactivated (pyruvate oxidase (*poxB*), and acetate kinase-phosphotransacetylase (*ackA-pta*)) (Figure 6-1). Once these two pathways were inactivated, acetate production decreased substantially, and more carbon flux was driven toward the TCA cycle.

The design of the aerobic production system involved redesigning the TCA cycle. To conserve the two carbons lost to CO₂ by the oxidative arm of the TCA cycle, the glyoxylate bypass was engineered as a route for succinate production. With this bypass, the two carbons were recovered as glyoxylate, which was then recycled back to malate to
replenish oxaloacetate (OAA). This was made possible by first inactivating the isocitrate dehydrogenase (*icd*) (Figure 6-1). Once *icd* was inactivated, succinate could no longer be produced by the oxidative arm of the TCA cycle. Next, the glyoxylate bypass was activated by inactivating the *aceBAK* operon repressor (*iclR*) (Figure 6-1). This caused the isocitrate lyase (*aceA*) and malate synthase (*aceB*) to be expressed constitutively. Upon these modifications, succinate could now be produced entirely through the glyoxylate bypass under absolute aerobic conditions.

The five mutations above were implemented, which together created a pentamutant of *E. coli*. Essentially the TCA cycle is now the glyoxylate cycle, where succinate is being produced aerobically as a major byproduct (Figure 6-2). This production system provides a new platform for further improving succinate production in *E. coli* by utilizing the redesigned aerobic central metabolic pathways. The system has a maximum theoretical yield of producing 1 mole of succinate for every mole of glucose consumed.

### 6.4.2 Effect of Mutations on Aerobic Metabolite Production and Cell Growth

Experiments were conducted on each mutant strain throughout the construction of the pentamutant strain. The results of the pentamutant strain and its precursor mutant strains are based on the strain HL26715k (Table 6-1). Each number in Figure 6-1 designates the specific gene that was inactivated, so each number in the strain name corresponds to a particular mutation. A total of five mutant strains and the parental strain (GJT001) were grown aerobically in flasks for 24 hours at 37°C with 10 g/L glucose. Figure 6-3 shows the results of succinate production and yield from the culture of each
**Figure 6-2:** The glyoxylate cycle, platform for aerobic succinate production.
Figure 6-3: Succinate production in modified strains. a) Succinate production at each incremental step of incorporating mutations into the parental strain. The strain containing the five mutations combined represents the aerobic succinate production system. Each number designates a specific knockout in the pathways as shown by Figure 1.; b) Succinate yield as a result of each incremental addition of mutation to the parental strain. Yield is mole of succinate produced per mole of glucose consumed. Mean and standard deviation were calculated based on duplicate experiments.

mutant strain and the parental strain. In the mutant strain HL2k with only $sdh$ inactivated, succinate accumulated during culture. Succinate accumulation was not possible in the wildtype (GJT001) as shown by zero succinate production (Figure 6-3a). Inactivation of the two acetate pathways, $poxB$ and $ackA-pta$, further increased succinate production and yield as shown by mutant strain HL267k. Next, as dictated by the design strategy, $icd$ was inactivated creating mutant strain HL2671k. When the $icd$ was inactivated, succinate production decreased as expected since the cell probably could no longer use the oxidative arm of the TCA cycle to produce succinate. The amount of succinate produced by HL2671k could be due to the glyoxylate bypass being partially active. The molar succinate yield of HL2671k increased significantly, though, but this was accompanied by a much lower glucose consumption (Figure 6-4a). Finally, when the glyoxylate bypass was activated by inactivating $iclR$, succinate production increased substantially to 14.28 mM with a molar yield of 0.344. This is over a 5-fold increase in
Figure 6-4: Glucose and acetate metabolism in modified strains. a) Glucose consumed by the parental strain and the five mutant strains after 24 hours of culture.; b) Acetate produced by the parental strain and the five mutant strains after 24 hours of culture. Mean and standard deviation were calculated based on duplicate experiments.

succinate production compared to HL2671k. The result is shown by the pentamutant strain HL26715k. At this point, a highly functioning glyoxylate cycle is created. This provides a detour to relieve the carbon flux from the TCA cycle bottleneck created in mutant strain HL2671k. Activating the glyoxylate bypass reconstituted the cycling and replenishment of OAA. As a result, HL26715k showed much higher glucose consumption than the previous three strains containing mutations due to a faster and more efficient carbon throughput (Figure 6-4a). The cell growth of HL26715k was also healthy again, and was similar to that of the wildtype parental strain GJT001 (Figure 6-5a).

Glucose consumption decreased throughout the first four mutant strains (Figure 6-4a). Once HL26715k was created, glucose consumption was much higher due to an active glyoxylate cycle, but still not as high as the wildtype parental strain. This was also the same trend observed for the OD, where HL26715k generated an OD similar to the parental strain (Figure 6-5a). The results of the OD yield study, Figure 6-5b, also show
that HL26715k has a healthy metabolism because of its glyoxylate cycle. OD yield relates to

Figure 6-5: Growth of modified strains in standard media. a) Growth of the parental strain and the five mutant strains after 24 hours. OD measured at 600nm.; b) OD yield after 24 hours of culture. Yield is OD generated per mole of glucose consumed. Mean and standard deviation were calculated based on duplicate experiments.

the amount of biomass generated per mole of glucose consumed. The OD yield of the parental strain is lower than the OD yield of the five mutant strains (Figure 6-5b). For the parental strain, this indicates that much of the consumed carbon source is being metabolized to end products rather than biomass. This would be due to the high carbon throughput of the central metabolism in the parental strain. In the mutant strain, the OD yield found in these cultures started to rise with each additional mutation up to the strain HL2671k. The OD yield for HL2671k was the highest among all the mutant strains. This is likely due to the inactivation of its two major acetate pathways and disruption of its entire TCA cycle. Because of these perturbations, the downstream part of HL2671k’s central metabolism is curtailed. This probably resulted in slow carbon throughput, therefore lower glucose uptake and more carbon flux driven toward biomass. When the iclR was inactivated in HL2671k creating the pentamutant strain, HL26715k, the OD
yield decreased significantly by 66% (Figure 6-5b). The OD yield of HL26715k is also now closer to the OD yield of the wildtype parental strain. This result shows that activating the glyoxylate bypass reconstituted the cycling effect of the TCA cycle. In HL26715k, restoration of the downstream metabolism again allowed faster carbon throughput, higher glucose consumption, and less biomass generation per mole of glucose consumed.

**Figure 6-6:** Pyruvate metabolism in modified strains. a) Accumulation of pyruvate by the parental strain and the five mutant strains after 24 hours of culture.; b) Pyruvate yield after 24 hours of culture. Yield is mole of pyruvate produced per mole of glucose consumed. Mean and standard deviation were calculated based on duplicate experiments.

Acetate production decreased significantly upon inactivation of *poxB* and *ackA-pta* (Figure 6-4b). This was shown to direct more carbon flux downstream to succinate production. The intermediate, pyruvate, was observed to be accumulating significantly in strains HL26k, HL267k, HL2671k, and HL26715k (Figure 6-6a). Inactivation of the two acetate pathways and disruption of the TCA cycle by *icd* knockout apparently caused a bottleneck at the pyruvate junction (Figure 6-1). This is why there is substantial pyruvate accumulation in mutant strains HL267k and HL2671k based on the amount of glucose they consumed. The pyruvate yield is 2.398 (mole pyruvate/mole glucose) for HL267k
and 2.274 (mole pyruvate/mole glucose) for HL2671k (Figure 6-6b). When HL26715k was created by activating the glyoxylate bypass, the pyruvate yield decreased significantly to 0.338 (mole pyruvate/mole glucose) indicating a substantial relief of pyruvate accumulation by the glyoxylate cycle. The maximum theoretical pyruvate yield is 2.0 (mole pyruvate/mole glucose) generated from glycolysis. The high pyruvate yield above 2.0 exhibited by strains HL267k and HL2671k can be due to the effect of nutrients originating from the complex medium such as phosphoenolpyruvate, which can be converted to pyruvate. In mutant strains with icd inactivated (HL2671k and HL26715k), there was also an accumulation of TCA cycle C₆ intermediates such as citrate and isocitrate (data not shown). Others have shown similar effects when icd was inactivated in E. coli (Aoshima et al., 2003). This accumulation is less, though, in HL26715k than in HL2671k due to the glyoxylate cycle. Nevertheless, the presence of pyruvate and TCA cycle C₆ intermediate accumulation in the pentamutant strain HL26715k shows that there are significant improvements that can be made to this strain to further increase the succinate yield. This aerobic succinate production system will serve as a novel platform for future metabolic engineering improvements on succinate production in E. coli.

6.4.3 Verification of Pentamutant Construction

Throughout the process of constructing the pentamutant strain, a library of hosts with individual and multiple mutations was created by multi-stage phage transduction. To confirm that pentamutant strain’s phenotype was correctly a result of the five mutations described in Figure 6-1 and not caused by artifacts from multiple phage transductions, seven different pentamutant strains were constructed with varying orders
of input of the five mutations. Experiments in flask cultures were performed on these seven pentamutant strains to compare their metabolite production. The succinate production and yield of the seven pentamutant strains were very similar and consistent (Table 2). These results show that the effect of the five mutations is indeed real and not affected by randomness of phage during transduction. This test further confirms the phenotype of the pentamutant strains.

Table 6-2: Analysis of seven differently constructed pentamutant strains. Construction of seven different pentamutant strains that vary in order of input of each mutation. The resulting succinate produced and succinate yield are similar among all seven strains. Yield is calculated as mole of succinate produced per mole of glucose consumed. The strains were grown using 250mL flasks with foam stoppers for 24 hours in LB+55 mM of glucose. Mean and standard deviation were calculated based on triplicate experiments.

<table>
<thead>
<tr>
<th>Pentamutant</th>
<th>Succinate Produced (mM)</th>
<th>Succinate Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL12675k</td>
<td>14.33 ± 0.14</td>
<td>0.336 ± 0.024</td>
</tr>
<tr>
<td>HL26715k</td>
<td>16.07 ± 1.12</td>
<td>0.406 ± 0.039</td>
</tr>
<tr>
<td>HL27615k</td>
<td>16.49 ± 0.05</td>
<td>0.383 ± 0.005</td>
</tr>
<tr>
<td>HL51267k</td>
<td>16.29 ± 0.29</td>
<td>0.377 ± 0.010</td>
</tr>
<tr>
<td>HL51276k</td>
<td>15.64 ± 1.51</td>
<td>0.370 ± 0.001</td>
</tr>
<tr>
<td>HL52167k</td>
<td>16.40 ± 0.33</td>
<td>0.373 ± 0.003</td>
</tr>
<tr>
<td>HL52176k</td>
<td>13.32 ± 1.97</td>
<td>0.304 ± 0.028</td>
</tr>
</tbody>
</table>

6.4.4 Aerobic Batch Reactor Characterization of the Pentamutant

The pentamutant strain was further characterized under controlled conditions in an aerobic batch reactor using strain HL27615k. This would also demonstrate the possibility of using this aerobic succinate production system in an industrial setting. In the bioreactor, 63 mM of glucose was added and 1% inoculum from an overnight culture grown from a single colony was used. Temperature and pH were maintained at 37°C and 7.0, respectively. The DO was maintained above 80% saturation.
The results show that at 24 hours, succinate production is 22 mM with a molar yield of 0.5 (Figure 6-7). This is better than the results from flask studies at 24 hours, which were 14 mM of succinate with a yield of 0.34. Using a bioreactor generates higher productivity due to a more controlled environment. Cells reached maximum OD of 9.12 after 12 hours with a specific growth rate of approximately 0.45 hr\(^{-1}\). At 24 hours, pyruvate accumulation reached a maximum of 48 mM and glucose consumed was 44 mM. After 24 hours, the cells started consuming the excreted pyruvate along with the remaining glucose. All the glucose was consumed by about 49 hours at which point the pyruvate was still being consumed. By 83 hours, the pyruvate was not completely consumed, but succinate production reached 43 mM with a yield of 0.7. There was also accumulation of TCA cycle C\(_6\) intermediates, which had not been consumed by the cells (data not shown). The results of the batch reactor study show that the pentamutant strain HL27615k has the potential to produce a large quantity of succinate under absolute aerobic conditions, and that there is potential to achieve the maximum succinate theoretical yield of 1.0. Future improvements to this platform would be to increase production rate and reduce the accumulation of intermediates. Various genetic modifications are underway to further enhance carbon throughput to succinate.

6.5 Conclusion

Although metabolic engineering of *E. coli* to enhance succinate production under anaerobic conditions has generated many promising improvements, they are still hampered by many inherent anaerobic constraints. This makes application on an industrial scale difficult due to low biomass generation and poor physiological state of
Figure 6-7: Aerobic batch reactor study on the pentamutant HL27615k. Solid diamond (♦) is glucose consumed. Solid square (■) is succinate produced. Solid triangle (▲) is acetate produced. Solid circle (●) is pyruvate produced. Open square (□) is the succinate yield (mole succinate produced per mole glucose consumed).
the cell. An aerobic succinate production system is being developed to provide an alternative to anaerobic fermentation. Aerobic conditions provide many advantages that favor implementation on an industrial scale due to higher biomass generation, faster carbon throughput and product formation.

The aerobic succinate production system is based on five mutations, \textit{sdhAB}, \textit{icd}, \textit{poxB}, \textit{(ackA-pta)}, and \textit{iclR}. With these mutations in \textit{E. coli}, a pentamutant strain is created with a new central metabolic network, i.e. the glyoxylate cycle. Through the glyoxylate cycle, succinate can be produced as a major byproduct under aerobic conditions. Experiments had shown that the pentamutant strain HL26715k could produce substantial succinate under absolute aerobic conditions. HL26715k shown by its glucose consumption and cell growth is healthy and robust due to its glyoxylate cycle. The substantial accumulation of pyruvate and TCA cycle C₆ intermediates hinders achieving the maximum succinate theoretical yield of 1.0. These accumulations also presumably slowed down the production rate of succinate. There are significant improvements that can still be made to the system. Nevertheless, the current system provides the first platform for enhancing succinate production in \textit{E. coli} under absolute aerobic conditions. Numerous metabolic engineering strategies can be further applied to this redesigned metabolic network for achieving the maximum succinate yield aerobically.

\section*{6.6 Acknowledgements}

The authors would like to thank Dr. Barry L. Wanner at Purdue University for providing plasmids pKD4, pKD46, and pCP20 that facilitate the construction of mutations in \textit{E. coli}. This work was supported by grants from the National Science
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Chapter 7

7. Metabolic Engineering of Aerobic Succinate Production Systems in *Escherichia coli* to Improve Process Productivity and Achieve the Maximum Theoretical Succinate Yield

* This chapter has been accepted for publication in *Metabolic Engineering* (Lin et al., 2005b).

7.1 Abstract

The potential to produce succinate aerobically in *E. coli* would offer great advantages over anaerobic fermentation in terms of faster biomass generation, carbon throughput, and product formation. Genetic manipulations were performed on two aerobic succinate production systems to increase their succinate yield and productivity. One of the aerobic succinate production systems developed earlier (Lin et al., 2004) was constructed with five mutations (*sdhAB, icd, iclR, poxB*, and *ackA-pta*), which created a highly active glyoxylate cycle. In this study, a second production system was constructed with four of the five above mutations (*sdhAB, iclR, poxB*, and *ackA-pta*). This system has two routes in the aerobic central metabolism for succinate production. One is the glyoxylate cycle and the other is the oxidative branch of the TCA cycle. Inactivation of *ptsG* and overexpression of a mutant *Sorghum pepe* in these two production systems showed that the maximum theoretical succinate yield of 1.0 mole per mole glucose consumed could be achieved. Furthermore, the two-route production system with *ptsG* inactivation and *pepe* overexpression demonstrated substantially higher succinate productivity than the previous system, a level unsurpassed for aerobic succinate
production. This optimized system showed remarkable potential for large-scale aerobic succinate production and process optimization.

