ENGINEERING THE METABOLISM OF *Escherichia coli* FOR THE SYNTHESIS OF OXIDIZED PRODUCTS: ACETATE AND PYRUVATE PRODUCTION

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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by

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This dissertation is dedicated to my wife Celeste and my family for their support through the years.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
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ENGINEERING THE METABOLISM OF *Escherichia coli* FOR THE SYNTHESIS OF OXIDIZED PRODUCTS: ACETATE AND PYRUVATE PRODUCTION

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To demonstrate a novel approach that combines attributes of fermentative metabolism and oxidative metabolism into a single biocatalyst for production of oxidized chemicals, *Escherichia coli* was genetically engineered for acetate and pyruvate production. Acetate producing *E. coli* TC36 was constructed by sequentially assembling chromosomal deletions to inactivate oxidative phosphorylation (Δ*atpFH*), disrupt the tricarboxylic acid cycle (Δ*sucA*), and eliminate fermentation pathways (Δ*focA-pflB* Δ*frdBC* Δ*ldhA* Δ*adhE*). StrainTC36 produced 572 mM acetate from 6% glucose (333 mM) and a maximum of 878 mM acetate with glucose excess. Although strain TC36 produced a maximum of 4 ATP (net) per molecule of glucose as compared to 33 ATP (net) for wild type strains, it grew in glucose minimal medium (μ = 0.49, 82% of W3110) without supplements. Glycolytic flux in strain TC36 was estimated to be 0.3 μmol min^{-1} (mg protein)$^{-1}$, twice that of the wild type parent (W3110). Pyruvate producing strain TC44 (Δ*focA-pflB* Δ*frdBC* Δ*ldhA* Δ*atpFH* Δ*adhE* Δ*sucA poxB*::*FRT*

Δ*ackA*) was constructed from strain TC36 by inserting additional chromosomal deletions in the acetate kinase (Δ*ackA*) and pyruvate oxidase (Δ*poxB*) genes. In mineral salts medium containing glucose as the sole carbon source, strain TC44 converted glucose to pyruvate with a yield of 0.75 g pyruvate per g glucose (77.9% of theoretical yield; 1.2 g pyruvate L^{-1} h^{-1}). The tolerance of *E. coli* to such drastic changes in metabolic flow and energy production implies considerable elasticity in permitted pool sizes for key metabolic intermediates such as pyruvate and acetyl~CoA. The basic principles demonstrated using strains TC36 and TC44 can be applied for the production of a variety of other chemicals, irrespective of their relative oxidation states.

CHAPTER 1
INTRODUCTION

Renewable Fuels and Chemicals

The current trend toward increasing demand for foreign petroleum in the United States is forecast to continue, reaching 70% imported petroleum in 2025 as opposed to 37% in 1980 (USDOE/EIA 2004). Subordination to foreign countries, some of which demonstrate mercenary ideals toward US interests, for such a critical component of the day-to-day functioning of society is a national security problem. The use of microbial biocatalysts for the conversion of renewable plant biomass to fuels and chemicals can significantly reduce the United States' dependence on imported petroleum.

The commercial production of commodity chemicals by microbial fermentation has been limited to reduced and redox neutral compounds such as ethanol and lactic acid, respectively, under anaerobic conditions where substrate loss to cell mass or CO_2 are minimal and product yields are high. In aerobic processes, *Escherichia coli* is widely used as a biocatalyst for production of higher value products such as recombinant proteins (Akesson et al. 2001; Aristidou et al. 1995; Contiero et al. 2000; Luli and Strohl 1990) and amino acids (Chotani et al. 2000; Eggeling et al. 2001). Traditional chemical companies have begun to develop infrastructure for the production of compounds using biocatalytic processes (reviewed in Schmid et al. 2001; Stringer 1996; Strohl, 2001). Considerable progress has been reported toward new processes for commodity chemicals (Danner and Braun 1999) such as ethanol (Ingram et al. 1999; Underwood et al. 2002), lactic acid (Chang et al. 1999; Dien et al. 2001; Zhou et al. 2003), 1,3-propanediol

(Nakamura et al. US Patent 6,013,494; Tong et al. 1991), and adipic acid (Niu et al. 2002). In addition, advances have been made in the genetic engineering of microbes for high-value specialty compounds such as polyketides (Beck et al. 2003; Dayem et al. 2002) and carotenoids (Lee and Shmidt-Dannert 2002; Wang et al. 2000).

Why Use *Escherichia coli* as a Biocatalyst?

Escherichia coli is a facultative anaerobe, possessing a robust metabolism, capable of growing on mineral salts media with only the addition of a carbon source. The capability *E. coli* to respire, exploiting a variety inorganic electron acceptors, and ferment, using internal metabolites as an electron sink, provide the metabolic engineer with flexibility to explore the production of many compounds regardless of redox state. *Escherichia coli* is capable of catabolizing hexose and pentose sugars which constitute the majority of carbohydrates in plant biomass commonly used as feedstock for fermentation processes. The efficient conversion of substrate into product is essential if a process is to be economically viable. The physiology and biochemistry of *E. coli* has been well documented, affording numerous tools for genetic manipulation. Many years of laboratory and industrial experience has demonstrated that *E. coli* is safe and reliable. Therefore, the broad substrate range and robust metabolism combined with years of technical experience makes *E. coli* an attractive biocatalyst for the production of fuels and chemicals.

Overview of Central Carbon Metabolism in *Escherichia coli*

Aerobic Versus Anaerobic Metabolism

Escherichia coli is a heterotrophic, facultative anaerobe. Hexose and pentose sugars are transported into the cell using either phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), ATP-binding cassette (ABC) transporter (the energy

for transport provided by ATP) or cation ($H^+$and Na^+) symporters (Linton and Higgins. 1998; Neidhardt et al. 1996). The PTS system transports sugars, such as glucose, using phosphoenolpyruvate (PEP) as the phosphoryl donor and as the energy source needed for translocation (Fig. 1-1).

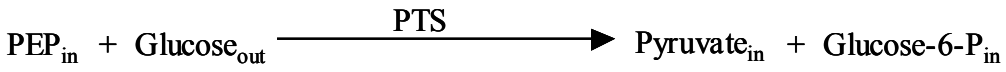

Figure 1-1. Reaction summary for glucose transport

Glucose-6-P can then be used as a carbon and energy source (glucose is also a source of oxygen for biosynthesis). Glucose catabolism can be directed through various pathways dependent on the biosynthetic needs of the cell. The research described in this document is primarily related to central carbon metabolism, therefore only these pathways are discussed in detail.