7.2 Introduction

The valuable specialty chemical succinate and its derivatives have extensive industrial applications. It can be used as an additive and flavoring agent in foods, a supplement for pharmaceuticals, a surfactant, a detergent extender, a foaming agent, and an ion chelator (Zeikus et al., 1999). Currently, succinate is produced through petrochemical processes that can be expensive and can lead to pollution problems. Much effort has shifted toward making biocatalysts a viable and improved alternative for the production of succinate. The success of microbial fermentation coupled with the use of renewable carbohydrates would significantly improve the economics of the succinate market (Schilling, 1995).

Various strains such as Anaerobiospirillum succinicivorans, Actinobacillus succinogenes, and Escherichia coli have been intensively studied for their potential as biocatalysts in succinate fermentation. The obligate anaerobe A. succinicivorans has shown high potential for industrial scale succinate production because of its high conversion yield and productivity when fermented with whey (Lee et al., 2000). However, A. succinicivorans is not practical for commercial fermentation because it is unstable due to its tendency to degenerate, and requires environments absolutely free of oxygen for cultivation (Nghiem et al., 1999). E. coli has also been extensively genetically engineered through the use of recombinant DNA technology in recent years to generate strains which showed promise for succinate fermentation. E. coli naturally
produces succinate as a minor fermentation product under anaerobic conditions (Clark, 1989). Under aerobic conditions, succinate is not produced as a byproduct in E. coli and acetate is the main byproduct. Numerous metabolic engineering strategies to enhance succinate production in E. coli have met with success. Strains in which enzymatic steps involved in the succinate pathway were amplified and the organism cultured under anaerobic conditions yielded higher succinate production. An example of this was shown when phosphoenolpyruvate carboxylase (pepc) from E. coli was overexpressed (Millard et al., 1996). Conversion of fumarate to succinate was improved by overexpressing native fumarate reductase (frd) in E. coli (Goldberg et al., 1983; Wang et al., 1998). Certain enzymes are not indigenous in E. coli, but can potentially help increase succinate production. By introducing pyruvate carboxylase (pyc) from Rhizobium etli into E. coli, succinate production was enhanced (Gokarn et al., 1998, 2000, 2001). Other metabolic engineering strategies also include inactivating competing pathways of succinate. When malic enzyme was overexpressed in a host with inactivated pyruvate formate lyase (pfl) and lactate dehydrogenase (ldh) genes, succinate became the major fermentation product (Stols and Donnelly, 1997; Hong and Lee, 2001). In cultures of this pfl and ldh mutant strain, there is a large pyruvate accumulation. Overexpression of malic enzyme in this mutant strain increased succinate production driven by the high pyruvate pool toward the direction of malate formation, which was subsequently converted to succinate. An inactive glucose phosphotransferase system (ptsG) in the same mutant strain (pfl and ldh) had also been shown to yield higher succinate production in E. coli and improve growth (Chatterjee et al., 2001).
The various genetic improvements described above for succinate production have all been done under anaerobic conditions utilizing the mixed-acid fermentation pathways of *E. coli*. Unfortunately, anaerobic fermentation has inherent disadvantages that are difficult to surmount. Anaerobic conditions often cause poor cell growth and slow carbon throughput, therefore generating low production rates. Succinate formation in mixed-acid fermentation is also hampered by the limitations of NADH availability, since 2 moles of NADH are required for every mole of succinate to be formed. Strategies to overcome the anaerobic barrier have included generating enough biomass under aerobic conditions, then switching to anaerobic conditions for succinate production. This was shown to be effective using a “dual-phase” fermentation system, in which initial aerobic growth phase was started then followed by an anaerobic production phase (Vemuri *et al.*, 2002a, 2002b).

Absolute aerobic production of succinate in *E. coli* until now has not been feasibly engineered (Lin *et al.*, 2004). *Saccharomyces cerevisiae* has increased succinate production when succinate dehydrogenase (*sdh*) is disrupted to utilize the oxidative pathway of the TCA cycle for aerobic production (Arikawa *et al.*, 1999). The capability to produce succinate under aerobic conditions would mean an active oxidative phosphorylation for generating energy with O₂ present as the electron acceptor. This would lead to higher biomass generation, faster carbon throughput and product formation. In this study, we seek to develop succinate production systems in *E. coli* that can function under absolute aerobic conditions. These systems would be robust and efficient with high succinate yield capability, and productivity.
Under aerobic conditions, the production of succinate is not naturally possible since it is only an intermediate of the TCA cycle. It is formed by succinyl-CoA synthetase, and then subsequently converted to fumarate by succinate dehydrogenase (SDH). Through the oxidation reaction by SDH, succinate provides electrons to the electron transport chain for oxidative phosphorylation. Because of this recycling process, succinate is never detected in aerobic cultures of *E. coli*. Acetate is the only major byproduct of *E. coli* under aerobic conditions. Previously, a novel aerobic succinate production system has been developed that can produce a substantial amount of succinate under aerobic conditions (Lin *et al.*, 2004). This aerobic succinate production system is based on the presence of five mutations (*sdhAB, icd, poxB, (ackA-pta)*, and *iclR*) that create an active glyoxylate cycle in the host strain (Figure 7-2). This pentamutant strain, with its glyoxylate cycle, can produce succinate as a major product aerobically, but there is still substantial accumulation of pyruvate and TCA cycle C₆ intermediates (citrate and isocitrate). Pathway modeling and simulation of aerobic metabolism shows that a maximum theoretical succinate yield of 1.0 mole per mole glucose consumed can be achieved. Because of the accumulation of pyruvate and TCA cycle C₆ intermediates, the pentamutant strain was hindered from achieving the maximum theoretical succinate yield.

In this study, further genetic modifications are made to the glyoxylate cycle system to improve succinate production and achieve the maximum theoretical succinate yield. Another aerobic succinate production platform was also created to examine its efficiency in succinate production. This system is a mutant strain with four mutations (*sdhAB, poxB, (ackA-pta)*, and *iclR*). These four mutations create two possible routes for
succinate production, the glyoxylate cycle and the oxidative branch of the TCA cycle (Figure 7-3). These two production systems both have the inherent capability to achieve the maximum theoretical succinate yield of 1.0 mol/mol. Therefore, the pathways of these two systems were further optimized through genetic manipulation to achieve the maximum theoretical succinate yield. Inactivation of the glucose

![Diagram of metabolic pathways]

**Figure 7-1**: Genetic engineering of glycolysis, TCA cycle, and glyoxylate bypass in the development of aerobic succinate production systems. 1 is *icd* knockout, 2 is *sdhAB* knockout, 5 is *iclR* knockout, 6 is *poxB* knockout, 7 is *ackA-pta* knockout, and 9 is *ptsG* knockout.
Figure 7-2: Aerobic succinate production platform, the glyoxylate cycle (mutant strain HL51276k).
phosphotransferase gene \textit{ptsG} and overexpression of a feedback inhibition resistant \textit{Sorghum} phosphoenolpyruvate carboxylase (PEPC) in the two production systems showed that the maximum theoretical succinate yield of 1.0 mol/mol could indeed be achieved. Furthermore, the platform with two routes for succinate production (glyoxylate cycle and oxidative branch of TCA cycle) demonstrated substantially higher succinate productivity than the other platform when \textit{ptsG} was inactivated and \textit{pepc} was overexpressed. This production system, in particular, has great potential for large-scale aerobic production of succinate and further process optimization.

7.3 Materials and Methods

7.3.1 Strains

Mutations were created in the laboratory wildtype GJT001, a spontaneous \textit{cadR} mutant of MC4100 (Tolentino \textit{et al}., 1992). A library of mutant strains was created during the process of constructing the final mutant strains for aerobic succinate production. A list of the final mutant strains that were studied is shown in Table 7-1. Knockouts were created in succinate dehydrogenase (\textit{sdhAB}), pyruvate oxidase (\textit{poxB}), acetate kinase-phosphotransacetylase (\textit{ackA-pta}), isocitrate dehydrogenase (\textit{icd}), \textit{aceBAK} operon repressor (\textit{iclR}), and the glucose phosphotransferase system (\textit{ptsG}). Each one of these mutations is designated by a number (1-\textit{icd}, 2-\textit{sdhAB}, 5-\textit{iclR}, 6-\textit{poxB}, 7-\textit{ackA-pta}, 9-\textit{ptsG}) used in the naming of the mutant strains (Figure 7-1). The kanamycin cassette was left in the final mutant strains to provide selective pressure during the fermentation experiments.
Figure 7-3: Aerobic succinate production platform, the two-route system with the glyoxylate cycle and the oxidative branch of the TCA cycle (mutant strain HL2765k).
Table 7-1: List of mutant strains and plasmids constructed and studied.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>GJT001</td>
<td>Spontaneous cadR mutant of MC4100(ATC35695) Δlac(arg-lac)U169rpsL150relA1ptsF Sm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Tolentino et al., 1992</td>
</tr>
<tr>
<td>HL51276k</td>
<td>GJT001(iclR, icd, sdhAB, (ackA-pta), poxB::Km&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Lin et al., 2004</td>
</tr>
<tr>
<td>HL512769k</td>
<td>GJT001(iclR, icd, sdhAB, (ackA-pta), poxB, ptsG::&lt;sup&gt;R&lt;/sup&gt;Km&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>HL2765k</td>
<td>GJT001(sdhAB, (ackA-pta), poxB, iclR::&lt;sup&gt;R&lt;/sup&gt;Km&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>HL27659k</td>
<td>GJT001(sdhAB, (ackA-pta), poxB, iclR, ptsG::&lt;sup&gt;R&lt;/sup&gt;Km&lt;sup&gt;R&lt;/sup&gt;)</td>
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<tr>
<td>HL51276k(pKK313)</td>
<td>HL51276k overexpressing S8D mutant <em>Sorghum pepc</em></td>
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Plasmids

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<th>Reference</th>
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<td>pKK313</td>
<td>S8D mutant <em>Sorghum pepc</em>, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Wang et al., 1992</td>
</tr>
<tr>
<td>pKK313C</td>
<td>Control vector of pKK313 with inactive <em>Sorghum pepc</em>, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

Plasmid pKK313, carrying the S8D mutation in the *Sorghum pepc* gene (Wang et al., 1992), was used to produce high levels of PEPC in the mutant strains (Table 7-1). Plasmid pKK313 confers ampicillin resistance. This altered *Sorghum* PEPC has a site-directed mutation that advantageously relieves malate feedback inhibition from the enzyme (Wang et al., 1992). A control plasmid was created from pKK313, designated pKK313C, by excising a 2.4 kb fragment from pepc using two *SacI* sites within the gene (Table 7-1). Plasmid pKK313C was then tested to show that it produced no PEPC activity.

7.3.2 Mutant Construction

Mutations were created using the one-step inactivation method of Datsenko and Wanner (Datsenko and Wanner, 2000). This method first requires the construction of the single mutations using the phage λ Red recombinase. P1 phage transduction was then
used to combine various mutations into one strain. Each mutation had to be added to the strain one at a time before the introduction of the next mutation because the kanamycin cassette had to be removed at each stage to enable selection of the next mutation. PCR products of the kanamycin cassette gene flanked by FRT (FLP recognition target) sites and homologous sequences to the gene of interest were made using pKD4 (Datsenko and Wanner, 2000) as the template. These PCR products were then transformed into the cells by electroporation (Bio-Rad Gene Pulser) for insertional inactivation of the gene of interest. These transformed cells carry the plasmid pKD46 (Datsenko and Wanner, 2000) that expresses the λ Red system (γ, β, exo) for recombination of the PCR product into the chromosome. Once the kanamycin cassette is inserted, it can be removed using the helper plasmid, pCP20 (Datsenko and Wanner, 2000), that expresses FLP. The removal of the FRT-flanked kanamycin cassette leaves behind an 84 base pair insertion cassette. At each stage of mutation, experiments were performed to test the intermediate mutant strain for the effect on metabolite production. Throughout the process of constructing the aerobic succinate production systems, a library of different mutant strains with varying types and numbers of mutations was created. All mutant strains were also verified with genomic PCR after construction to ensure that the gene of interest had been disrupted.

7.3.3 Bioreactor Culture Medium and Conditions

Aerobic batch reactor experiments were conducted for all the mutant strains. The medium used is LB with 2 g/L NaHCO₃ and approximately 60 mM of glucose. The medium used for inoculum preparation is also LB, except glucose was not supplemented. NaHCO₃ was added to the culture medium because it yielded better cell growth and
succinate production due to its pH-buffering capacity and its ability to supply CO₂. Kanamycin was added to the medium at a concentration of 50 mg/L for strains not harboring plasmids. In strains harboring pKK313 or pKK313C, ampicillin, carbenicillin, and oxacillin were added to the medium at a concentration of 200 mg/L each. Studies have shown that the use of methicillin and ampicillin is effective as a selective pressure in the cultivation of recombinant *E. coli* (Lee and Kim, 1996). Oxacillin is an analog of methicillin. The use of ampicillin, carbenicillin, and oxacillin in combination during the experiments enforced that the plasmids were retained throughout the aerobic fermentation. IPTG was added at 1 mM to the medium to induce gene expression for plasmids pKK313 and pKK313C.

The initial medium volume is 600 ml in a 1.0-L New Brunswick Scientific Bioflo 110 fermenter. A 1% (v/v) inoculum was used from an overnight culture grown from a single colony for 12 hours. The pH was measured using a glass electrode and controlled at 7.0 using 1.5N HNO₃ and 2N Na₂CO₃. The temperature was maintained at 37°C, and the agitation speed was constant at 800 rpm. The inlet airflow used was 1.5 L/min. The dissolved oxygen was monitored using a polarographic oxygen electrode (New Brunswick Scientific) and was maintained above 80% saturation throughout the experiment. This dissolved oxygen level was to demonstrate that the succinate production systems were working under absolute aerobic conditions.

7.3.4 Analytical Techniques

Optical density was measured at 600nm with a spectrophotometer (Bausch & Lomb Spectronic 1001); the culture was diluted to the linear range with 0.15 M NaCl.
For analyzing the extracellular metabolites, 1 ml of culture was centrifuged and the supernatant was then filtered through a 0.45-µm syringe filter for HPLC analysis. The HPLC system (Shimadzu-10A Systems, Shimadzu, Columbia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A) and a differential refractive index (RI) detector (Waters 2410, Waters, Milford, MA). A 0.6 mL/min mobile phase using 2.5 mM H₂SO₄ solution was applied to the column. The column was operated at 55 °C. Standards were prepared for glucose, succinate, acetate, and pyruvate for both the RI detector and UV detector, and calibration curves were created. Glucose, succinate, and acetate were measured by the RI detector and pyruvate was measured by the UV detector at 210nm.

7.4 Results and Discussion

7.4.1 Comparison of Two Aerobic Succinate Production Systems

Previously, an aerobic succinate production platform was developed, which could produce succinate exclusively through the glyoxylate cycle (Figure 7-2) (Lin et al., 2004). Five mutations (sdhAB, icd, iclR, poxB, and (ackA-pta)) (Figure 7-1) were strategically created in the glycolysis, TCA cycle, and glyoxylate bypass to create a pentamutant strain of E. coli, HL51276k (Table 7-1). These mutations together created an active glyoxylate cycle, which was shown to produce a substantial amount of succinate under aerobic conditions (Lin et al., 2004). This system was the first platform developed in E. coli to show the feasibility of producing succinate entirely under aerobic conditions. Strain HL51276k, although it produced a substantial amount of succinate
also exhibited accumulation of pyruvate and TCA cycle C₆ intermediates, such as citrate and isocitrate. Because of the accumulation, the maximum theoretical yield of one mole succinate produced per mole glucose consumed could not be achieved and the productivity was hampered.

In the current study, another aerobic succinate production system was constructed in *E. coli* to improve upon the platform developed in strain HL51276k. This system has four mutations created (*sdhAB, iclR, poxB*, and *(ackA-ptai)*) (Figure 7-1). It is essentially the same as HL51276k, except *icd* is not inactivated. These four mutations create the strain HL2765k (Table 7-1), which has two pathways opened for succinate production (Figure 7-3). One pathway is the glyoxylate cycle leading to succinate formation. The second pathway utilizes the oxidative arm of the TCA cycle leading to succinate. Both pathways branch from isocitrate to form succinate (Figure 7-3).