The first stage in glucose-6-P metabolism is the transformation of glucose-6-P to fructose-6-P followed by phosphorylation. (Fig. 1-3A). The additional phosphate is supplied by ATP, therefore the first stage of glycolysis results in an investment of two ATP equivalents (one from PEP and the other from ATP). The net reaction for stage 1 is summarized in Figure 1-2.

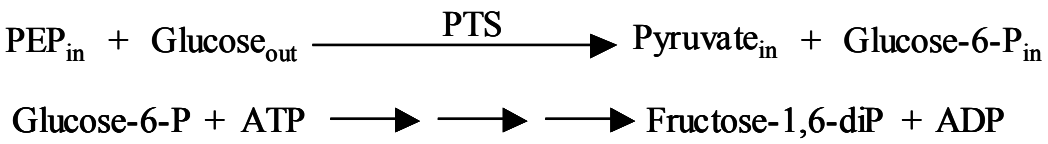

Figure 1-2. Reaction summary for the energy consuming reactions of glycolysis

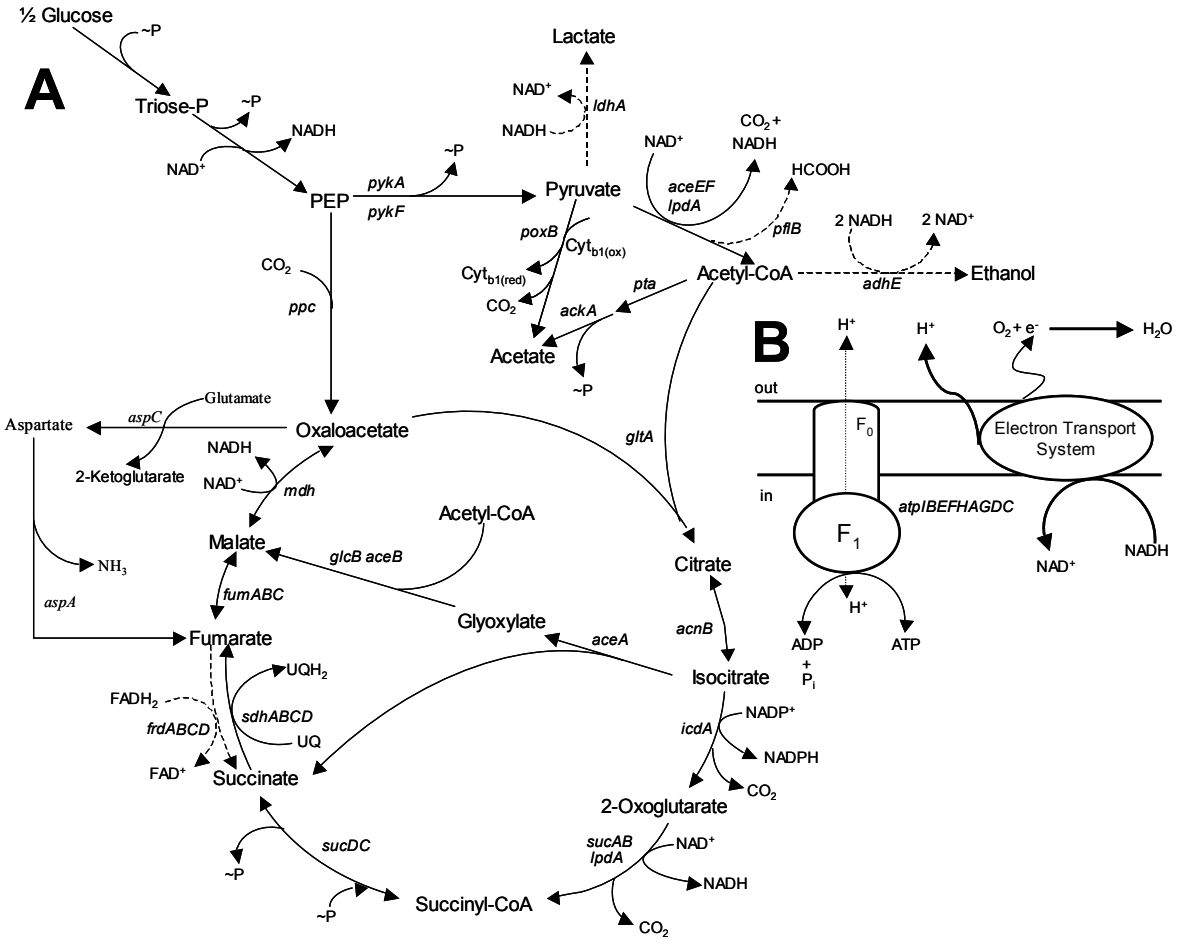

Figure 1-3. Central carbon metabolism in *Escherichia coli.* Dashed arrows represent fermentative pathways. A) Central carbon metabolism B) Oxidative phosphorylation. Genes: *aceA*, isocitrate lyase; *aceB*, malate synthase B; *aceEF,* E1p and lipoate acetyltransferase/dihydrolipoamide acetyltransferase component of the PDHc; *ackA*, acetate kinase; *acnB*, aconitase; *adhE*, alcohol/aldehyde dehydrogenase pyruvate formate-lyase deactivase; *atpIBEFHAGDC*, $(F_1F_0)H^+$-ATP synthase; *frdABCD*, fumarate reductase; *glcB*, malate synthase G; *gltA*, citrate synthase; *icdA*, isocitrate dehydrogenase; *ldhA*, lactate dehydrogenase; *lpdA*, dihydrolipoate dehydrogenase/dihydrolipoamide dehydrogenase component of the PDHc and 2-oxoglutarate dehydrogenase; *pflB*, pyruvate formate-lyase; *poxB*, pyruvate oxidase; *pta*, phosphotransacetylase; *pykA*, pyruvate kinase II; *pykF*, pyruvate kinase I; *sdhABCD*, succinate dehydrogenase; *sucAB*, 2-oxoglutarate dehydrogenase; *sucDC*, succinyl-CoA synthetase.

Glyceraldehyde-3-P oxidation to pyruvate is the energy yielding stage (stage 2) of glycolysis. Glyceraldehyde-3-P dehydrogenase incorporates an inorganic phosphate with the concomitant reduction of NAD^+ and the formation of 1,3-diphosphoglycerate. The acyl phosphate is then used to phosphorylate ADP to ATP. The incorporation of the inorganic phosphate results in the net gain in ATP from glycolysis. Another ATP is formed from PEP during the production of pyruvate. A reducing equivalent is produced during the oxidation of glyceraldehyde-3-P (Fig. 1-3A and1-4).

$$\text{Glucose} + 2\text{ADP} + 2\ P_i + 2\ NAD^+ \longrightarrow$$
$$2\ \text{Pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ H^+$$

Figure 1-4. Reaction Summary for glycolysis

Glycolysis is not only an energy generating pathway but also provides precursors for the biosynthesis of several amino acids, lipids, polysaccharide, and components of peptidoglycan.

The fate of pyruvate produced during glycolysis is dependent upon oxygen available in the environment. When *E. coli* is growing in an aerobic environment, pyruvate is oxidatively decarboxylated to acetyl-CoA by the pyruvate dehydrogenase complex (PDHc) with the concomitant production of NADH. Acetyl-CoA can then enter the tricarboxylic acid cycle (TCA cycle), serve as the substrate for acetate production (phosphotransacetylase and acetate kinase reactions) yielding an ATP by substrate level phosphorylation, or serve as the basic building block for fatty acid biosynthesis. Entry into the TCA cycle is the major route for acetyl-CoA during aerobic metabolism which, like glycolysis, serves energetic and biosynthetic functions. The cyclic functioning of the TCA cycle results in the production of four reducing equivalents, two carbon dioxides,

and one GTP (Fig 1-3A and 1-5). Therefore, four ATP equivalents are produced by substrate-level phosphorylation from catabolism of one glucose molecule by glycolysis and the TCA cycle.

$$\text{Acetyl-CoA} + 2\,H_2O + GDP + P_i + FAD + NADP^+ + 2\,NAD^+ \longrightarrow$$
$$2\,CO_2 + GTP + FADH_2 + NADPH + 2\,NADH + 3\,H^+ + CoASH$$

Figure 1-5. Reaction summary of TCA cycle reactions

The reducing equivalents produced during aerobic growth are re-oxidized via the electron transport chain with molecular oxygen as the final electron acceptor (Fig.1-3B and 1-6). Electrons from NADH enter the electron transport chain by reduction of ubiquinone (UQ) to ubiquinol (UQH_2). The NADH dehydrogenases, NDH I and NDH II, catalyze the reduction of UQ to UQH_2 (Campbell and Young 1983; Ingledew and Poole 1984; Jaworowski et al. 1981; Meinhardt et al. 1989; Weidner et al. 1992; Young et al. 1981). There are two terminal cytochrome oxidases, cytochrome *bo* oxidase and cytochrome *bd* oxidase, in *E. coli* that transfer the electrons from UQH_2 to oxygen (Ingledew and Poole 1984; Koland et al. 1984; Miller and Gennis 1985; Neidhardt et al.1990; Puustinen et al. 1991; Rice and Hempfing 1978). Cytochrome *o* oxidase is predominant under aerobic conditions and has a low affinity, with a K_m for oxygen of 0.20 μM (Rice and Hempfing 1978). Cytochrome *bo* oxidase functions as a scalar transporter of protons as well as a proton pump (Neidhardt et al. 1996; Puustinen et al. 1989; Puustinen et al. 1991). Therefore, the H^+/e^- for cytochrome *bo* oxidase is 2. The high affinity cytochrome *bd* complex, which is predominant under micro-aerobic conditions (Tseng et al. 1996), has a K_m for oxygen of 0.024 μM (Rice and Hempfling 1978). The respiratory efficiency of *E. coli* depends on the stoichiometry of the NADH dehydrogenases and cytochrome oxidases (Fig. 1-6). Cytochrome *bd* oxidase can only

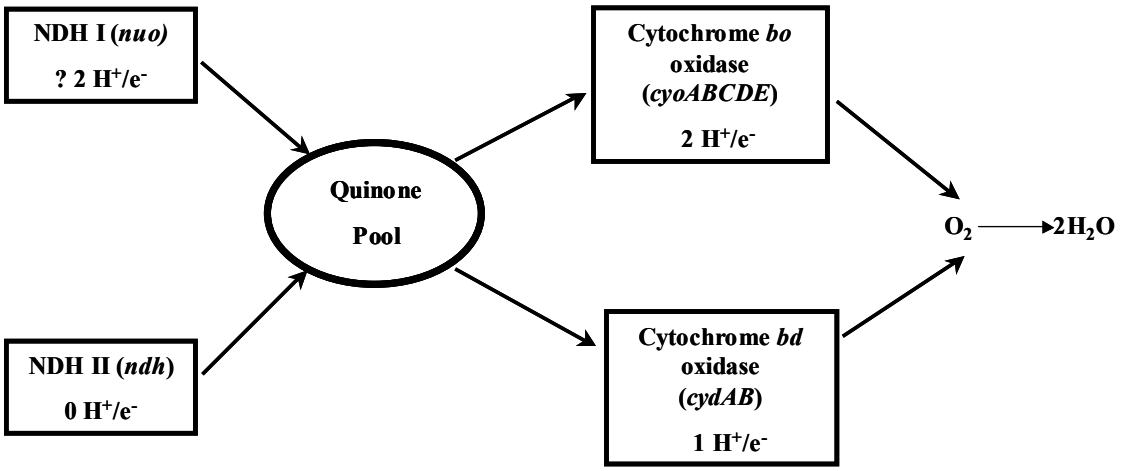

Figure 1-6. Electron flow from NADH in *Escherichia coli* (figure adapted from Neidhardt, F. C. et al. 1996). NADH is oxidized by the NADH deydrogenases, *nuo* and *ndh*. Quinones serve as intermediate electron carriers which are oxidized by the terminal cytochrome oxidases, *cyoABCDE* and *cydAB*.

transport protons by scalar chemistry resulting in a H^+/e^- of 1 (Neidhardt et al. 1990; Puustinen et al. 1991). Figure 1-6 is a simplified diagram of the process and summarizes the H^+/e^- for each redox reaction.