Strains HL51276k and HL2765k were grown in bioreactors at 37°C where the dissolved oxygen was maintained above 80% saturation throughout the experiment. Their metabolite profiles were compared. Results showed that strain HL2765k had a higher succinate production than HL51276k. At approximately 48 hours, the succinate concentration in the HL2765k culture was 40 mM compared to that of the HL51276k culture, which had 31 mM succinate (Figure 7-4a). Succinate molar yields at the highest concentration produced were 0.67 for HL2765k and 0.65 for HL51276k (Table 7-2). HL2765k also had 65% higher volumetric succinate productivity and 12% higher specific succinate productivity than HL51276k (Table 7-2).

Strain HL2765k grew to a higher OD (14.27 OD) than strain HL51276k (9.21 OD). HL2765k also had a faster biomass generation rate (0.60 g/l-hr) than HL51276k
Table 7-2: Results of succinate yield and productivity of cultures of various mutant strains in aerobic batch reactor experiments. $Y_{SG}$ is the molar succinate yield at the end of fermentation (mole of succinate produced per mole of glucose consumed); $Q_p$ is the average volumetric succinate productivity at the end of fermentation (mass concentration of succinate (g/l) over time (hr)); $q_p$ is the average specific succinate productivity at the end of fermentation (mass of succinate (mg) per mass of biomass (g) over time (hr)).

<table>
<thead>
<tr>
<th>Strain</th>
<th>$Y_{SG}$ (mol/mol)</th>
<th>$Q_p$ (g/l-hr)</th>
<th>$q_p$ (mg/g-hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL51276k</td>
<td>0.65</td>
<td>0.057</td>
<td>24.04</td>
</tr>
<tr>
<td>HL512769k</td>
<td>0.87</td>
<td>0.086</td>
<td>35.47</td>
</tr>
<tr>
<td>HL2765k</td>
<td>0.67</td>
<td>0.094</td>
<td>26.84</td>
</tr>
<tr>
<td>HL27659k</td>
<td>0.78</td>
<td>0.130</td>
<td>32.82</td>
</tr>
<tr>
<td>HL51276k(pKK313C)</td>
<td>0.61</td>
<td>0.048</td>
<td>27.54</td>
</tr>
<tr>
<td>HL51276k(pKK313)</td>
<td>1.09</td>
<td>0.140</td>
<td>44.26</td>
</tr>
<tr>
<td>HL512769k(pKK313C)</td>
<td>0.85</td>
<td>0.083</td>
<td>38.99</td>
</tr>
<tr>
<td>HL512769k(pKK313)</td>
<td>0.96</td>
<td>0.094</td>
<td>45.23</td>
</tr>
<tr>
<td>HL2765k(pKK313C)</td>
<td>0.71</td>
<td>0.113</td>
<td>28.33</td>
</tr>
<tr>
<td>HL2765k(pKK313)</td>
<td>0.75</td>
<td>0.111</td>
<td>35.54</td>
</tr>
<tr>
<td>HL27659k(pKK313C)</td>
<td>0.74</td>
<td>0.106</td>
<td>31.14</td>
</tr>
<tr>
<td>HL27659k(pKK313)</td>
<td>0.95</td>
<td>0.270</td>
<td>73.66</td>
</tr>
</tbody>
</table>

(0.24 g/l-hr), because its glucose consumption rate is faster than HL51276k. There was pyruvate accumulation in cultures of both strains, which was produced and then consumed (Figure 7-4c). HL2765k had a higher pyruvate accumulation in the beginning of fermentation than HL51276k. This is because HL2765k has faster glucose consumption and cell mass generation than HL51276k. The pyruvate was taken up and consumed faster by HL2765k than HL51276k (Figure 7-4c). HL2765k also produced acetate faster than HL51276k due to its more rapid glucose consumption rate (Figure 7-4d). Strain HL2765k did not have any accumulation of TCA cycle C_6 intermediates, whereas strain HL51276k did (data not shown). Lactate and ethanol were not detected in the cultures of either strain. Comparison of these two strains showed that HL2765k was
more robust than HL51276k. Strain HL2765k with two pathways engineered for succinate production.

![Graphs showing metabolite levels over time](image)

**Figure 7-4**: Metabolite comparison in cultures of strains HL51276k, HL512769k, HL2765k, and HL27659k. a) succinate production graph; b) glucose remaining graph; c) pyruvate production graph; d) acetate production graph. Solid square (■): HL27659k; solid triangle (▲): HL2765k; open square (○): HL512769k; open triangle (Δ): HL51276k. Cultivation medium is LB with 2 g/L NaHCO₃ and approximately 60 mM of glucose.

has a faster succinate productivity and glucose consumption rate than HL51276k, which only utilizes the glyoxylate cycle for succinate production.

### 7.4.2 Effect of *ptsG* Inactivation in the Two Aerobic Succinate Production Systems

The glucose phosphotransferase system (PTS) was studied in the two strains HL2765k and HL51276k to examine the possibility of reducing pyruvate and acetate
accumulation (Chou et al., 1994). By inactivating the phosphotransferase uptake system, pyruvate can no longer be formed from phosphoenolpyruvate (PEP) through the transport of glucose. This genetic manipulation can potentially reduce pyruvate accumulation. Acetate formation occurs because of excess consumption of glucose that the cell is unable to utilize for biomass synthesis or energy requirements, leading to repression of enzymes in the TCA cycle by glucose (Yang et al., 1999). The secretion of acetate leads to an uncoupled metabolism (Doelle et al., 1981). Inactivating *ptsG* can slow glucose uptake and possibly allow a more balanced glucose metabolism. Inactivation of *ptsG* has been shown to increase succinate production in pyruvate formate lyase and lactate dehydrogenase mutant strains (Chatterjee et al., 2001; Chou et al., 1994). This effect is probably due to more PEP being conserved and available for the succinate synthesis pathway, while also generating a slower glucose uptake rate. *ptsG* was inactivated in the strain HL2765k to form HL27659k (Table 7-1), and in the strain HL51276k to form HL512769k (hexamutant strain of *E. coli*) (Table 7-1). The number 9 represents the inactivation of *ptsG* (Figure 7-1).

Strains HL27659k and HL512769k were grown aerobically under the same batch reactor conditions described earlier for strains HL2765k and HL51276k. The results showed *ptsG* inactivation did improve succinate production. At approximately 48 hours, HL27659k produced 49 mM succinate compared to HL2765k, which produced 40 mM, and HL512769k produced 44 mM succinate compared to HL51276k, which produced 31 mM (Figure 7-4a). At the highest succinate concentration produced, the molar yield was also higher when *ptsG* was inactivated (0.78 for HL27659k compared to 0.67 for HL2765k, and 0.87 for HL512769k compared to 0.65 for HL51276k) (Table 7-2). The
succinate volumetric productivity and specific productivity at the highest succinate concentration were also higher from cultures of those strains with \textit{ptsG} inactivation. HL27659k had 38% higher succinate volumetric productivity and 22% higher specific productivity than HL2765k (Table 7-2). HL512769k had 51% higher succinate volumetric productivity and 48% higher specific productivity than HL51276k (Table 7-2). The effects of \textit{ptsG} inactivation improve succinate production more in HL51276k than in HL2765k; this is because there are more bottlenecks in the TCA pathways of HL51276k than in HL2765k (HL51276k has TCA cycle C$_6$ accumulation and HL2765k does not).

\textit{ptsG} inactivation caused cell growth to be lower due to slower glucose consumption during the exponential phase. HL27659k grew to an OD of 12.59 at the end of its exponential phase compared to HL2765k, which grew to 14.27 OD. HL512769k grew to an OD of 8.31 at the end of its exponential phase compared to HL51276k, which grew to an OD of 9.21. By the end of the exponential phase, the biomass generation rate of strain HL2765k was 0.60 g/l-hr compared to strain HL27659k, which was 0.27 g/l-hr. For strain HL51276k, the biomass generation rate at the end of the exponential phase was 0.24 g/l-hr compared to strain HL512769k, which has 0.13 g/l-hr.

Inactivation of \textit{ptsG} did reduce pyruvate accumulation in cultures of strains HL2765k and HL51276k. Pyruvate accumulation in HL27659k only reached a maximum concentration of 48 mM compared to HL2765k, which reached 72 mM (Figure 7-4c). Strain HL512769k produced a maximum pyruvate concentration of 23 mM, compared to HL51276k, which produced 48 mM (Figure 7-4c). In strain HL512769k, there was no pyruvate accumulation after 48 hours. All the glucose was consumed by
cultures of all four strains by the end of fermentation. Inactivation of *ptsG* also reduced acetate production. HL27659k had a lower acetate production rate than HL2765k in the first 24 hours. By the end of the fermentation though when all the glucose was consumed in the cultures, HL27659k had slightly higher acetate production than HL2765k (Figure 7-4d). Acetate production was lower in HL512769k than in HL51276k throughout the fermentation (Figure 7-4d). Strain HL512769k accumulated TCA cycle C₆ intermediates and strain HL27659k did not. Both lactate and ethanol were not detected in the cultures of these strains. Results showed that *ptsG* inactivation did improve succinate yield and productivity in cultures of the two strains, HL2765k and HL51276k. Because of the *ptsG* inactivation, glucose consumption was slowed, which helped reduce pyruvate and acetate accumulation by providing a more balanced metabolism.

### 7.4.3 Effect of *pepc* Overexpression with *ptsG* Inactivation in the Aerobic Succinate Production Systems

*ptsG* inactivation has been shown to improve succinate yield and productivity. Yet in strains HL27659k and HL512769k, the maximum theoretical succinate yield of one mole produced per mole glucose consumed has not been obtained. This indicates that the aerobic production systems can be further optimized. PEPC converts PEP to OAA through a carboxylation reaction with CO₂ (Figure 7-1). OAA is an important precursor for the synthesis of succinate. Overexpression of PEPC in *E. coli* has been shown to increase succinate production (Millard *et al.*, 1996). Therefore, overexpression of PEPC in the aerobic succinate production systems should improve succinate yield and
productivity, and at the same time further reduce pyruvate and acetate accumulation. A mutant PEPC from *Sorghum* was overexpressed on plasmid pKK313 (Table 7-1) in the four strains HL51276k, HL512769k, HL2765k, and HL27659k. This mutant PEPC is feedback inhibition resistant to malate (Wang *et al.*, 1992). This mutation is advantageous for the aerobic succinate production systems because even though succinate is not being formed by the reduction of malate as in anaerobic conditions, malate is still present and required in the glyoxylate cycle as the precursor of OAA (Figures 7-2 and 7-3). The control plasmid for pKK313 is pKK313C (Table 7-1), which was also transformed into the four mutant strains. The mutant strains carrying the plasmids were grown aerobically in bioreactors at 37°C where the dissolved oxygen was maintained above 80% saturation throughout the experiment. Mutant strains harboring pKK313 were compared in terms of their metabolite production and succinate yield and productivity with the same mutant strains harboring pKK313C.

Overexpression of the mutant *Sorghum* PEPC in strains HL51276k, HL512769k, HL2765k, and HL27659k was effective in increasing succinate production. The succinate production of cultures of strain HL51276k(pKK313) was 130% higher than those of strain HL51276k(pKK313C) at the end of the fermentation when all the glucose had been consumed (Figure 7-5a). Cultures of HL27659k(pKK313) had 37% higher succinate production than its control strain HL27659k(pKK313C) (Figure 7-8a). The increase in succinate production of cultures of strains HL512769k(pKK313) and HL2765k(pKK313) compared to their respective controls was not as substantial as that found for HL51276k(pKK313), although their succinate concentrations were
continuously higher than their controls throughout the production phase (Figures 7-6a and 7-7a). In strains with high levels of PEPC, the molar succinate yields for strains

![Graphs](image)

**Figure 7-5:** Metabolite comparison in cultures of strains HL51276k(pKK313) and HL51276k(pKK313C). a) succinate production graph; b) glucose remaining graph; c) pyruvate production graph; d) acetate production graph. Solid diamond (♦): HL51276k(pKK313); solid square (■): HL51276k(pKK313C). Cultivation medium is LB with 2 g/L NaHCO₃ and approximately 60 mM of glucose.

HL51276k(pKK313), HL512769k(pKK313) and HL27659k(pKK313) all reached the maximum theoretical value of one mole of succinate produced per mole of glucose consumed (Table 7-2). The molar succinate yield for strain HL2765k(pKK313) was 0.75 compared to 0.71 for its control strain HL2765(pKK313C) (Table 7-2). PEPC overexpression, therefore, was not as effective in increasing the succinate yield in strain
HL2765k as in the other strains. The specific succinate productivity was higher in all the strains overexpressing PEPC; strain HL51276k(pKK313) was 61% higher than its control; HL512769k(pKK313) was 16% higher, HL2765k(pKK313) was 25% higher, and HL27659k(pKK313) was 137% higher than respective their controls carrying pKK313C (Table 7-2). These results showed that high expression of mutant *Sorghum* PEPC was very effective in improving succinate yield in the mutant *E. coli* host strains and successfully optimized three of the aerobic production systems (HL51276k, HL512769k, and HL27659k) to produce the maximum theoretical succinate yield of 1.0 mole per 1.0 mole glucose consumed. Although cultures of strains HL51276k(pKK313), HL512769k(pKK313), and HL27659k(pKK313) all achieved the maximum theoretical succinate yield, they still varied in their efficiency. Among the three strains, HL27659k(pKK313) was the most efficient. Fermentation showed substantially higher volumetric succinate productivity (0.27 g/l-hr) and specific productivity (73.66 mg/g-hr) than the other two strains (Table 7-2). The volumetric succinate productivity for cultures of HL27659k(pKK313) was 93% and 187% higher than strains HL51276k(pKK313) and HL512769k(pKK313), respectively. As for specific succinate productivity, cultures of HL27659k(pKK313) were 66% and 63% higher than those of strains HL51276k(pKK313) and HL512769k(pKK313), respectively. These results demonstrate that fermentations of strain HL27659k(pKK313), with its 1.0 mol/mol succinate yield, is a more efficient and robust aerobic succinate production system for large-scale production than the other systems.

Overexpression of PEPC was also effective in reducing pyruvate production. Maximum pyruvate produced in cultures of strains HL51276k(pKK313) and
HL2765k(pKK313) was lower than their controls carrying pKK313C (Figures 7-5c and 7-7c). In cultures of strains HL512769k(pKK313) and HL27659k(pKK313), pyruvate accumulation was virtually eliminated (Figures 7-6c and 7-8c). These results demonstrate that overexpression of pepc coupled with ptsG inactivation was the most effective in reducing pyruvate accumulation, thus providing more efficient carbon throughput.

**Figure 7-6:** Metabolite comparison in cultures of strains HL512769k(pKK313) and HL512769k(pKK313C). a) succinate production graph; b) glucose remaining graph; c) pyruvate production graph; d) acetate production graph. Solid diamond (♦): HL512769k(pKK313); solid square (■): HL512769k(pKK313C). Cultivation medium is LB with 2 g/L NaHCO₃ and approximately 60 mM of glucose.
Acetate production was reduced in cultures of the mutant strains with the high levels of PEPC. Cultures of strain HL512769k(pKK313) had a 47% reduction, HL2765k(pKK313) had a 29% reduction, HL27659k(pKK313) had an 82% reduction compared to cultures of their respective control strains carrying pKK313C (Figures 7-6d, 7-7d, and 7-8d). Acetate production for strain HL51276k(pKK313) was lower than HL51276k(pKK313C) throughout production, but their final acetate concentrations at the end of fermentation after all the glucose was consumed were similar (Figure 7-5d). Cultures of strain HL27659k(pKK313) exhibited the lowest acetate level produced among all the four strains (below 5 mM) and also had little pyruvate accumulated (below 2 mM). Its main product was succinate at 60 mM at the end of fermentation when all the glucose was consumed, equivalent to approximately 1.0 mol succinate/mol glucose. Lactate and ethanol were not detected in the cultures of any of these mutant strains.

The culture conditions of the mutant strains developed and studied for aerobic succinate production, thus far, have not been optimized for production. The calculation of succinate yield and productivity (Table 7-2) has included both the growth phase (biomass generation) and the production phase. These aerobic systems are efficient and practical because they do not require separation of the growth phase from the production phase for succinate production as in conventional anaerobic succinate production systems (Vemuri et al., 2002a, 2002b; Nghiem et al., 1999). Nevertheless, the culture conditions of the aerobic succinate production systems can be further optimized to improve process productivity.