The NADH dehydrogenases, like the cytochrome oxidases, differ in the number of protons that are extruded (H^+/e^-) on reduction of the substrate (UQ or O_2, respectively). NDH I has a calculated H^+/e^- of 1.5-2.0 (Bogachev et al. 1996; Calhoun et al. 1993) and NDH II does not contribute to the membrane potential gradient (Young et al. 1981). The number of protons extruded by NDH I is yet to be established. Difficulty in obtaining evidence for the actual H^+/e^- arises from the many respiratory chain linked dehydrogenases present in *E. coli* (Ingledew and Poole 1984). Oxygen pulse experiments performed under anaerobic conditions conducted by Calhoun et al. (1993) and Bogachev et al. (1996) have indicated a $H^+/e^- = 2$ and $H^+/e^- = 1.5$, respectively. Since, a $H^+/e^- = 1.5$ is unlikely, two protons transported per electron is a reasonable assumption. Another line

of evidence which suggests the $H^+/e^- = 2$ is that NDH I is the homolog of the mitochondrial complex I (Meinhardt et al. 1989; Weidner et al. 1992) which has been demonstrated to extrude two protons per electron (Pozzan et al. 1979; Wikström and Penttilä 1982).

The stoichiometry of the NADH dehydrogenases and cytochrome oxidases, which depend on the growth conditions (Spiro and Guest 1991), result in 1 H^+/e^- to as many as 4 H^+/e^- delivered to the periplasm under aerobic conditions (Fig. 1-6). The greater the H^+/e^- the higher the respiratory efficiency i.e., increasing the membrane potential difference results in more ATP produced per mole of oxygen consumed (P/O). If all of the electrons originating from glucose catabolism are transported through NDH I and cytochrome *bo* oxidase then a maximum of 33 ATP can be produced. However, growth yield experiments conducted by Stouthamer (1979) have demonstrated that a maximum of 26 ATP are produced per molecule of glucose when *E. coli* is grown under optimal conditions. This lower value is the result of 1) differential electron flux through the NADH dehydrogenases and terminal oxidases, 2) the maintenance energy requirement (Pirt 1982; Pirt 1987) and 3) proton leakage reducing the energized state of the cytoplasmic membrane (Stouthamer and Bettenhaussen 1977). The large amount of ATP produced and the high respiratory efficiency of *E. coli,* grown under aerobic conditions and limiting glucose, result in the production of carbon dioxide and cell mass in a 50:50 ratio (Calhoun et al. 1993; Neidhardt et al. 1990).

When *E. coli* is grown anaerobically on glucose with out an exogenous terminal electron acceptor, NAD is regenerated by reduction of internal organic compounds (Fig.1-3A). The major end products of glucose fermentation are acetate, ethanol, lactate,

and formate in a 1:1:2:2 ratio (Neidhardt et al. 1996). Ten percent of the glucose consumed is converted to succinate and cell mass (Neidhardt et al. 1996). In contrast to the large amount of ATP produced during aerobic metabolism, only two net ATP are produced (by substrate-level phosphorylation) during fermentation of glucose to pyruvic acid (Fig.1-3A). An additional ATP is produced with the formation of acetate through acetate kinase. In addition to the low ATP yield and low cell mass, fermentative metabolism also differs from aerobic metabolism in that the TCA cycle functions in a non-cyclic manner. The transcription of 2-oxoglutarate dehydrogenase (*sucAB* gene product) is repressed under anaerobic conditions (Amarasingham and Davis 1965; Cunningham and Guest. 1998; Park et al. 1997) which seperates the TCA cycle into reductive and oxidative branches. The anaerobic TCA cycle functions only in a biosynthetic capacity.

During fermentative growth, many of the genes involved in aerobic metabolism are repressed and expression of genes involved in anaerobic metabolism are activated. Two global regulatory systems play a major role in the transition to fermentative metabolism. The ArcAB (aerobic respiratory control) two-component signal transduction pathway (ArcA is the response-regulator and ArcB is the sensor kinase) and the redox-sensitive protein, FNR (fumarate and nitrate reduction) function to regulate gene expression in response to oxygen availability (Iuchi and Weiner 1996; Kiley and Beinert 1999). The ArcAB system functions mainly to repress expression of aerobic genes under anaerobic conditions (Spiro and Guest 1991; Iuchi and Weiner 1996). Under micro-aerobic conditions the expression of the *cyoABCDE* (cytochrome *bd*, the high affinity terminal oxidase) operon is enhanced by ArcAB (Iuchi and Weiner. 1996). The

expression of both oxidases is repressed by FNR under anaerobic conditions (Kiley and Beinert 1999; Spiro and Guest 1991). FNR is known to activate 22 operons and repress eight operons under anaerobic conditions (Spiro and Guest 1991). When *E. coli* is growing fermentatively, FNR activates the transcription of *pflB*, pyruvate formate-lyase. The products of pyruvate formate-lyase are acetyl-CoA and formate (Fig 1-3A). There are no reducing equivalents produced during the decarboxylation of pyruvate under fermentative conditions. The lipoamide dehydrogenase (*lpdA*) protein, shared by PDHc and 2-oxoglutarate dehydrogenase, is repressed by ArcAB under anaerobic conditions (Cunningham and Guest 1998). Transcription of *sucAB*, which codes for the E1 and E2 subunits of 2-oxoglutarate dehydrogenase, is repressed by FNR from the *sdhC* promoter (Park et al. 1997). The repression of *sucAB* under anaerobic conditions results in the non-cyclic functioning of the TCA cycle mentioned above.

In summary, high growth rate and conversion of glucose into high levels of cell mass and large amounts of carbon dioxide, are characteristic of aerobic metabolism in *E. coli*. During anaerobic metabolism, approximately 95% of the glucose is converted to organic acids and ethanol, while less than 5% is converted to cell mass. Figure 1-7 is a simplified diagram of carbon flow in *E. coli* during aerobic and anaerobic metabolism. Typically, the growth rate is also decreased during anaerobic conditions.