The reproducibility of the bioreactor experiment results of the various mutant strains was demonstrated by repeating the experiments for a select few mutant strains that are
prominent for aerobic succinate production (HL51276k, HL51276k(pKK313), and HL27659k(pKK313)) (Figure 7-9). The results of these duplicate experiments showed that the trends of the metabolite concentrations were the same, therefore, reproducible. Analysis of metabolite samples was performed in triplicates in HPLC to ensure accuracy.

![Figure 7-7: Metabolite comparison in cultures of strains HL2765k(pKK313) and HL2765k(pKK313C). a) succinate production graph; b) glucose remaining graph; c) pyruvate production graph; d) acetate production graph. Solid diamond (♦): HL2765k(pKK313); solid square (■): HL2765k(pKK313C). Cultivation medium is LB with 2 g/L NaHCO₃ and approximately 60 mM of glucose.](image-url)
Figure 7-8: Metabolite comparison in cultures of strains HL27659k(pKK313) and HL27659k(pKK313C). a) succinate production graph; b) glucose remaining graph; c) pyruvate production graph; d) acetate production graph. Solid diamond (♦): HL27659k(pKK313); solid square (■): HL27659k(pKK313C). Cultivation medium is LB with 2 g/L NaHCO₃ and approximately 60 mM of glucose.

the trends of the metabolite concentrations were the same, therefore, reproducible. Analysis of metabolite samples was performed in triplicates in HPLC to ensure accuracy.

7.5 Conclusion

Although metabolic engineering of *E. coli* to enhance succinate production under anaerobic conditions has generated many promising improvements, it is still hampered by inherent anaerobic constraints, such as cofactor NADH requirement and slow cell
Figure 7-9: Duplicate batch reactor experiments of select mutant strains to show result reproducibility. a) open square (○): HL51276k glucose consumption (data from Figure 7-4); open triangle (Δ): HL51276k glucose consumption (repeated experiment); solid square (■): HL51276k succinate production (data from Figure 7-4); solid triangle (▲): HL51276k succinate production (repeated experiment). b) open square (○): HL51276k pyruvate production (data from Figure 7-4); open triangle (Δ): HL51276k pyruvate production (repeated experiment); solid square (■): HL51276k acetate production (data from Figure 7-4); solid triangle (▲): HL51276k acetate production (repeated experiment). c) open square (○): HL51276k(pKK313) glucose consumption (data from Figure 7-5); open triangle (Δ): HL51276k(pKK313) glucose consumption (repeated experiment); solid square (■): HL51276k(pKK313) succinate production (data from Figure 7-5); solid triangle (▲): HL51276k(pKK313) succinate production (repeated experiment). d) open square (○): HL27659k(pKK313) glucose consumption (data from Figure 7-8); open triangle (Δ): HL27659k(pKK313) glucose consumption (repeated experiment); solid square (■):HL27659k(pKK313) succinate production (data from Figure 7-8); solid triangle (▲):HL27659k(pKK313) succinate production (repeated experiment).

growth. This makes application on an industrial scale difficult due to low biomass generation and the poor physiological state of the cell. Aerobic conditions provide many
advantages that favor implementation on an industrial scale due to faster biomass generation, carbon throughput and product formation. Aerobic succinate production systems were thus developed in this study to demonstrate promising and feasible alternatives to anaerobic fermentation. Production systems were optimized to achieve the maximum theoretical succinate yield of 1.0 mol/mol of glucose.

Two aerobic succinate production platforms were presented, HL51276k (Lin et al., 2004) and HL2765k (this study). Strain HL51276k’s central metabolism was the glyoxylate cycle (Figure 7-2), and strain HL2765k had the glyoxylate cycle and the oxidative branch of the TCA cycle for synthesizing succinate (Figure 7-3). HL2765k, with higher succinate productivity, produced more succinate than HL51276k. It also had faster biomass generation and glucose consumption than HL51276k. Strain HL2765k also did not accumulate any TCA cycle C₆ intermediates, whereas strain HL51276k did.

With ptsG inactivated in strains HL512769k and HL27659k, succinate concentration and productivity increased compared to fermentations of strains HL51276k and HL2765k, respectively. Inactivation of ptsG also reduced pyruvate and acetate accumulation in the two strains. Overexpression of the mutant Sorghum pepe in HL51276k, HL512769k, HL2765k, and HL27659k promoted a significant increase in succinate yield and productivity. It also reduced pyruvate and acetate production in cultures of all four strains. Cultures of three strains, HL51276k(pKK313), HL512769k(pKK313), and HL27659k(pKK313), achieved the maximum theoretical succinate yield of 1.0 mol/mol. Among these three production systems, HL27659k(pKK313) had substantially higher succinate productivity than the other two. It also had minimal pyruvate and acetate accumulation throughout the fermentation.
Strain HL27659k(pKK313) is the most efficient aerobic succinate production system developed and cultures demonstrate a succinate yield of 1.0 mol/mol. It is a promising system for large-scale succinate production and process optimization.

7.6 Acknowledgments

The authors would like to thank Dr. Jean Vidal for providing the *Sorghum pepc* plasmid (pKK313). The authors would also like to thank Dr. Barry L. Wanner for providing plasmids pKD4, pKD46, and pCP20 that facilitate the construction of mutations in *E. coli*. This work was supported by grants from the National Science Foundation (BES-0222691 and BES-0000303). Henry Lin was supported by a training grant from the National Science Foundation (DGE0114264).
Chapter 8

8. Fed Batch Culture of a Metabolically Engineered *Escherichia coli* Strain Designed for High Level Succinate Production and Yield under Aerobic Conditions

* This chapter has been accepted for publication in *Biotechnology and Bioengineering* (Lin *et al.*, 2005c).

8.1 Abstract

An aerobic succinate production system developed by Lin *et al.* (2005b) is capable of achieving the maximum theoretical succinate yield of 1.0 mol/mol glucose for aerobic conditions. It also exhibits high succinate productivity. This succinate production system is a mutant *E. coli* strain with five pathways inactivated: *sdhAB*, *(ackA-pta)*, *poxB*, *iclR*, and *ptsG*. The mutant strain also overexpresses the *Sorghum vulgare pepc*. This mutant strain is designated HL27659k(pKK313). Fed batch reactor experiments were performed for the strain HL27659k(pKK313) under aerobic conditions to determine and demonstrate its capacity for high level succinate production. Results showed that it could produce 58.3 g/l of succinate in 59 hrs under complete aerobic conditions. Throughout the whole fermentation, the average succinate yield was 0.94 ± 0.07 mol/mol glucose, the average productivity was 1.08 ± 0.06 g/l-hr, and the average specific productivity was 89.77 ± 3.40 mg/g-hr. The strain HL27659k(pKK313) is, thus, capable of large scale succinate production under aerobic conditions. The results also showed that the aerobic succinate production system using the designed strain
HL27659k(pKK313) is more practical than conventional anaerobic succinate production systems. It has remarkable potential for industrial scale succinate production and process optimization.

8.2 Introduction

The valuable specialty chemical succinate and its derivatives have extensive industrial applications. It can be used as an additive and flavoring agent in foods, a supplement for pharmaceuticals, a surfactant, a detergent extender, a foaming agent, and an ion chelator (Zeikus et al., 1999). *E. coli* has also been extensively genetically engineered through the use of recombinant DNA technology in recent years to generate strains that show promise for succinate production. *E. coli* naturally produces succinate as a minor fermentation product under anaerobic conditions (Clark, 1989). Under aerobic conditions, succinate is not produced in *E. coli* and acetate is the main byproduct. Numerous metabolic engineering strategies to enhance succinate production in *E. coli* have met with success. Strains in which enzymatic steps involved in the succinate pathway were amplified and the organism cultured under anaerobic conditions yielded higher succinate production. An example of this was shown when phosphoenolpyruvate carboxylase (*pepc*) from *E. coli* was overexpressed (Millard et al., 1996). Conversion of fumarate to succinate was improved by overexpressing native fumarate reductase (*frd*) in *E. coli* (Goldberg et al., 1983; Wang et al., 1998). Certain enzymes are not indigenous in *E. coli*, but can potentially help increase succinate production. By introducing pyruvate carboxylase (*pyc*) from *Rhizobium etli* into *E. coli*, succinate production was enhanced (Gokarn et al., 1998, 2000, 2001). Other metabolic engineering strategies also include
inactive competing pathways of succinate. When malic enzyme was overexpressed in a host with inactivated pyruvate formate lyase (pfl) and lactate dehydrogenase (ldh) genes, succinate became the major fermentation product (Stols and Donnelly, 1997; Hong and Lee, 2001). In cultures of this pfl and ldh mutant strain, there is a large pyruvate accumulation. Overexpression of malic enzyme in this mutant strain increased succinate production driven by the high pyruvate pool toward the direction of malate formation, which subsequently was converted to succinate. An inactive glucose phosphotransferase system (ptsG) in the same mutant strain (pfl' and ldh') had also been shown to yield higher succinate production in E. coli and improve growth (Chatterjee et al., 2001).

The various genetic improvements described above for succinic acid production have all been done under anaerobic conditions utilizing the mixed-acid fermentation pathways of E. coli. Unfortunately, anaerobic fermentation has inherent disadvantages that are difficult to surmount. Anaerobic conditions often cause poor cell growth and slow carbon throughput, therefore generating low production rates. Succinic acid formation in mixed-acid fermentation is also hampered by the limitations of NADH availability, since 2 moles of NADH are required for every mole of succinate to be formed. Strategies to overcome the anaerobic barrier have included generating enough biomass under aerobic conditions, then switching to anaerobic conditions for succinate production. This was shown to be effective using a “dual-phase” fermentation system, in which initial aerobic growth phase was started then followed by an anaerobic production phase (Vemuri et al., 2002a, 2002b).
Ultimately, the solution for overcoming the anaerobic condition constraints is to produce succinate completely under aerobic conditions. Recently, aerobic production of succinate in *E. coli* has been demonstrated to be feasible by Lin *et al.* (2005a). A novel *E. coli* aerobic succinate production system was developed by Lin *et al.* (2005b) that could produce succinate at a yield of 1.0 mole per mole glucose. This yield based on pathway modeling and optimization is the maximum theoretical yield achievable for succinate in *E. coli* under aerobic conditions (Lin *et al.*, 2005b). This succinate production system was purposely designed to function under absolute aerobic conditions. Aerobic fermentation is advantageous over anaerobic fermentation in that oxidative phosphorylation is active for generating energy with O$_2$ present as the electron acceptor. This would lead to higher biomass generation, faster carbon throughput and product formation.

After various genetic designs of the aerobic central metabolism in *E. coli*, only one exhibited high succinate productivity, while achieving a succinate yield of 1.0 mol/mol glucose under aerobic batch reactor conditions (Lin *et al.*, 2005b). The strain also has minimal pyruvate and acetate accumulation during aerobic fermentation (Lin *et al.*, 2005b). This aerobic succinate production system was constructed through genetic reconstruction of the aerobic central metabolic pathways. Five genes were strategically inactivated in *E. coli*. One is succinate dehydrogenase (*sdhAB*), which allows the cell to accumulate succinate under aerobic conditions. Then inactivation of pyruvate oxidase (*poxB*) and acetate kinase-phosphotransacetylase (*ackA-pta*) in the host channels more carbon flux toward the oxidative branch of the TCA cycle for succinate synthesis, and significantly reduces acetate production. The *aceBAK* operon repressor (*iclR*) was
inactivated in the \textit{E. coli} strain in order to activate the glyoxylate bypass for succinate synthesis. The glucose phosphotransferase system (\textit{ptsG}) was also inactivated in the altered host to increase phosphoenolpyruvate (PEP) pool for succinate synthesis and to improve carbon-throughput. Phosphoenolpyruvate carboxylase (\textit{pepc}) was also overexpressed in the system in order to drive more carbon flux toward oxaloacetate (OAA), a major precursor for succinate synthesis. In the end, this aerobic succinate production system has two possible routes for production. One is the oxidative branch of the TCA cycle and the other is the glyoxylate cycle (Figure 8-1). \textit{E. coli} (\textit{sdhAB, ackA-pta}), \textit{poxB, iclR, ptsG}) overexpressing \textit{pepc} constitutes the aerobic succinate production system. This mutant strain is designated HL27659k(pKK313) (Figure 8-1).

The strain HL27659k(pKK313) has potential for large-scale succinate production because of its high productivity under aerobic conditions. In this study, this strain is studied in fed batch reactor experiments under aerobic conditions to determine and demonstrate its high capacity for succinate production. Succinate productivity, yield and absolute production concentration are examined to show feasibility for large-scale production.

\textbf{8.3 Materials and Methods}

\textbf{8.3.1 Strain}

Mutant strain HL27659k(pKK313) constructed by Lin \textit{et al.} (2005b) was used for all succinate production experiments. The parental wildtype strain for HL27659k(pKK313) is GJT001, a spontaneous \textit{cadR} mutant of MC4100 (Tolentino \textit{et}
al., 1992). Knockouts were created in succinate dehydrogenase (sdhAB), pyruvate oxidase (poxB), acetate kinase-phosphotransacetylase (ackA-pta), aceBAK operon repressor (iclR), and the glucose phosphotransferase system (ptsG). Plasmid pKK313, carrying the mutant Sorghum vulgare pepc gene (Wang et al., 1992), was used to produce

**Figure 8-1:** The aerobic succinate production system featuring two routes for succinate synthesis. One is the oxidative branch of the TCA cycle and the other is the glyoxylate cycle. This *E. coli* mutant strain (sdhAB, ackA-pta, poxB, iclR, ptsG) with pepc overexpression is designated HL27659k(pKK313).
high levels of PEPC in the mutant strain HL27659k. Plasmid pKK313 confers ampicillin resistance.

8.3.2 Bioreactor Culture Medium and Conditions

Reactor medium used is SB (Ausubel et al., 1988) with 2 g/L NaHCO₃. SB consists of 5 g/l NaCl, 20 g/l yeast extract, 32 g/l tryptone, 7 g/l Na₂HPO₄, 3 g/l KH₂PO₄, and 1 g/l NH₄Cl. NaHCO₃ was added to the culture medium because it yielded better cell growth and succinate production due to its pH-buffering capacity and its ability to supply CO₂. Ampicillin, carbenicillin, and oxacillin were added to the medium at a concentration of 200 mg/l each. Studies have shown that the use of methicillin and ampicillin is effective as a selective pressure in the cultivation of recombinant E. coli (Lee and Kim, 1996). Oxacillin is an analog of methicillin. The use of ampicillin, carbenicillin, and oxacillin in combination during the experiments enforced that the plasmids were retained throughout the aerobic fermentation. IPTG was added at 1.0 mM to the medium to induce gene expression of pepe for plasmid pKK313.

Batch reactor experiments were conducted under aerobic conditions. The initial medium volume is 600 ml in a 1.0-L New Brunswick Scientific Bioflo 110 fermenter. A 1% (v/v) inoculum was used from an overnight culture grown from a single colony for 12 hours. The pH was measured using a glass electrode and controlled at 7.0 using 1.5N HNO₃ and 2N Na₂CO₃. The temperature was maintained at 37°C, and the agitation speed was constant at 800 rpm. The inlet airflow used was 2.0 l/min. The dissolved oxygen was monitored using a polarographic oxygen electrode (New Brunswick Scientific,
Edison, New Jersey) and was maintained above 50% saturation throughout the experiment.

Aerobic fed batch experiments were also performed under the same conditions as batch experiments, except the initial medium volume in the reactor was 400 ml. Glucose was fed exponentially according to the specific growth rate of strain HL27659k(pKK313), obtained from batch experiment results. The program used for glucose feeding was BioCommand Plus BioProcessing Software from New Brunswick Scientific.

### 8.3.3 Analytical Techniques

Optical density was measured at 600nm with a spectrophotometer (Bausch & Lomb Spectronic 1001); the culture was diluted to the linear range with 0.15 M NaCl. For analyzing the extracellular metabolites, 1 ml of culture was centrifuged and the supernatant was then filtered through a 0.45-μm syringe filter for HPLC analysis. The HPLC system (Shimadzu-10A Systems, Shimadzu, Columbia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A) and a differential refractive index (RI) detector (Waters 2410, Waters, Milford, MA). A 0.6 mL/min mobile phase using 2.5 mM H₂SO₄ solution was applied to the column. The column was operated at 55 °C. Standards were prepared for glucose, succinate, acetate, and pyruvate for both the RI detector and UV detector, and calibration curves were created. Glucose, succinate, and acetate were measured by the RI detector and pyruvate was measured by the UV detector at 210nm.
8.4 Results

8.4.1 Comparison of Different Inoculum Sizes on Succinate Production

Three different inoculum sizes of strain HL27659k(pKK313) were studied for aerobic production of succinate. The experiments were carried out in batch reactor settings under aerobic conditions. The small inoculum resulted in a starting culture concentration of approximately 0.02 OD_{600 nm}, the medium inoculum resulted in a starting culture concentration of 2.4 OD_{600 nm}, and the large inoculum resulted in a starting culture concentration of 22.6 OD_{600 nm}. Results show that the larger the inoculum size, the lower the succinate molar yield (Figure 8-2). Strain HL27659k(pKK313) has been shown to be capable of generating the maximum theoretical succinate yield of 1.0 mol/mol glucose in batch cultures (Lin et al., 2005b). Cultures of strain HL27659k(pKK313) starting at approximately 0.02 OD generated a succinate yield of 1.0 mol/mol glucose through the first 26 hrs, and then the yield decreased to approximately 0.81 after 57 hrs of fermentation (Figure 8-2). The succinate concentration obtained at the end of fermentation was 367.0 mM, equivalent to 43.0 g/l. Cultures starting at 2.4 OD generated succinate yield of 1.0 mol/mol glucose only for the first 10 hrs (Figure 8-2). Then the succinate yield decreased to approximately 0.75 after 47 hrs of fermentation, and the succinate concentration obtained was 356.0 mM, equivalent to 42.0 g/l. When cultures of strain HL27659k(pKK313) was started at 22.6 OD to increase productivity, the starting succinate yield was approximately 0.85 mol/mol glucose, and then the yield decreased to approximately 0.66 after 27 hrs of fermentation (Figure 8-2). The succinate concentration obtained was 380.0 mM, equivalent to 45.0 g/l. Results show a significant
Figure 8-2: Comparison of three different inoculum sizes (small (starting OD of 0.02), medium (starting OD of 2.42) and large (starting OD of 22.57)) for the strain HL27659k(pKK313) in aerobic batch reactor experiments. (■) Succinate production (mM) of small inoculum culture. (▲) Succinate production (mM) of medium inoculum culture. (●) Succinate production (mM) of large inoculum culture. (□) Succinate yield (mol/mol glucose) of small inoculum culture. (△) Succinate yield (mol/mol glucose) of medium inoculum culture. (○) Succinate yield (mol/mol glucose) of large inoculum culture.

reduction in succinate yield when large inoculum is used, indicating glucose is not being fully utilized through the succinate synthesis pathways.

The large inoculum culture (starting OD of 22.6) did achieve a substantially high succinate productivity of 2.3 g/l-hr at the beginning of fermentation, and then decreased to 1.7 g/l-hr at the end of the 27-hour fermentation. This succinate productivity is substantially higher than that of the two lower inoculum cultures (starting OD of 0.02 and 2.4). Succinate productivity of the medium inoculum culture (starting OD of 2.4) was approximately 0.90 g/l-hr at the end of 47-hour fermentation. Succinate productivity of the small inoculum culture (starting OD of approximately 0.02) was approximately 0.72
g/l-hr at the end of 57-hour fermentation. The high succinate productivity of the large inoculum culture was obtainable apparently due to higher biomass and faster glucose consumption rate. The final biomass generated at the end of the 27-hour fermentation was 76.6 OD. The final biomass for the small inoculum culture (starting OD of approximately 0.02) and the medium inoculum culture (starting OD of 2.4) were 45.1 OD and 37.0 OD, respectively.

No pyruvate was produced by any of the three different size inoculum cultures. Acetate was the only other product. The final molar ratio of succinate to acetate for the small inoculum (starting OD of approximately 0.02), medium inoculum (starting OD of 2.4) and large inoculum (starting OD of 22.6) cultures were 9.5 mol/mol, 7.4 mol/mol and 8.8 mol/mol, respectively. This infers that more carbon flux is being directed for succinate production than acetate production when starting the culture with a small inoculum. The large inoculum probably caused the culture to have uncoupled metabolism because of faster glucose consumption rate that results in higher acetate production (Doelle et al., 1981); this, therefore, caused lower succinate yield. Fast glucose consumption rate incites high acetate production because of the excess influx of carbon from glucose that the cells are unable to thoroughly utilize for biomass synthesis and energy requirements (Yang et al., 1999). Total glucose consumed for the small inoculum, medium inoculum and large inoculum cultures were 453.0 mM, 471.5 mM and 567.5 mM, respectively.

8.4.2 Fed Batch Fermentation under Aerobic Conditions
The results of the comparison of the three different size inoculum cultures of strain HL27659k(pKK313) show that the larger the inoculum, the lower the succinate yield. Thus for the aerobic fed batch experiment of strain HL27659k(pKK313), a small inoculum size (starting OD of approximately 0.02) was chosen. The experiment started first with batch growth for 14 hrs, and then it was switched to fed batch growth. At the time of transition, the OD was 30.1. The total fermentation time was 59 hrs. The fed batch experiment was performed three times and the results showed that the high level succinate production could be reproduced. Only one set of data is presented.

The results for the fed batch experiment are shown in Figure 8-3. By the end of the fermentation, 493.6 mM of succinate was produced in 59 hrs, equivalent to 58.3 g/l of succinate. Total glucose consumed was 583.2 mM. The accumulative succinate yield at end of fermentation was approximately 0.85 mol/mol glucose. Succinate yield was maintained at 1.0 mol/mol glucose for the first 26 hrs before it started decreasing. The acetate produced after 59 hrs was 50.0 mM. The molar ratio of succinate to acetate was 9.9 mol/mol. Interestingly, 69.3 mM of pyruvate was produced after 59 hrs as a result of fed batch. No pyruvate was detected under batch conditions. The final OD after 59 hrs was 35.0 (not shown in Figure 8-3).

Throughout the whole fermentation, the average succinate yield was 0.94 ± 0.07 mol/mol glucose, the average productivity was 1.08 ± 0.06 g/l-hr, and the average specific productivity was 89.77 ± 3.40 mg/g-hr. Accounting for only the fed batch phase of the fermentation which started after 14 hrs, the average succinate yield was 0.90 ± 0.09 mol/mol glucose, the average productivity was 1.29 ± 0.19 g/l-hr, and the average specific productivity was 105.80 ± 15.65 mg/g-hr.
Figure 8-3: Aerobic fed batch reactor experiment of strain HL27659k(pKK313). Glucose (mM) (♦), succinate (mM) (■), pyruvate (mM) (●), acetate (mM) (▲), and succinate yield (mol/mol glucose) (□) are shown.

The results of the fed batch show that this aerobic production system is highly efficient for succinate production. It also requires only 1% (v/v) inoculum from an overnight culture to initiate the whole fermentation process (starting culture OD is approximately 0.02). A high level of biomass can be generated rapidly from the aerobic environment. This fast growth rate is not possible with anaerobic conditions; thus, generation of high biomass is often a major constraint of anaerobic conditions.

8.5 Discussion

The aerobic succinate production system developed by Lin et al. (2005b) was further studied for its capability to achieve a high concentration of succinate. This novel production system takes advantage of the aerobic environment, which provides faster cell growth, carbon throughput, and product formation than the anaerobic environment. Five
mutations (sdhAB, (ackA-pta), poxB, iclR, and ptsG) were strategically created in wildtype E. coli, and pepc was overexpressed. This mutant strain designated HL27659k(pKK313) represents the aerobic succinate production system.

Fed batch culture of HL27659k(pKK313) showed that it could produce 58.3 g/l of succinate in 59 hrs under aerobic conditions. Throughout the whole fermentation, the average succinate yield was 0.94 ± 0.07 mol/mol glucose, the average productivity was 1.08 ± 0.06 g/l-hr, and the average specific productivity was 89.77 ± 3.40 mg/g-hr.

The results of the fed batch experiment of strain HL27659k(pKK313) show that substantial succinate production can be achieved under aerobic conditions. More importantly, this is the first time that succinate can be produced at such a high level under aerobic conditions. This aerobic succinate production system is also more practical than anaerobic production systems. Anaerobic succinate production typically requires separation of the growth phase from the production phase. The growth phase is carried out under aerobic conditions for generating high biomass, and then the atmosphere is switched to anaerobic conditions for producing succinate (Vemuri et al., 2002a, 2002b; Ngkiem et al., 1999). The aerobic succinate production system, thus, would not require this procedure since its fermentation is carried out only under aerobic conditions. The aerobic succinate production system of strain HL27659k(pKK313) is a practical, efficient and viable alternative to conventional anaerobic succinate production systems. It is promising for industrial large-scale succinate production and process optimization.

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Chapter 9

9. Chemostat culture characterization of *Escherichia 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

* This chapter has been submitted to *Metabolic Engineering* for publication (Lin et al., 2005e).

9.1 Abstract

Various *E. coli* mutant strains designed for succinate production under aerobic conditions were characterized in chemostat. The metabolite profiles, enzyme activities, and gene expression profiles were studied to better understand the metabolic network operating in these mutant strains. The most efficient succinate producing mutant strain HL27659k was able to achieve a succinate yield of 0.91 mol/mol glucose at a dilution rate of 0.1 hr⁻¹. This strain has the five following mutations: *sdhAB*, *(ackA-pta)*, *poxB*, *iclR*, and *ptsG*. Four other strains involved in this study were HL2765k, HL276k, HL2761k, and HL51276k. Strain HL2765k has mutations in *sdhAB*, *(ackA-pta)*, *poxB* and *iclR*, strain HL276k has mutations in *sdhAB*, *(ackA-pta)* and *poxB*, strain HL2761k has mutations in *sdhAB*, *(ackA-pta)*, *poxB* and *icd*, and strain HL51276k has mutations in *iclR*, *icd*, *sdhAB*, *(ackA-pta)* and *poxB*. Enzyme activity data showed strain HL27659k has substantially higher citrate synthase and malate dehydrogenase activities than the other four strains. The data also showed that only *iclR* mutation strains exhibited
isocitrate lyase and malate synthase activities. Gene expression profiles also complemented the studies of enzyme activity and metabolites from chemostat cultures. The results showed that the succinate synthesis pathways engineered in strain HL27659k were highly efficient, yielding succinate as the only major product produced under aerobic conditions. Strain HL27659k was the only strain without pyruvate accumulation, and its acetate production was the least among all the mutant strain examined.

9.2 Introduction

The production of succinate has been an area of recent interest due to its value as a precursor to various commodity chemicals used in industries like food, pharmaceutical, detergent, and polymer (Zeikus et al., 1980, 1999). Metabolic engineering to enhance succinate production in bacteria has the potential to significantly improve the economics of the succinate market, especially when coupled with the use of renewable carbohydrates (Schilling, 1995). Examples include the high yield production of succinic acid from wood hydrolysate, whey, or glycerol by Anaerobiospirillum succiniciproducens through improvement of its fermentation conditions (Lee et al., 2000, 2001, 2003b; Samuelov et al., 1999). Other organisms that also have the innate capability for high yield succinic acid production include the facultative anaerobe Actinobacillus succinogenes (Van der Werf et al., 1997), and the organism from bovine rumen Mannheimia succiniciproducens, which had been shown to be able to produce succinic acid from whey and corn steep liquor (Lee et al., 2002, 2003a).

Various metabolic engineering strategies have been applied to improve succinate production in Escherichia coli (Chatterjee et al., 2001; Gokarn et al., 1998, 2000, 2001;
Goldberg et al., 1983; Hong and Lee, 2001; Sánchez et al., 2004a b; Stols and Donnelly, 1997; Vemuri et al., 2002a; Wang et al., 1998). Because E. coli, naturally, only produces succinate under anaerobic conditions in minimal quantities (Clark, 1989), numerous genetic modifications have been performed on E. coli to enhance succinate production. Genetic engineering coupled with optimization of production conditions has shown promising results for large-scale production of succinate from E. coli. This makes succinate production in E. coli. competitive with that of other organisms like Anaerobiospirillum succiniciproducens. A genetically improved E. coli mutant strain (AFP111/pTrc99A-pyc) grown anaerobically in optimized fed batch conditions was shown to achieve succinate production of 99.2 g/L with yield of 110% and productivity of 1.3 g/L h (Vemuri et al., 2002b). Because of anaerobic fermentation process disadvantages that include poor biomass generation, slow carbon throughput, and, therefore, slow product formation, E. coli was also genetically engineered to produce succinate under aerobic conditions (Lin et al., 2005a b). Aerobic fed batch culture of a genetically modified E. coli mutant strain (HL27659k(pKK313)) was shown to produce 58.3 g/L of succinate with yield of 94% and productivity of 1.1 g/L h (Lin et al., 2005c).

In this study, several E. coli mutant strains constructed by Lin et al. during the development of aerobic succinate production systems were selected for characterization to further understand their metabolic functions, as a result of multiple pathway inactivations. This is important for understanding how the pathway manipulations in aerobic central metabolism affected the metabolic network and enabled aerobic succinate production in E. coli. These mutant strains were characterized in chemostat culture for their metabolite profiles, enzyme activities, and gene expression profiles. Enzyme
activities and gene expression patterns were examined for those involved in pathways that affect the aerobic succinate production system. Combining gene expression profiles with enzyme activity and metabolite profiles provides a holistic approach for better understanding the connections between genes, proteins, and metabolites.

The mutations in the mutant strains were strategically created during the development process to enable aerobic succinate production in *E. coli*. Based on pathway modeling and optimization, the maximum theoretical succinate yield that can be achieved in *E. coli* under aerobic conditions is 1.0 mole of succinate per mole of glucose consumed (Lin et al., 2005a b). Following the design for optimal succinate production under aerobic conditions, genetic manipulation was carried out in *E. coli*. As a result, several engineered *E. coli* mutant strains were able to achieve the maximum theoretical succinate yield of 1.0 mol/mol glucose under aerobic conditions in batch reactors (Lin et al., 2005b). Although the maximum theoretical succinate yield was achieved by these strains, they still varied significantly in succinate productivity (Lin et al., 2005b). One of the mutant *E. coli* strains, though, exhibited significantly higher succinate productivity than the other strains, while still reaching the maximum succinate yield of 1.0 mol/mol glucose. This strain, HL27659k(pKK313), mentioned earlier was shown to have high capacity for succinate production in aerobic fed batch cultures. Before strain HL27659k(pKK313) was developed, this level of succinate production under aerobic conditions was never possible in *E. coli*.