ATP and Glycolytic Flux

Studies conducted by Patnaik et al. (1992) and Chao and Liao (1994) have demonstrated that creation of futile cycles in *E. coli*, which consume ATP, cause a two-fold increase in glycolytic flux. Over-expression of phosphoenolpyruvate synthase (*pps*) (Fig. 1-8A) by 30-fold resulted in a two-fold increase in glycolytic flux (Patnaik et al. 1992). Low induction levels of Pps resulted in increased respiration to compensate for

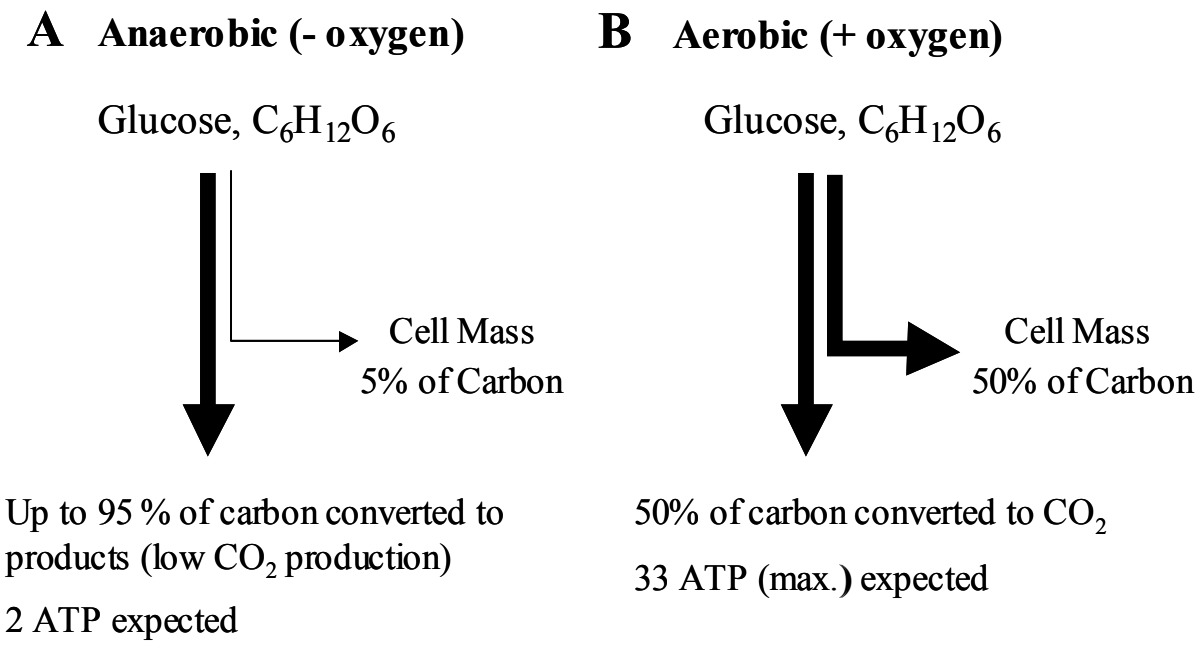

Figure 1-7. Summary of anerobic and aerobic metabolism in *E.* coli. A) Anaerobic metabolism. B) Aerobic metabolism

the loss of ATP. High levels of Pps induction resulted in both increased respiration and increased glycolytic flux. The authors explained the observation at high Pps induction levels by 1) saturation of the respiratory chain and 2) increased glucose uptake due to a high phosphoenolpyruvate/pyruvate ratio resulting in an increase in the phosphorylated forms of enzyme I, Hpr, and enzyme III^{glc} of the PTS (Patnaik et al. 1992). Although the increase in glucose transport may contribute to the increased glycolytic flux in the mutant, it is unlikely the only determinant. Phosphoenolpyruvate is an allosteric inhibitor of phosphofructokinase which may become a "bottleneck" at high phosphoenolpyruvate levels. The Pyk-Pps futile cycle resulted in a net loss of ATP concomitant with elevated respiration (Patnaik et al. 1992), indicative of a reduction in respiratory efficiency. Therefore, the increased glycolytic flux may be due to decreased ATP production coupled with increased availability of ADP, an allosteric activator of phosphofructokinase (Deville-Bonne et al. 1991), under aerobic conditions.

Introduction of a futile cycle between phosphoenolpyruvate and oxaloacetate by over-expression of phosphoenolpyruvate carboxykinase (*pck*) and phosphoenolpyruvate carboxylase (*ppc*) also resulted in an increase in glycolytic flux (Fig. 1-8B) (Chao and Liao 1994). Increasing the magnitude of the futile cycle for ATP consumption was followed by (in order) an increase in respiration rate, increase in glucose consumption and excretion of acetate and pyruvate (Chao and Liao 1994). These results suggest that *E. coli* compensates for decrease in ATP by first, increasing respiration to form ATP at a higher rate, secondly, by increasing glycolytic flux and the formation of ATP by substrate-level phosphorylation. Another interesting observation was that inactivation of 2-oxoglutarate dehydrogenase in addition to the futile cycle resulted in even greater increase in glycolytic flux than the futile cycle alone (Chao and Liao 1994). This is a result of the decreased amount of electrons available for electron transport and oxidative phosphorylation. The authors comment that, "cells with an incomplete TCA cycle cannot effectively compensate for the energy stress caused by the induced futile cycle" (Chao and Liao 1994).Our observations with the *atpFH* mutant are contrary to those of Chao and Liao and will be discussed in Chapter 2.

Deletion of the $(F_1F_0)H^+$-ATP synthase has also been found to increase glycolytic flux as well as acetate production (Jensen and Michelsen 1992). A two-fold increase in glycolytic flux was observed in an *E. coli* strain carrying a deletion in the *atpIBEFHAGDC* operon, consistent with the experiments using artificially induced futile cycles. By inactivating the $(F_1F_0)H^+$-ATP synthase *E. coli* can no longer derive ATP from the proton motive force generated during oxidative respiration. The growth rate for the $(F_1F_0)H^+$-ATP synthase mutants was decreased to ~74% of the wild type level and the