Strain HL27659k(pKK313) is the most efficient and optimal *E. coli* strain created thus far for aerobic succinate production. Plasmid pKK313 provides the overexpression of a mutant *Sorghum vulgare* phosphoenolpyruvate carboxylase (PEPC) to enhance
carbon flux to oxaloacetate (OAA) for the succinate synthesis pathways. Background strain HL27659k has five mutations created in genes encoding enzymes of aerobic central metabolism. These five mutations are *sdhAB*, inactivation of succinate dehydrogenase, (*ackA-pta*), inactivation of acetate kinase-phosphotransacetylase, *poxB*, inactivation of pyruvate oxidase, *iclR*, inactivation of *aceBAK* operon repressor, and *ptsG*, inactivation of glucose phosphotransferase system (Figure 9-1). Naturally, under aerobic conditions, succinate is only an intermediate of the tricarboxylic acid (TCA) cycle and it is formed by succinyl-CoA synthetase and subsequently oxidized to fumarate by succinate dehydrogenase (SDH). Because of this, succinate is never detected in aerobic cultures of *E. coli*. Inactivation of SDH is, therefore, essential for accumulation of succinate under aerobic conditions. By inactivating SDH, the TCA cycle becomes branched with the oxidative branch capable of producing succinate as a product under aerobic conditions. Inactivation of the two acetate producing pathways *ackA-pta* and *poxB* increases the carbon flux toward the branched TCA cycle for succinate production. Inactivation of the *aceBAK* operon repressor (*iclR*) activates the glyoxylate bypass for succinate production (Gui *et al.*, 1996). The glyoxylate bypass consists of two steps. The first step is carried out by isocitrate lyase (ICL), which converts isocitrate (C₆) to succinate (C₄) and glyoxylate (C₂). The second step is carried out by malate synthase (MS), which condenses acetyl-CoA (C₂) with glyoxylate (C₂) to form malate (C₄). With the inactivation of *iclR* and *sdhAB*, two routes are created for aerobic succinate production. One route is the oxidative branch of the TCA cycle and the second route is the glyoxylate cycle. Inactivation of *ptsG* increases the phosphoenolpyruvate (PEP) pool for succinate
synthesis, since glucose transport would no longer require the conversion of PEP to pyruvate for phosphorylation. This would also improve the balanced carbon metabolism

**Figure 9-1:** Genetic engineering of glycolysis, TCA cycle, and glyoxylate bypass in the development of aerobic succinate production systems (Lin et al., 2005b). 1 is icd knockout, 2 is sdhAB knockout, 5 is iclR knockout, 6 is poxB knockout, 7 is ackA-pta knockout, and 9 is ptsG knockout.
by slowing down the glucose uptake, since acetate is formed when there is excess influx of glucose that the cell is unable to utilize for biomass synthesis (Doelle et al., 1981; Yang et al., 1999). The five mutations in strain HL27659k (Figure 9-2) together create an aerobic succinate production platform that can produce substantial levels of succinate with high productivity.

Another strain that was also shown to reach the maximum theoretical succinate yield of 1.0 mol/mol glucose under aerobic conditions in batch reactor was strain HL51276k(pKK313) (Lin et al., 2005b). In batch cultures, strain HL51276k(pKK313) and strain HL27659k(pKK313) both achieved the succinate yield of 1.0 mol/mol glucose, but strain HL51276k(pKK313) had much lower productivity than strain HL27659k(pKK313) (Lin et al., 2005b). The background strain HL51276k has five mutations, \textit{sdhAB}, \textit{(ackA-pta)}, \textit{poxB}, \textit{iclR}, and \textit{icd}. It differs from strain HL27659k in that it only has the glyoxylate cycle for succinate production (Figure 9-3). Because of the inactivation of isocitrate dehydrogenase (\textit{icd}), the oxidative branch of the TCA cycle is not active for succinate production. The TCA cycle of strain HL51276k, in essence, is reduced to the glyoxylate cycle.

Certain mutant strains constructed during the process of developing an aerobic succinate production system in \textit{E. coli} were selected for chemostat characterization. The most prominent mutant strain created for aerobic succinate production is strain HL27659k. Therefore, this strain was selected for further study in chemostat cultures. Four additional mutant strains were selected to complement strain HL27659k in understanding how the genetic manipulations affected the metabolic functions among the related strains. One mutant strain is HL2765k (\textit{sdhAB}, \textit{(ackA-pta)}, \textit{poxB}, and \textit{iclR}), has
Figure 9-2: Aerobic succinate production platform, the two-route system with the glyoxylate cycle and the oxidative branch of the TCA cycle (mutant strain HL27659k).
Figure 9-3: Aerobic succinate production platform, the glyoxylate cycle (mutant strain HL51276k) (Lin et al., 2005b).
the same mutations as strain HL27659k, except for \(\text{ptsG}\). Strain HL2765k will provide
the basis for studying the effect of \(\text{ptsG}\) inactivation on the glucose metabolism in strain
HL27659k. Strain HL276k \((\text{sdhAB, (ackA-pta), poxB})\), has three mutations, serves as the
base control for strains HL2765k and HL27659k. Strain HL276k does not have the \(\text{iclR}\)
inactivated; therefore, the glyoxylate bypass operon is not constitutively expressed.
Strain HL276k can produce succinate only from the oxidative branch of the TCA cycle.
Another mutant strain selected for study, described earlier, is strain HL51276k \((\text{iclR, icd,}
\text{sdhAB, (ackA-pta), and poxB})\). The TCA cycle in this strain has been reconstructed to
become just the glyoxylate cycle. Succinate produced by this strain should thus originate
only from the ICL pathway of the glyoxylate cycle. Strain HL51276k provides an
interesting comparison with strain HL2765k, which has both the glyoxylate cycle and the
oxidative branch of the TCA cycle active for succinate production. The last mutant strain
selected for characterization is strain HL2761k \((\text{sdhAB, (ackA-pta), poxB, icd})\). This
strain does not have either the glyoxylate cycle or the oxidative branch of the TCA cycle
active for succinate production. An \(\text{icd}\) mutation in the strain HL2761k inactivates the
TCA cycle, and without a mutation in the \(\text{iclR}\), there is no bypass for the carbon flux
entering the TCA cycle. Strain HL2761k provides an interesting comparison with strain
HL51276k, since they differ in that strain HL51276k has an active glyoxylate cycle and
strain HL2761k does not. Strain HL276k also serves as the control for strains HL2761k
and HL51276k. Comparison of strain HL2761k with strain HL276k provides
understanding of the effect of \(\text{icd}\) inactivation on the carbon flux toward the branched
TCA cycle. The five mutant strains selected for study in chemostat culture, and their
genotypes are summarized in Table 9-1.
9.3 Materials and Methods

9.3.1 Strains

Mutations were created in the laboratory wildtype GJT001, a spontaneous cadR mutant of MC4100 (Tolentino \textit{et al.}, 1992). A library of mutant strains was created during the process of constructing the final mutant strains for aerobic succinate production (Table 9-2). Knockouts were created in succinate dehydrogenase (\textit{sdhAB}), pyruvate oxidase (\textit{poxB}), acetate kinase-phosphotransacetylase (\textit{ackA-pta}), isocitrate dehydrogenase (\textit{icd}), \textit{aceBAK} operon repressor (\textit{iclR}), and the glucose phosphotransferase system (\textit{ptsG}). Each one of these mutations is designated by a number (1-\textit{icd}, 2-\textit{sdhAB}, 5-\textit{iclR}, 6-\textit{poxB}, 7-(\textit{ackA-pta}), 9-\textit{ptsG}) used in the naming of the mutant strains (Figure 9-1). The kanamycin cassette was left in the final mutant strains to provide selective pressure during the fermentation experiments. The mutant strains selected for study in chemostat were HL27659k, HL2765k, HL276k, HL2761k, and HL51276k. Strain HL276k is the background control strain for all the other strains.

9.3.2 Chemostat Culture Medium and Conditions

The medium used was Luria-Bertani broth (LB), 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl (Sambrook \textit{et al.}, 1989), added with 2 g/L NaHCO$_3$ and 20 g/L of glucose. The medium used for inoculum preparation was also the same medium, except glucose was not added. NaHCO$_3$ was added to the culture medium because it promoted cell growth and succinate production due to its pH-buffering capacity and its
Table 9-1: List of mutant strains studied in chemostat and their mutations. *Background control strain for all the other strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>sdhAB</th>
<th>ackA-pta</th>
<th>poxB</th>
<th>iclR</th>
<th>icd</th>
<th>ptsG</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL27659k</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL2765k</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>HL276k*</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL2761k</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>HL51276k</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 9-2: Library of mutant strains constructed and studied.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GJT001</td>
<td>Spontaneous cadR mutant of MC4100(ATCC35695) Δlac(lac–lac)U169ptsL150relA1ptsF SmR</td>
<td>Tolentino et al., (1992)</td>
</tr>
<tr>
<td>HL2k</td>
<td>GJT001(sdhAB::KmR)</td>
<td>Lin et al. (2005a)</td>
</tr>
<tr>
<td>HL27k</td>
<td>GJT001(sdhAB, ackA-pta::KmR)</td>
<td>Lin et al. (2005a)</td>
</tr>
<tr>
<td>HL276k</td>
<td>GJT001(sdhAB, (ackA-pta), poxB::KmR)</td>
<td>Lin et al. (2005a)</td>
</tr>
<tr>
<td>HL2761k</td>
<td>GJT001(sdhAB, (ackA-pta), poxB, icd::KmR)</td>
<td>Lin et al. (2005a)</td>
</tr>
<tr>
<td>HL2765k</td>
<td>GJT001(sdhAB, (ackA-pta), poxB, iclR::KmR)</td>
<td>Lin et al. (2005b)</td>
</tr>
<tr>
<td>HL27659k</td>
<td>GJT001(sdhAB, (ackA-pta), poxB, iclR, ptsG::KmR)</td>
<td>Lin et al. (2005b)</td>
</tr>
<tr>
<td>HL5k</td>
<td>GJT001(iclR::KmR)</td>
<td>Lin et al. (2005a)</td>
</tr>
<tr>
<td>HL51k</td>
<td>GJT001(iclR, icd::KmR)</td>
<td>Lin et al. (2005a)</td>
</tr>
<tr>
<td>HL512k</td>
<td>GJT001(iclR, icd, sdhAB::KmR)</td>
<td>Lin et al. (2005a)</td>
</tr>
<tr>
<td>HL5127k</td>
<td>GJT001(iclR, icd, sdhAB, (ackA-pta)::KmR)</td>
<td>Lin et al. (2005a)</td>
</tr>
<tr>
<td>HL51276k</td>
<td>GJT001(iclR, icd, sdhAB, (ackA-pta), poxB::KmR)</td>
<td>Lin et al. (2005a)</td>
</tr>
</tbody>
</table>
ability to supply CO₂. Kanamycin was added to the medium at a concentration of 50 mg/L.

Chemostat experiments were performed under aerobic conditions at a dilution rate of 0.1 hr⁻¹. This dilution rate was selected based on specific growth rates of the five mutant strains, obtained from log phase growth data of previous batch culture studies. The working volume was maintained at 600 mL in a 1.0-L New Brunswick Scientific Bioflo 110 fermenter. The pH was measured using a glass electrode and controlled at 7.0 using 1.5 N HNO₃ and 2 N Na₂CO₃. The temperature was maintained at 37°C, and the agitation speed was constant at 500 rpm. The inlet airflow used was 0.6 L/min. The dissolved oxygen was monitored using a polarographic oxygen electrode (New Brunswick Scientific) and was maintained above 50% saturation throughout the experiment. A 1% (v/v) inoculum from an overnight culture grown from a single colony for 12 hours was used to inoculate the bioreactor. The culture was allowed to grow in batch mode for 12 to 14 hours before the feed pump and waste pump were turned on to start the chemostat. The continuous culture reached steady state after 5 residence times. Optical density and metabolites were measured from samples at 5 and 6 residence times and then compared to ensure that steady state had been established. Actual samples used for obtaining the results presented were taken after 6 residence times.

9.3.3 Analytical Techniques

Optical density (OD) was measured at 600 nm with a spectrophotometer (Bausch & Lomb Spectronic 1001); the culture was diluted to the linear range with 0.15 M NaCl and the dilution factor was accounted in the calculation of OD. The measured OD was
correlated with dry cell weight. For analyzing the extracellular metabolites, 1 mL of culture was centrifuged at 8000g for 5 min and the supernatant was then filtered through a 0.2 μm PVDF membrane syringe filter and stored frozen at -20°C until analysis.

For analyzing intracellular metabolites and acetyl-CoA, 40 OD units (OD\textsubscript{660nm} vol (mL) = 40) of cell culture were taken into a precooled centrifuge tube, immediately chilled on ice, and centrifuged at 5000g at 4°C for 10 min. The pellet was cold washed and resuspended in 1 mL of cold 6% perchloric acid to lyse the cells and then placed on an ice bath for 10 min. Then 0.3 mL of cold 3 M potassium carbonate was added while vortexing to neutralize the acid. The solution was then centrifuged again at 4°C for 10 min and the supernatant was filtered through a 0.2 μm PVDF membrane syringe filter. The filtered supernatant was then stored at -80°C until analysis. Approximate intracellular concentrations relative to the cell volume were calculated based on the cell volume constant of 2.7 μL/mg dry weight (Winkler and Wilson, 1966).

The HPLC system (Shimadzu-10A Systems, Shimadzu, Columbia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A) and a differential refractive index (RI) detector (Waters 2410, Waters, Milford, MA). A 0.6 mL/min mobile phase using 2.5 mM H\textsubscript{2}SO\textsubscript{4} solution was applied to the column. The column was operated at 55 °C. Standards were prepared for glucose, succinate, acetate, pyruvate, and glyoxylate for both the RI detector and UV detector, and calibration curves were created. Glucose, succinate, acetate, and glyoxylate were measured by the RI detector and pyruvate was measured by the UV detector at 210 nm. Citrate, isocitrate, and malate were measured by enzyme assay kits obtained from R-Biopharm. All samples were measured three times for each metabolite.
The quantification of acetyl-CoA was based on a modified protocol of Boynton (Boynton et al., 1994) and used previously by others (Vadali et al., 2004; Lin et al., 2004). Acetyl-CoA was analyzed by HPLC (Thermofinnigan, San Jose, CA) using a UV detector set at 254 nm. The column used was a 5 μm octyldecyl silane column (Cell Technologies, Inc., Houston, TX) preceded by an Allsphere ODS-2 (C18) guard column (Alltech, Deerfield, IL). The column was operated at room temperature. Two mobile phases of buffer were used at a flow rate of 1 mL/min. One buffer was 0.2 M sodium phosphate (pH 5.0) and the other buffer was 800 mL of 0.25 M sodium phosphate (pH 5.0) mixed with 200 mL of 100% acetonitrile. The run profile was adopted from Boynton (Boynton et al., 1994). All samples were measured three times for acetyl-CoA.

9.3.4 Enzyme Assays

Crude extracts for all enzyme assays were prepared by taking 20 OD units of culture (OD_{660nm} * vol (mL) = 20) and centrifuging the appropriate volume at 5000g and 4°C for 20 min. The cell pellet was then washed once in 15 mL of the appropriate buffer for each type of enzyme assay. The pellet was then centrifuged again and resuspended in 10 mL of that buffer. The cells were then subjected to sonication for 10 min in an ice bath. The sonicated cells were centrifuged at 5000g and 4°C for 60 min to remove cell debris. The supernatant was then used for the enzyme assay. Total protein concentration of the crude extract was measured by Lowry’s method (Sigma Lowry Reagent, Modified) using bovine serum albumin as standard. All enzyme activities were expressed as U/mg, μmol of substrate converted to product per min per mg protein. All enzyme assays for each sample were performed in triplicate.
Citrate synthase activity was measured by a modified method of Aoshima et al., 2003. Citrate synthase produces citrate and CoA from the condensation of acetyl-CoA and oxaloacetate. DTNB, 5,5-dithiobis(2-nitrobenzoic acid), was added to react with the sulfhydryl group of the CoA, which could be measured at 412 nm. The molar extinction coefficient used was 13.6 mM\(^{-1}\) cm\(^{-1}\). The rate of increase in absorbance was used to calculate citrate synthase activity.

Malate dehydrogenase was measured by a modified method of Zeikus et al., 1977. Malate dehydrogenase reduces oxaloacetate to malate through the oxidation of NADH to NAD\(^+\). This reaction was measured at 340 nm and the molar extinction coefficient used was 6.22 mM\(^{-1}\) cm\(^{-1}\). The rate of decrease in absorbance was used to calculate malate dehydrogenase activity.

Phosphoenolpyruvate carboxylase was measured by a modified method of Terada et al., 1991. This assay is a coupled enzyme assay with malate dehydrogenase. Phosphoenolpyruvate carboxylase converts phosphoenolpyruvate to oxaloacetate through a carboxylation reaction. Malate dehydrogenase then reduces oxaloacetate to malate with the oxidation of NADH. The rate of decrease in absorbance was used to calculate phosphoenolpyruvate carboxylase activity.

Isocitrate dehydrogenase was measured by a modified method of Soundar et al., 1996. Isocitrate dehydrogenase oxidizes isocitrate to 2-ketoglutarate with a concomitant release of CO\(_2\) and reduction of NADP\(^+\) to NADPH. The reaction was measured at 340 nm and the molar extinction coefficient used was 6.22 mM\(^{-1}\) cm\(^{-1}\). The rate of increase in absorbance was used to calculate isocitrate dehydrogenase activity.
Malate synthase was measured by the method of de Jong-Gubbels et al., 1995. The assay is a coupled enzyme assay in which the malate formed by malate synthase is oxidized to oxaloacetate by malate dehydrogenase with the concomitant reduction of \( \text{NAD}^+ \) to NADH. The reaction was measured at 340 nm and the molar extinction coefficient used was 6.22 mM\(^{-1}\) cm\(^{-1}\). The rate of increase in absorbance was used to calculate malate synthase activity.

Isocitrate lyase was measured by a modified method of Dixon and Kornberg, 1959. Isocitrate lyase catalyzes the hydrolysis of isocitrate into glyoxylate and succinate. The glyoxylate formed in the presence of phenylhydrazine was measured as glyoxylic acid phenylhydrazone at 324 nm. The molar extinction coefficient used was 17 mM\(^{-1}\) cm\(^{-1}\). The rate of increase in absorbance in the linear range was used to calculate isocitrate lyase activity.

9.3.5 Gene Expression Analysis

9.3.5.1 RNA Preparation

Total RNA was isolated with the Promega SV Total RNA Isolation System (Promega Corporation, Madison, WI) according to the manufacturer’s protocol. The isolated RNA was then treated again with DNase (Promega) and RNase Inhibitor (Promega). The reaction was incubated at 37°C for 30 min. The RNA was then extracted with phenol once, phenol/chloroform (50:50) once, and then chloroform twice. The RNA was then precipitated with ethanol and resuspended in RNase-free H\(_2\)O (DEPC treated). The RNase-free H\(_2\)O (DEPC treated) was prepared by adding DEPC (1\(\mu\)L/mL) to
distilled water, then storing it overnight at 37°C, and then autoclaving it. The concentration of RNA was quantified by measuring the absorbance at 260 nm and applying the formula, concentration (μg/mL) = A260 * 40 * dilution factor. The purity of the RNA was determined by reading the absorbance at 260 nm and 280 nm. RNA samples used were ensured to have the A260/A280 ratio between 1.8-2.1 for clean RNA.

9.3.5.2 cDNA Synthesis and Quantitative Real-time PCR

Quantitative real-time RT-PCR was performed in a two-step process. The first step is the cDNA synthesis, and the second step is the quantitative real-time PCR using the cDNA synthesized. cDNA was synthesized by reverse transcription using the RNA prepared as template. This was performed by using the Promega Reverse Transcription System (Promega, Madison, WI) and the reaction was carried out in a RoboCycler Gradient 96 (Stratagene, La Jolla, CA). The cDNA was synthesized in a total reaction mixture volume of 60 μL containing 1 μg of RNA template. The reaction mixture was incubated for 10 min at room temperature for primer extension, 30 min at 50°C for reverse transcription, and then 5 min at 95°C and 10 min at 6°C for inactivation of the reverse transcriptase. Control samples of the cDNA were also prepared following the same protocol, except reverse transcriptase was not added to the reaction mixture. The single-stranded cDNA sample was then diluted into the working range with nuclease free H2O and stored at -20°C until further use.

Quantitative real-time PCR was performed in a ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Direct detection of PCR product is
monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA (SYBR Green PCR Master Mix and RT-PCR Protocol, Applied Biosystems). Forward and reverse primers were designed for each gene studied. The list of genes studied and primers designed is shown in Table 9-3. Reactions were carried out in a 96-well plate using the cDNA prepared as template. Each well contains a reaction mixture consisting of cDNA, forward and reverse primers, SYBR Green PCR Master Mix, and nuclease free water. PCR reaction was performed three times under identical reaction conditions for every gene using a particular strain’s cDNA in order to calculate a standard deviation; reaction using the control cDNA sample of that strain was also performed concurrently to ensure that there was no contamination.

Following the manufacturer’s protocol, the comparative $C_T$ method for relative quantification of gene expression was used (ABI Prism 7700 Sequence Detection System User Bulletin #2, Applied Biosystems). The threshold cycle, $C_T$, was the data obtained. The $rrsA$ gene encoding rrnA 16S ribosomal RNA was used as an endogenous control in order to standardize the amount of sample DNA added to a reaction. This gene is not subjected to variable expression because its expression is abundant and relatively constant in most cells. In addition, since ribosome level varies with cell growth rate, the chemostat culture of $\mu=0.1 \, \text{hr}^{-1}$ should maintain a steady level of this expression between strains. The difference between the $C_T$ of the studied gene and the $rrsA$ gene was calculated ($\Delta C_T$) and a sensitivity test to confirm that the $\Delta C_T$ was independent of the RNA concentration was performed for each gene. The $\Delta \Delta C_T$ was then calculated by taking the difference between the $\Delta C_T$ of a gene in one strain and the $\Delta C_T$ of the same gene in the control strain. The relative expression of a particular gene between a strain
Table 9-3: List of genes studied and primers designed for quantitative PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>aceA</td>
<td>Forward: 5'-ATCTGATCACCCTCGGATTG-3' &lt;br&gt; Reverse: 5'-CACAGACCAAGGTCAGATA-3'</td>
</tr>
<tr>
<td>aceB</td>
<td>Forward: 5'-TCTGAGTGAGCTGGTCGACG-3' &lt;br&gt; Reverse: 5'-GCGAATTCTCAATACGAT-3'</td>
</tr>
<tr>
<td>aceE</td>
<td>Forward: 5'-ACGCAGGCTGACGACTAC-3' &lt;br&gt; Reverse: 5'-CTTATCGATTTGCCACGTT-3'</td>
</tr>
<tr>
<td>aceF</td>
<td>Forward: 5'-AAGTGACCAGAAATCCTGGTG-3' &lt;br&gt; Reverse: 5'-CATTGTGACCCACGTTC-3'</td>
</tr>
<tr>
<td>gltA</td>
<td>Forward: 5'-ATGATTCTTTTCCGTCATG-3' &lt;br&gt; Reverse: 5'-TTCCAGCTCCATAGCCACTT-3'</td>
</tr>
<tr>
<td>icd</td>
<td>Forward: 5'-TCCTGAAAAACTCGGAAGAC-3' &lt;br&gt; Reverse: 5'-TCCGAACACGGCTTAATACC-3'</td>
</tr>
<tr>
<td>mdh</td>
<td>Forward: 5'-GGCGTATTATACCTGACG-3' &lt;br&gt; Reverse: 5'-GTGACGAAACAGACGACG-3'</td>
</tr>
<tr>
<td>ppc</td>
<td>Forward: 5'-GGTCCGGTTATCTTCTGAG-3' &lt;br&gt; Reverse: 5'-CGACAGTCCGAAACCGACG-3'</td>
</tr>
<tr>
<td>ptsG</td>
<td>Forward: 5'-AGTGCCGTAAAATCGCTGAT-3' &lt;br&gt; Reverse: 5'-ATGCTTTCGAAAGACGACC-3'</td>
</tr>
<tr>
<td>pykA</td>
<td>Forward: 5'-ACTGACGCTGTGATGCTGTC-3' &lt;br&gt; Reverse: 5'-CCACATTTGCGAATGACG-3'</td>
</tr>
<tr>
<td>pykF</td>
<td>Forward: 5'-TATCCGTCAGCTCAAGCTG-3' &lt;br&gt; Reverse: 5'-TTACCTTTTGCCGATTCACC-3'</td>
</tr>
<tr>
<td>rrsA</td>
<td>Forward: 5'-CTGCTGTCAGCTGTTGTG-3' &lt;br&gt; Reverse: 5'-AGGGCCATGATGACTTG-3'</td>
</tr>
</tbody>
</table>
and its control strain is given by the formula $2^{-\Delta\Delta T}$. Using this method, it is important to emphasize that the expression levels of different genes cannot be compared. Expression levels can only be compared between different strains for the same gene, since the primers in those reactions would be the same.

9.4 Results and Discussion

9.4.1 Comparison of Metabolite Profiles

The production of succinate, pyruvate, and acetate under aerobic conditions was compared between the five mutant strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k in chemostat cultures at 0.1 hr$^{-1}$ dilution rate. Succinate production is the focus of this study, since it is the valuable product that *E. coli* was genetically engineered to produce under aerobic conditions. The biomass concentrations of the five mutant strains were HL27659k (3.4 g/L), HL2765k (3.6 g/L), HL276k (3.4 g/L), HL2761k (2.4 g/L) and HL51276k (2.6 g/L). Succinate production reached substantial levels that were similar in strains HL27659k, HL2765k, and HL276k (Figure 9-4b). Strain HL27659k produced 57 mM of succinate, strain HL2765k produced 61 mM, and strain HL276k produced 58 mM. Succinate production was significantly lower for strains HL2761k and HL51276k than the other three strains (Figure 9-4b). Strain HL2761k only produced 2 mM of succinate and strain HL51276k produced only 3 mM of succinate. Strain HL27659k achieved the highest succinate yield among all the strains. It obtained 0.91 mole succinate per mole glucose (Figure 9-5a). This is 91% of the maximum theoretical succinate yield, which is 1.0 mol/mol glucose under aerobic conditions. Strains
Figure 9-4: Glucose consumed and extracellular metabolite production by strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k.
HL2765k, HL276k, HL2761k, and HL51276k reached succinate yields of 0.74, 0.73, 0.02 and 0.03, respectively (Figure 9-5a).

The results obtained for strains HL27659k and HL51276k in chemostat cultures are different from results obtained in batch reactor cultures. In chemostat culture, strain HL27659k achieved significantly higher succinate yield than in batch culture (0.91 mol/mol yield compared to 0.78 mol/mol yield (Lin et al., 2005b)). Strain HL51276k achieved significantly lower succinate yield in chemostat culture than in batch culture (0.03 mol/mol yield compared to 0.65 mol/mol yield (Lin et al., 2005b)). A possible explanation why strain HL51276k did not obtain similar succinate yield in batch culture and chemostat culture is that the set specific growth rate of 0.1 hr\(^{-1}\) did not allow the culture enough growth time to have complete carbon-throughput through the glyoxylate cycle. Instead, a majority of the metabolized glucose accumulated as pyruvate for strain HL51276k. Strain HL51276k produced 151 mM of pyruvate with a yield of 1.38 mol/mol glucose (Figures 9-4c and 9-5c). The acetate produced was 14 mM with a yield of 0.13 mol/mol glucose; this is higher than any of the other four strains (Figures 9-4d and 9-5b). Strain HL2761k also exhibited the same metabolite production characteristics as strain HL51276k. It produced 146 mM of pyruvate, yield of 1.35 mol/mol glucose, and 12 mM of acetate, yield of 0.11 mol/mol glucose (Figures 9-4c, 9-4d, 9-5b, and 9-5c). Both strains HL51276k and HL2761k produce pyruvate as their major product. The similarity can be explained by the icd inactivation in both strains, rendering the TCA cycle inactive at a specific growth rate of 0.1 hr\(^{-1}\). Inactivation of icd severely hampered succinate production and caused pyruvate to become the major product in chemostat cultures.
Figure 9-5: Succinate, acetate, and pyruvate yields of strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k. Yield is mole of product produced per mole of glucose consumed.
Strains HL2765k and HL276k produced 67 mM and 74 mM of pyruvate, respectively, at yields of 0.80 mol/mol glucose and 0.93 mol/mol glucose, respectively (Figures 9-4c and 9-5c). Strains HL2765k and HL276k, precursors to strain HL27659k, accumulated significant amounts of pyruvate, which decreased their succinate yields. Strain HL27659k did not produce any pyruvate (Figure 9-4c) and its acetate production was the lowest among all the strains (Figure 9-4d). Succinate was the only major product of strain HL27659k. This demonstrates the efficiency of the pathway design in strain HL27659k in allowing a majority of the carbon flux to be channeled toward the succinate pathways.

The inactivation of ptsG was pivotal in improving carbon-throughput and succinate yield in strain HL27659k in chemostat cultures. Once ptsG was disrupted in strain HL2765k, pyruvate accumulation decreased from 67 mM to 0 mM (Figure 9-4c). Succinate yield also increased from 0.73 to 0.91 mol/mol glucose, allowing strain HL27659k to obtain the highest succinate yield among the five mutant strains (Figure 9-5a). There was also minimal acetate production, and no pyruvate accumulation. The inactivation of ptsG did decrease the amount of glucose consumed as shown by Figure 9-4a. Strain HL27659k consumed 64 mM of glucose where its precursor strain HL2765k consumed 85 mM of glucose. Presumably, the lower glucose consumption allowed more balanced glucose metabolism and more efficient carbon-throughput. It is interesting to find that strains HL2761k and HL51276k consumed the most amount of glucose (110 and 111 mM, respectively). Since these two strains have inactivation in icd that inhibits carbon flux through the oxidation pathways of the TCA cycle, it would be logical to assume that their glucose consumption would be suppressed. On the contrary, glucose
consumption in strains HL2761k and HL51276k was higher than the glucose consumption in strains HL276k, HL2765k, and HL27659k.

Citrate, isocitrate, glyoxylate and malate were also measured in the cultures of the five mutant strains, but, interestingly, none of these metabolites was detected. Strains HL51276k and HL2761k were previously observed to accumulate citrate and isocitrate when grown in the batch reactor mode (Lin et al., 2005b). Apparently, growth of these two strains in chemostat at 0.1 hr\(^{-1}\) dilution rate caused pyruvate to be accumulated, instead, as the major product. At this dilution rate, the TCA cycle of strains HL51276k and HL2761k does not contribute to glucose metabolism.

The results of specific glucose consumption rate and specific productivities of succinate, pyruvate, and acetate correspond well with the results of the metabolite profiles and yields. Strain HL27659k has the lowest specific glucose consumption rate among all five mutant strains, since it is inactivated in \(ptsG\) (Figure 9-6a). The specific succinate productivity of strain HL27659k was equivalent to the specific succinate productivities of strains HL2765k and HL276k (Figure 9-6b). This is because although strain HL27659k obtained a higher succinate yield than the other two strains, its specific glucose consumption rate was lower. Specific pyruvate and acetate productivities follow the same trend as pyruvate and acetate production for all five mutant strains (Figures 9-6c and 9-6d).

Figure 9-7 shows the results of intracellular metabolite molar concentrations in the five mutant strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k. All intracellular metabolite concentrations are higher than their respective extracellular metabolite concentrations, associated with concentration gradients required for transport.
Figure 9-6: Specific glucose consumption rate and specific metabolite productivities of strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k. Specific rate is mmole of metabolite per gram of biomass per hour time.
**Figure 9-7**: Intracellular metabolite concentrations of strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k. Intracellular metabolite concentrations are relative to cell volume.
Strains HL27659k, HL2765k, and HL276k retained higher intracellular succinate concentrations than strains HL2761k and HL51276k (Figure 9-7a). This is because substantially more succinate was produced and excreted by strains HL27659k, HL2765k, and HL276k than strains HL2761k and HL51276k (Figure 9-4b). Perhaps, engineering an active succinate transport system in these mutant strains could increase the extracellular level of succinate and reduce the amount accumulated intracellularly. Intracellular pyruvate concentrations were the highest in strains HL2761k and HL51276k, in accordance with their large accumulation of extracellular pyruvate (Figures 9-7b and 9-4c). Strain HL27659k not only did not excrete any pyruvate, but also there was no detected intracellular pyruvate accumulation (Figures 9-4c and 9-7b). Intracellular acetate concentrations follow the same trend as extracellular acetate concentrations for all mutant strains, except strain HL27659k (Figures 9-7c and 9-4d). Strain HL27659k did not accumulate any intracellular acetate. The intracellular acetyl-CoA concentration results did not present any significant comparisons between the five mutant strains (Figure 9-7d). The levels of intracellular acetyl-CoA concentration were similar for all five strains.