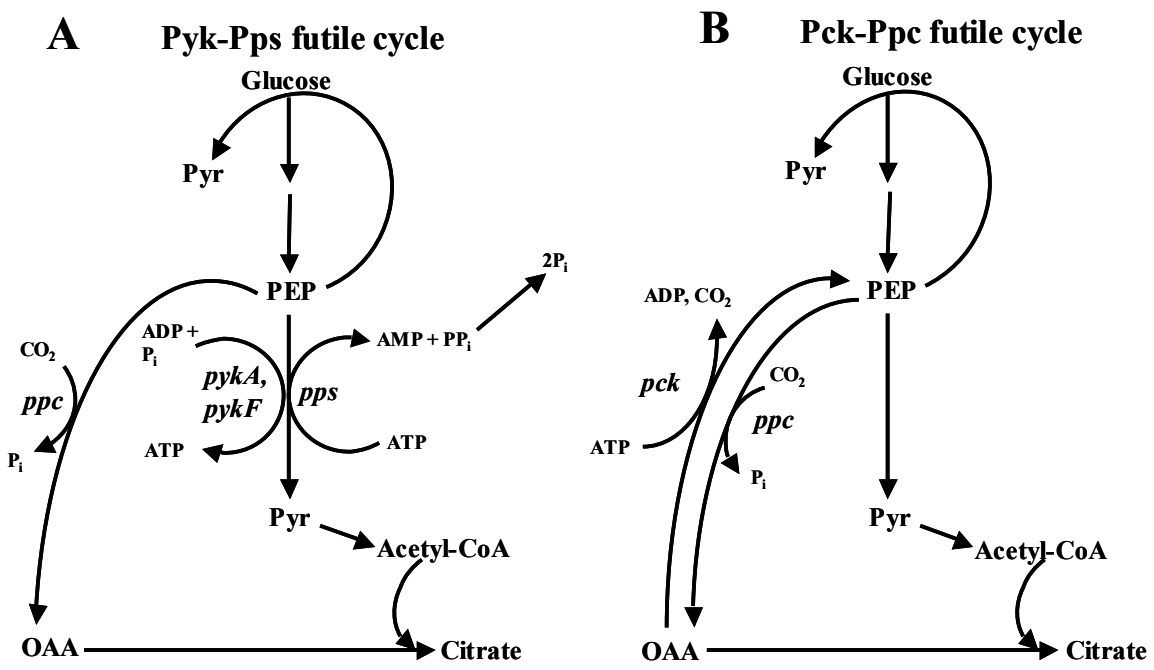

Figure 1-8. Futile cycles constructed in *E. coli*. A) The *pps* gene was cloned into a plasmid behind an IPTG-inducible bacteriophage T7 promoter modified to overlap two *lac* operators. Over-expression of *pps* during growth on glucose results in net loss of ATP due to formation of a futile cycle involving Pps and Pyk. Abbreviations: *ppc*, phosphoenolpyruvate carboxylase; *pps*, phosphoenolpyruvate synthase; *pykA* and *pykF*, major and minor pyruvate kinases, respectively (adapted from Patnaik, R. et al. 1992). B) The structural genes of *ppc* and *pck* onto a plasmid behind the IPTG inducible *tac* promoter. Simultaneous expression of *ppc* and *pck* result in a net loss of ATP forming a futile cycle. Abbreviations: *pck*, phosphoenolpyruvate carboxykinase; *ppc*, phosphoenolpyruvate carboxylase (Chao, Y., and J. C. Liao. 1994).

ATP/ADP ratio was decreased from 19 in the wild type to 7 in the mutant strain (Jensen and Michelsen 1992). Deletion of the *atp* operon also resulted in increased respiration proceeded by accumulation of acetate. The following observations comparing the growth characteristics between wild type *E. coli* and the Δ*atpIBEFHAGDC* mutant:

1. increased respiration
2. increased glycolytic flux
3. accumulation of acetate

led the investigators to conclude that *E. coli* will oxidize NADH by respiration even if it is not coupled to ATP synthesis leading to enhanced substrate level phosphorylation (Jensen and Michelsen 1992).

The approaches described above demonstrate that limiting ATP by enhancing futile cycles or suppression of the oxidative phosphorylation results in increased glycolytic flux. Recently, Koebmann et al. (2002) describe a genetic approach that induces ATP hydrolysis to elucidate the nature of control on glycolytic flux. The genes *atpAGD,* encoding the F_1 subunit of the $(F_1F_0)H^+$-ATP synthase, were cloned behind synthetic constituative promoters of different strengths. These investigators discovered that control of glycolytic flux varies with ATP/ADP ratio (Koebmann et al. 2002). During growth of wild type *E. coli* on glucose minimal medium the anabolic (ATP consuming) reactions exert a majority of the control on glycolytic flux. When the ATP/ADP increases flux control shifts to the catabolic (ATP producing) reactions. These findings are consistent with mathematical models that indicate greater than 75% of glycolytic flux is controlled by ATP/ADP in *E. coli* (Hofmeyer and Cornish-Bowden. 2000).

Combine Beneficial Effects of Aerobic and Anaerobic Metabolism.

It is possible to combine the beneficial effects of aerobic and anaerobic metabolism into a single biocatalyst for the production of oxidized and redox neutral compounds. This can be accomplished by combining the high rate of glycolysis and low cell yield of fermentative metabolism with the high growth rate and availability of an external electron acceptor of aerobic metabolism. The advantages for production of oxidized compounds are expected to be increased product yield and rate of product formation combined with decreased production of carbon dioxide and cell mass. The major advantage for production of reduced compounds would be the increase in process stability due to the

elimination of the energy advantage of NADH oxidation by the electron transport chain. To demonstrate this concept, the metabolism of *Escherichia coli* was engineered for the production of acetate and pyruvate.

CHAPTER 2
ENGINEERING THE METABOLISM OF *Escherichia coli* W3110 FOR THE CONVERSION OF SUGAR TO ACETATE

Introduction

In all aerobic and anaerobic processes, acetate production by the native *E. coli* pathway (phosphotransacetylase and acetate kinase) has been generally regarded as an undesirable consequence of excessive glycolytic flux (Akesson et al. 2001; Aristidou et al. 1995; Contiero et al. 2000; Farmer and Liao 1997; Underwood et al. 2002; Zhou et al. 2003). Although acetate is equivalent to glucose in redox state, pathways in *E. coli* that produce acetate also produce CO_2 resulting in a combined redox state of carbon more oxidized than in glucose with the production of NADH. To facilitate expansion into more oxidized commodity chemicals, such as acetate, we developed a novel genetic approach that combines the attributes of fermentative metabolism and oxidative metabolism (rapid growth, external electron acceptor for NADH oxidation) into a single biocatalyst.

The biological production of acetic acid is currently limited to food uses. Petrochemical routes have largely displaced biological processes as uses expanded to plastics, solvents, and road de-icers (Berraud 2000; Cheryan et al. 1997; Freer 2002). There are two major petrochemical routes for commercial acetic acid production; methanol carbonylation and liquid-phase oxidation of n-butane. Approximately 35% of commercial acetic acid is recovered from other processes, most significantly, from the production of cellulose acetates and polyvinyl alcohol (Kirschner 2003). Synthesis of acetate in the United States is estimated at 7.8 billion pounds per year, with a projected

growth of 2% per year through 2008 (Kirschner 2003). In the current biological process, sugars are fermented to ethanol by *Saccharomyces*. Ethanol in the resulting beer is subsequently oxidized to acetic acid by *Acetobacter sp.* under aerobic conditions (Berraud 2000; Cheryan et al. 1997; Freer 2002). This process can summarized as follows:

$$C_6H_{12}O_6 + 2\,H_2O \longrightarrow 2\,C_2H_4O_2 + 2\,CO_2 + 8\,[H]$$

$$8\,[H] + 2\,O_2 \longrightarrow 4\,H_2O$$

Figure 2-1.Reaction summary for the conversion of hexose to acetate

In our study, we have genetically modified *E. coli* W3110 to produce acetic acid as the primary product from glycolysis. Although ethanol was eliminated as an intermediate, the stoichiometry of the reaction remained as shown above. The resulting biocatalyst (TC36) contains multiple chromosomal alterations (Fig. 2-2A and B) that direct carbon flow to acetate and minimize carbon loss to cell mass, CO_2, and alternative products. This strain is devoid of plasmids, foreign genes, and antibiotic resistance markers and grows well in mineral salts medium without complex nutrients.

Materials and Methods

Microorganisms and Media

Strains and plasmids used in our study are listed in Table 1. Working cultures of *E. coli* W3110 (ATCC 27325) derivatives were maintained on minimal medium containing mineral salts (per liter: 3.5 g KH_2PO_4; 5.0 g K_2HPO_4; 3.5 g $(NH_4)_2HPO_4$, 0.25 g $MgSO_4 \bullet 7\,H_2O$, 15 mg $CaCl_2 \bullet 2\,H_2O$, 0.5 mg thiamine, and 1 mL of trace metal stock), glucose (2% in plates; 3% in broth), and 1.5% agar. The trace metal stock was prepared in 0.1 M HCl (per liter: 1.6 g $FeCl_3$, 0.2 g $CoCl_2 \bullet 6\,H_2O$, 0.1 g $CuCl_2$, 0.2 g

Table 2-1. Sources and characteristics of strains

Strains	Relevant Characteristics	Reference
W3110	K12 wild type	ATCC 27325
TOP10F'	*lacI*^{q} (episome)	Invitrogen
LY01	*E. coli* B, *frd pfl*::*pdo*$_{ZM}$ *adhE*$_{ZM}$ *cat*	Yomano et al. 1998
LY74	W3110, Δ*poxB*::*FRT-tet-FRT*	Causey et al. 2004
LY117	W3110, *ycaC*::*FRT-aap-FRT*::*ycaD*	This study
SE2279	MG1655, *pflB ldhA*::Tn*10*	Causey et al. 2003
SZ33	W3110, *ldhA*::Tn*10*	Causey et al. 2003
SZ40	W3110, Δ*(focA-pflB)*::*FRT* Δ*frdBC*	Zhou et al. 2003
SZ46	W3110, Δ(*focA-pflB)*::*FRT* Δ*frdBC ldhA*::Tn*10*	Causey et al. 2003
SZ47	W3110, Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA*	Causey et al. 2003
TC20	W3110, Δ*adhE*::*FRT-tet-FRT*	Underwood et al. 2002
TC21	W3110, Δ*atpFH*::*FRT-tet-FRT*	Causey et al. 2003
TC23	W3110, Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT-tet-FRT*	Causey et al. 2003
TC24	W3110, Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT*	Causey et al. 2003
TC25	W3110, Δ*sucA*::*FRT-tet-FRT*	Causey et al. 2003
TC30	W3110, Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT* Δ*adhE*::*FRT-tet-FRT*	Causey et al. 2003
TC31	W3110, Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT* Δ*adhE*::*FRT*	Causey et al. 2003
TC32	W3110, (Succ$^-$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT-tet-FRT*	Causey et al. 2003
TC35	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT-tet-FRT*	Causey et al. 2003
TC36	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT*	Causey et al. 2003
TC41	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*poxB*::*FRT-tet-FRT*	Causey et al. 2004
TC42	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*poxB*::*FRT*	Causey et al. 2004
TC45	W3110, Δ*atpA*::*FRT-tet-FRT*	This study
TC49	W3110, (Succ$^+$), *ycaC*::*FRT-aap-FRT*::*ycaD focA*$^+$ *pflB*$^+$ Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT*	This study
TC51	W3110, (Succ$^+$), *ycaC*::*FRT*::*ycaD focA*$^+$ *pflB*$^+$ Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT*	This study
TC55	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*atpA*::*FRT-tet-FRT*	This study
TC56	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp(FH)*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*atpA*::*FRT*	This study

Table 2-2. Sources and characteristics of plasmids

Plasmids	Relevant Characteristics	Reference
pCR2.1-TOPO	*bla kan,* TOPO^{TM} TA cloning vector	Invitrogen
pFT-A	*bla flp* low-copy vector containing recombinase and temperature-conditional pSC101 replicon	Posfai et al. 1997
pKD46	*bla* γ β *exo* low-copy vector containing red recombinase and temperature-conditional pSC101 replicon	Datsenko and Wanner 2000
pLOI2065	*bla, Sma*I fragment with *FRT* flanked *tet* gene	Underwood et al. 2002
pLOI2075	*bla kan poxB*	Causey et al. 2004
pLOI2078	*bla poxB*	Causey et al. 2004
pLOI2080	*bla poxB*::*FRT-tet-FRT*	Causey et al. 2004
pLOI2403	*bla*	Martinez-Morales et al. 1999
pLOI2745	*kan* temperature conditional replicon	This study
pLOI2800	*bla kan sucA*	Causey et al. 2003
pLOI2801	*bla kan sucA*::*FRT-tet-FRT*	Causey et al. 2003
pLOI2802	*bla kan adhE*	Underwood et al. 2002
pLOI2803	*bla kan adhE*::*FRT-tet-FRT*	Causey et al. 2003
pLOI2805	*bla kan atpEFH*	Causey et al. 2003
pLOI2820	*bla kan atpA*	This study
pLOI2821	*bla kan atpA*::*FRT-tet-FRT*	This study
pLOI3421	*EcoRI* fragment containing *FRT* flanked *aap* gene	This study
pLOI3430	*bla kan ycaC ycaD*	This study
pLOI3439	*kan ycaC ycaD* temperature conditional replicon	This study
pLOI3447	*bla kan ycaC FRT-aac-FRT ycaD* used for repairing *focA pflB* deletion	This study