9.4.2 Comparison of Enzyme Activities

Crucial enzyme activities of TCA cycle pathways involved in the design of the aerobic succinate production system were measured and examined for the mutant strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k. Examining the enzyme activities can provide a better understanding of how each mutant strain’s metabolite profile is correlated with its metabolic pathways. The following pathways relevant to
succinate synthesis through the TCA cycle were examined: citrate synthase (CS), isocitrate dehydrogenase (ICDH), isocitrate lyase (ICL), malate synthase (MS), malate dehydrogenase (MDH), and phosphoenolpyruvate carboxylase (PPC).

Strain HL27659k, the most efficient and highest yield succinate producing mutant strain, has significantly more CS activity than the other four mutant strains (Figure 9-8a). Strain HL27659k has approximately five-fold higher CS activity than strains HL2765k and HL276k. This result shows that inactivation of \textit{ptsG} may be significantly related to the increase in CS activity in strain HL27659k. The high CS activity can explain why succinate is so efficiently produced by strain HL27659k and there is no pyruvate accumulation in strain HL27659k. CS drives carbon flux toward the succinate synthesis pathways of the TCA cycle thus reduces the amount of carbon accumulated at the pyruvate node (Figure 9-2). Strains HL2765k and HL276k, although they accumulate pyruvate, can produce substantially more succinate than strains HL2761k and HL51276k. This can also be attributed to the CS activities of strains HL2765k and HL276k being approximately twice that of strains HL2761k and HL51276k. Inactivation of \textit{icd} seems to result in a decrease in CS activity as shown by the lower CS activity of strain HL2761k compared to strain HL276k.

No ICDH activities were measured in strains HL2761k and HL51276k, as expected, since the \textit{icd} was disrupted in both strains (Figure 9-8b). Strains HL2765k and HL276k both showed similar ICDH activities, which were two-fold higher than that of strain HL27659k. This implies that the oxidative branch of the TCA cycle is more active in strains HL2765k and HL276k than in strain HL27659k. The inactivation of \textit{ptsG} may be related to the decrease in ICDH activity in strain HL27659k.
Figure 9-8: Specific enzyme activities of strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k. Specific enzyme activities were expressed in U/mg, which is μmol of substrate converted to product per min per mg protein.
Strains HL27659k, HL2765k, and HL51276k all have ICL and MS activities because *iclR* was inactivated in these strains (Figures 9-8c and 9-8d). This shows that the disruption of *iclR* induces expression of the *aceA* and *aceB* for ICL and MS, respectively, when the culture is grown on glucose. This was also shown by Gui *et al.*, 1996. Strains HL276k and HL2761k, which do not have the *iclR* mutation, show no ICL and MS activities. The ICL and MS activities do vary among strains HL27659k, HL2765k, and HL51276k. Strains HL27659k and HL2765k both have higher ICL and MS activities than strain HL51276k. Strain HL27659k has lower ICL activity than strain HL2765k, but equivalent MS activity with strain HL2765k.

Strain HL27659k has the highest MDH activity among all five mutant strains (Figure 9-8e). This may infer that strain HL27659k has a more efficient glyoxylate cycle for succinate production than the other two mutant strains HL2765k and HL51276k with glyoxylate cycle activity. This is because MDH is required to oxidize the malate produced from MS back to OAA (Figure 9-1). The OAA can then be utilized by CS to continuously drive carbon flux through the glyoxylate cycle. Considering only strains with glyoxylate cycle activity, strain HL27659k has approximately twice the MDH activity of strain HL2765k and approximately eleven-fold higher MDH activity than strain HL51276k. Inactivation of *icd* seems significantly related to decrease in MDH activity since strain HL2761k has much lower MDH activity than strain HL276k.

The PPC activities of strains HL27659k, HL2765k, and HL276k are all significantly higher than strains HL2761k and HL51276k (Figure 9-8f). Strains HL27659k, HL2765k, and HL276k also produced substantially more succinate than strains HL2761k and HL51276k (Figure 9-4b). PPC is essential for increasing the OAA
pool, therefore increasing succinate production (Millard et al., 1996). The inactivation of *icd* was observed to cause a decrease in PPC activity, since strain HL2761k has lower PPC activity than strain HL276k. This negative effect on PPC activity by the inactivation of *icd* was also observed for CS and MDH.

### 9.4.3 Comparison of Gene Expression Profiles

The expression of genes involved in the design of the aerobic succinate production system was profiled by quantitative real-time RT-PCR for the mutant strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k. Gene expression profiles combined with enzyme activities and metabolite production can provide a more holistic understanding of the connections between genes, proteins, and metabolites. For the purpose of studying the aerobic succinate production system designed, this methodology was narrowed down to a select few genes for expression profiling. The genes examined were some TCA cycle genes, *mdh, gltA, icd, aceA* and *aceB*, and some glycolysis genes, *ptsG, ppc, aceE, aceF, pykA*, and *pykF*. The genes *mdh, gltA, icd, aceA, aceB, ptsG*, and *ppc* refer to malate dehydrogenase, citrate synthase, isocitrate dehydrogenase, isocitrate lyase, malate synthase, glucose phosphotransferase system, and phosphoenolpyruvate carboxylase, respectively. The genes *aceE* and *aceF* are core components of the pyruvate dehydrogenase complex. Genes *pykA* and *pykF* express isoenzymes of pyruvate kinase. Expression of a particular gene was compared between the five mutant strains. The expression of a gene in the strains HL27659k, HL2765k, HL2761k and HL51276k is relative to the expression of that gene in the control strain HL276k. The results do not allow cross comparison of different genes.
The *gltA* expression profile shows that strain HL2761k has the highest level of *gltA* expression among the five strains (Figure 9-9c). The result does not correspond with the enzyme activity profile of CS. Strain HL27659k was shown to exhibit the highest CS activity whereas strains HL2761k and HL51276k exhibited the lowest CS activities (Figure 9-8a). This shows that gene expression does not necessary correlate with enzyme activity because the amount of protein expressed may not be proportional to the amount of RNA transcripts. The *gltA* expression in strain HL27659k is higher than that of its precursor strain HL2765k and the control strain HL276k. This is the same trend observed for the CS activity of strain HL27659k compared to strains HL2765k and HL276k. The *mdh* expression profile also did not correspond to the MDH activities of the five strains. Figure 9-9a shows that strain HL2761k has the highest *mdh* expression, even though it has the lowest MDH activity (Figure 9-8e). Strain HL27659k has the highest MDH activity, although its *mdh* expression does not correlate with this. The high MDH activity contributes to the efficiency of the glyoxylate cycle in strain HL27659k.

The *icd* expression profile corresponded well with the ICDH activities of the five mutant strains. Strains HL2761k and HL51276k did not have any *icd* expression because of the *icd* mutation (Figure 9-9e). Strain HL27659k has lower *icd* expression than strains HL2765k and HL276k and this result correlates with the lower ICDH activity of strain HL27659k than that of strains HL2765k and HL276k. Figures 9-9b and 9-9d confirm that there is no expression of the glyoxylate bypass genes *aceA* and *aceB* in strains HL276k and HL2761k, since there is functional IclR in these strains. Strains HL27659k, HL2765k, and HL51276k, which have *iclR* mutation, are the only strains with *aceA* and *aceB* expressed, and thus possess ICL and MS activities.
Figure 9-9: Gene expression profiles (mdh, gltA, icd, aceA, and aceB) of strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k. Expression levels of all strains are relative to that of the control strain HL276k. Expression levels cannot be compared between different genes.
The *ppc* expression profile did not correspond with the PPC activities of the five mutant strains. Strains HL27659k, HL2765k and HL276k showed higher PPC activities than strains HL2761k and HL51276k, but their *ppc* expression values did not follow this trend (Figures 9-8f and 9-10b). Strain HL2761k had the highest *ppc* expression. The *ptsG* expression profile shows that strain HL27659k did not have *ptsG* expression because of its *ptsG* mutation, and strain HL2761k had the highest *ptsG* expression (Figure 9-10a). Strain HL2761k, with *icl* inactivation, has higher *ptsG* and *ppc* expression than the control strain HL276k. Strain HL27659k, with *iclR* inactivation, has higher *mdh* and *icd* expression and lower *gltA* expression than its precursor strain HL2765k (Figures 9-9a, 9-9c, and 9-9e).

The components of pyruvate dehydrogenase complex, *aceE* and *aceF*, were expressed together in the same proportion in the five mutant strains since both are required to form pyruvate dehydrogenase (Figures 9-10c and 9-10d). Gene expression profiles of *aceE* and *aceF* follow the same trend for the five strains. Strain HL2761k has the highest level of *aceE* and *aceF* expression among the five strains. The expression profiles of *pykA* and *pykF*, both isoenzymes of pyruvate kinase, did not follow the same trend as each other for the five mutant strains. Strain HL2761k possessed the highest *pykA* expression among the five strains, whereas strain HL276k possessed the highest *pykF* expression among the five strains (Figures 9-10e and 9-10f). The dissimilarity in the expression profiles of *pykA* and *pykF* may be explained by the different pyruvate kinase enzymes they encode. The *pykF* isoenzyme has been shown to play a greater role than the *pykA* isoenzyme in the activity of pyruvate kinase when *E. coli* is grown on glucose (Ponce *et al.*, 1995). Higher *pykA* and lower *pykF* expression levels were
Figure 9-10: Gene expression profiles (ptsG, ppc, aceE, aceF, pykA, and pykF) of strains HL27659k, HL2765k, HL276k, HL2761k, and HL51276k. Expression levels of all strains are relative to that of the control strain HL276k. Expression levels cannot be compared between different genes.
observed when *icd* was inactivated. Higher *gltA* expression level was also observed when *icd* was inactivated. Lower *pykA* and *pykF* expression levels were observed when *ptsG* was inactivated. Lower expression levels were observed for *mdh, icd, ptsG, ppc, aceE, aceF, pykA* and *pykF* when *iclR* was inactivated. These observations were based on the comparison of gene expression profiles between strain HL2765k and strain HL276k, and between strain HL51276k and strain HL2761k.

### 9.5 Conclusion

Aerobic succinate production platforms were developed to allow further improvement of succinate production in *E. coli* under aerobic conditions (Lin *et al.*, 2005a b). The maximum theoretical succinate yield of 1.0 mol/mol glucose under aerobic conditions had been achieved through pathway optimization and engineering (Lin *et al.*, 2005b). The optimal *E. coli* strain engineered for aerobic succinate production was strain HL27659k(pKK313). This strain in aerobic fed batch cultures could produce 58.3 g/L of succinate in 59 hrs with a specific productivity of approximately 90 mg/g hr, while also achieving the maximum succinate yield of 1.0 mol/mol glucose (Lin *et al.*, 2005c). The background strain HL27659k was thus further characterized in chemostat to getter a better understanding of its metabolite profile in relation to its enzyme activities and gene expression profiles.

Several mutant strains involved in the development of the aerobic succinate production systems were also characterized to provide comparison with strain HL27659k. Chemostat results distinctly showed that strain HL27659k was the most efficient aerobic succinate production strain. At a dilution rate of 0.1 h\(^{-1}\), this strain could reach 91% of the
maximum theoretical succinate yield. Strain HL27659k consumed less glucose, which allowed more balanced glucose metabolism and efficient carbon-throughput to the desired end product succinate. Strain HL27659k did not accumulate any pyruvate and it produced the least acetate among all the mutant strains. Enzyme activity measurements showed that citrate synthase and malate dehydrogenase were substantially more active in strain HL27659k than in any of the other four mutant strains studied. The results also showed that strain HL27659k did have an active glyoxylate cycle with the inactivation of iclR. These results imply that strain HL27659k possesses a more efficient metabolism than the other mutant strains, allowing it to produce succinate as its major product with a minimal level of acetate and no pyruvate.

Gene expression profiles using quantitative RT-PCR show that the amount of protein expressed is not always strictly proportional to the level of RNA transcripts. Examples of this were observed between gene expression and enzyme activity for citrate synthase, malate dehydrogenase, and phosphoenolpyruvate carboxylase. Although strain HL27659k exhibited the highest CS and MDH activities among the mutant strains, the levels of gltA and mdh gene expression were not the highest. Strains with icd inactivation were observed to have higher expression levels of gltA, ppc, ptsG, aceE, aceF and pykA, and lower expression level of pykF. The strain with ptsG inactivation was observed to have higher expression level of gltA, and lower expression levels of mdh, icd, aceE, aceF, pykA and pykF. Inactivation of iclR induces the expression of aceA and aceB. Strains with iclR inactivation were also observed to have lower expression levels of mdh, icd, ptsG, ppc, aceE, aceF, pykA, and pykF. Because the background of all these mutant
strains has mutations in sdhAB, ackA-pta, and poxB, this might have also influenced the gene expression patterns observed.

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Chapter 10

9. Recommendations

Novel metabolic networks designed for optimal succinate production under aerobic conditions were created in this work. The most efficient aerobic succinate production system developed was shown to achieve the maximum theoretical succinate yield of 1.0 mol/mol glucose. In fed batch cultures under aerobic conditions, this system could produce 58 g/L of succinate in 59 hrs with a specific productivity of 90 mg/g cell-hr. This level of succinate production has never been achieved before under aerobic conditions, until now. The success of the aerobic succinate production system in *E. coli* exemplifies the efficacy of metabolic engineering in creating purposeful metabolic networks.

Various *E. coli* mutant strains constructed throughout the development of the aerobic succinate production systems were characterized in chemostat cultures to further understand their metabolic function as a result of multiple pathway manipulations. The metabolite profiles, enzyme activities, and gene expression profiles of the various mutant strains were examined. This provides a holistic approach to understanding the intrinsic connections between genes, proteins, and metabolites. Genetic perturbation on the pathways of the aerobic central metabolism was observed to cause significant changes in the expression level of various genes. The expression level of multiple genes can be affected by a single gene perturbation because all genes are interconnected by common regulators. The changes in gene expression levels were also observed to significantly affect the enzyme activities of pathways and the final metabolite characteristics.
Gene expression profiles in the characterization study were performed by quantitative real-time RT-PCR. Additional investigation with DNA microarrays can also be insightful, since global gene expression patterns of the *E. coli* mutant strains can be examined. These global gene expression patterns would provide a clear insight into how genetic manipulations affect the overall gene expression profile. Specifically, a bigger picture of the regulation of genes in the genome, such as the ones involved in glycolysis, pentose pathway, TCA cycle, biosynthetic pathways, regulatory pathways, and protein processing, can be accessed. This will allow an overall understanding of the dynamic regulation of every gene as the metabolic network is subjected to design alterations. With DNA microarrays, differential transcription levels of the complete *E. coli* genome can be analyzed with robust statistical methods that warrant high confidence level for gene expression ratios. Good examples of this type of work on the global gene expression profiling of *E. coli* for metabolic engineering applications are the recent studies by Oh *et al.*, 2002a, 2002b, 2002.

For more comprehensive analysis of the cellular metabolism, gene expression profile is not sufficient to dictate the outcome of specific alterations to the metabolic network. The metabolic flux distribution profiles are also needed to thoroughly comprehend the metabolic response to pathway perturbations. Disruption of genes, for example, not only will change the expression levels of genes, but also cause ensuing effects on the final cellular functions and metabolic processes. Therefore, the metabolic flux distribution of a perturbed metabolic network is the ultimate piece of information for evaluating if the intended modification has achieved the desired outcome. Conventional analysis of metabolic flux distribution using stoichiometric equations and measured
specific rates is limited when it comes to analyzing the complete metabolic network. This is because the complete metabolic network will include bidirectional reactions, parallel reaction pathways, and cyclic pathways, such as the glyoxylate cycle, that cannot be differentiated using the stoichiometric mass balance. An alternative method for overcoming this is the use of isotope balance based on the data of nuclear magnetic resonance (NMR) or gas chromatography-mass spectrometry (GC-MS). In general, experiments are carried out by using substrates, such as glucose, labeled with $^{13}$C. The intracellular metabolites incorporating the $^{13}$C from these substrates can then be used to determine the multidimensional network fluxes. Further investigation of the *E. coli* mutant strains constructed for aerobic succinate production with $^{13}$C tracer experiments would significantly improve the understanding of the metabolic processes of these mutant strains. Good examples of this type of work using $^{13}$C-labeling experiments on *E. coli* mutant strains can be found in recent studies by Jiao *et al.*, 2003, Yang *et al.*, 2003, and Siddiquee *et al.*, 2004.
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