$ZnCl_2$ ● 4 H_2O, 0.2 g $NaMoO_4$, and 0.05 g H_3BO_3). MOPS (0.1 M, pH 7.1) was added to both liquid and solid media (filter-sterilized) when needed for pH control, but was not included in medium used for 10 L fermentations. Minimal medium was also prepared using succinate (1 g L^{-1}) as a sole source of carbon (nonfermentable substrate). Succinate (1 g L^{-1}) was also added as a supplement to glucose-minimal medium when needed. During plasmid and strain construction, cultures were grown in Luria-Bertani (LB) broth or on LB agar plates (1.5% agar) (Miller 1992). Glucose (2%) was added to LB medium for all strains containing mutations in (F1F0)H+-ATP synthase. Antibiotics were included as appropriate (kanamycin, 50 mg L^{-1}; ampicillin, 50 mg L^{-1}; and tetracycline,

12.5 or 6.25 mg L^{-1}). Fusaric acid plates were used to select for loss of Tn10-encoded tetracycline resistance (Zhou et al. 2003).

Genetic Methods

Standard methods were used for plasmid construction, phage P1 transduction, electroporation, and polymerase chain reaction (PCR) (Miller 1992; Sambrook and Russell 2001). Chromosomal DNA from *E. coli* W3110 (and derivatives) served as a template to amplify genes using primers complementary to coding regions (ORFmers) purchased from the Sigma-Genosys, Inc. (The Woodlands, TX). PCR products were initially cloned into plasmid vector pCR2.1-TOPO. During plasmid constructions, restriction products were converted to blunt ends using either the Klenow fragment of DNA polymerase I (5' overhang) or T4 DNA polymerase (3' overhang) as needed. Integration of linear DNA was facilitated by using pKD46 (temperature conditional) containing an arabinose-inducible Red recombinase (Datsenko and Wanner 2000). Integrants were selected for tetracycline resistance (6.25 mg L^{-1}) and screened for appropriate antibiotic resistance markers and phenotypic traits. At each step, mutations were verified by analyses of PCR products and fermentation products. *FRT*-flanked antibiotic resistance genes used for selection were deleted using a temperature-conditional plasmid (pFT-A) expressing FLP recombinase from a chlortetracycline-inducible promoter (Martinez-Morales et al. 1999; Posfai et al. 1997).

Fermentation

Acetate production was examined in glucose-minimal medium containing 167 mM glucose using a New Brunswick Bioflow 3000 fermentor (New Brunswick Scientific, Edison, NJ) with a 10 L working volume (37°C, dual Rushton impellers, 450 rpm). Dissolved oxygen was maintained at 5% of air saturation (unless otherwise stated) by

altering the proportion of N_2 and O_2. Broth was maintained at pH 7.0 by the automatic addition of 11.4 M KOH. During fed batch experiments, additional glucose was added from a sterile 60% stock. Three fed batch regimes were investigated: A. 3% glucose initially with the addition of 3% after 12 h (6% total); B. 6% glucose initially with the addition of 4% glucose after 16 h (10% total); C. 3% glucose initially with multiple additions to maintain glucose levels above 100 mM. Five liter fermentations (7 L vessel, 350 rpm using dual Rushton impellers) were used to examine the effects of deletion of *atpFH* and *atpA* or the effect(s) of restoring pyruvate formate-lyase activity. For these fermentations the dissolved oxygen was allowed to fall from 100% to 5% of air saturation at which time O_2 was mixed with air to maintain a dissolved oxygen concentration higher than 5%.

Seed cultures were prepared by inoculating colonies from a fresh plate (48 h) into 3 mL of glucose-minimal medium (13 x 100 mm tube) containing 0.1 M MOPS. After incubation for 14 h (120 rpm rotator), cultures were diluted 400-fold into 1 L baffled flasks containing 200 mL of mineral salts medium (37°C, 280 rpm). When cells reached 1.5-2.2 OD_{550nm}, sufficient culture volume was harvested (5000 x g, 25°C) to provide an inoculum of 33 mg dry cell weight L^{-1} in the 10 L working volume.

Broth samples were removed to measure organic acids, residual glucose, and cell mass. Volumetric and specific rates were determined from measured values for glucose and acetate using GraphPad Prism (GraphPad Software, San Diego, CA). A smooth curve was generated with 10 points per min (Lowess method) to fit measured results. The first derivative (acetate or glucose versus time) of each curve served as an estimate of

volumetric rate. Specific rates (mmoles L^{-1} h^{-1} [mg dry cell weight]$^{-1}$) were calculated by dividing volumetric rates by respective values for cell mass.

ATPase Assay

Cells were grown for enzyme assays as described above for seed cultures. Upon reaching 0.75-1.0 OD_{550nm} , cultures were chilled on ice and harvested by centrifugation (8000 x g, 5 min at 4^{o}C). Cell pellets were washed 5 times with 0.1 M Tris-HCl (pH 7.55), resuspended in 1 mL of this buffer, and broken using a model W220F ultrasonic cell disruptor (Heat Systems Ultrasonics, Plainview, NY). Total ATPase activity in disrupted cell preparations was assayed at pH 7.55 essentially as described by Evans (Evans 1969). Inorganic phosphate was measured by the method of Rathbun and Betlach (Rathbun and Betlach 1969). Results represent an average of three cultures for each strain. Specific activity is expressed as μmol P_i released min^{-1} (mg protein)$^{-1}$.

Analyses

Organic acids and glucose concentrations were determined using a Hewlett Packard HPLC (HP 1090 series II) equipped with a UV monitor (210 nm) and refractive index detector. Products were separated using a Bio-Rad HPX-87H column (10 μl injection) with 4 mM H_2SO_4 as the mobile phase (0.4 mL min^{-1}, 45°C). Cell mass was estimated by measuring OD_{550nm} (1.0 OD_{550nm} is equivalent to 0.33 g dry cell weight L^{-1}) using a Bausch & Lomb Spectronic 70 spectrophotometer with 10 x 75 mm culture tubes as cuvettes. Protein concentration was determined using the BCA Protein Assay Kit from Pierce (Rockford, IL).

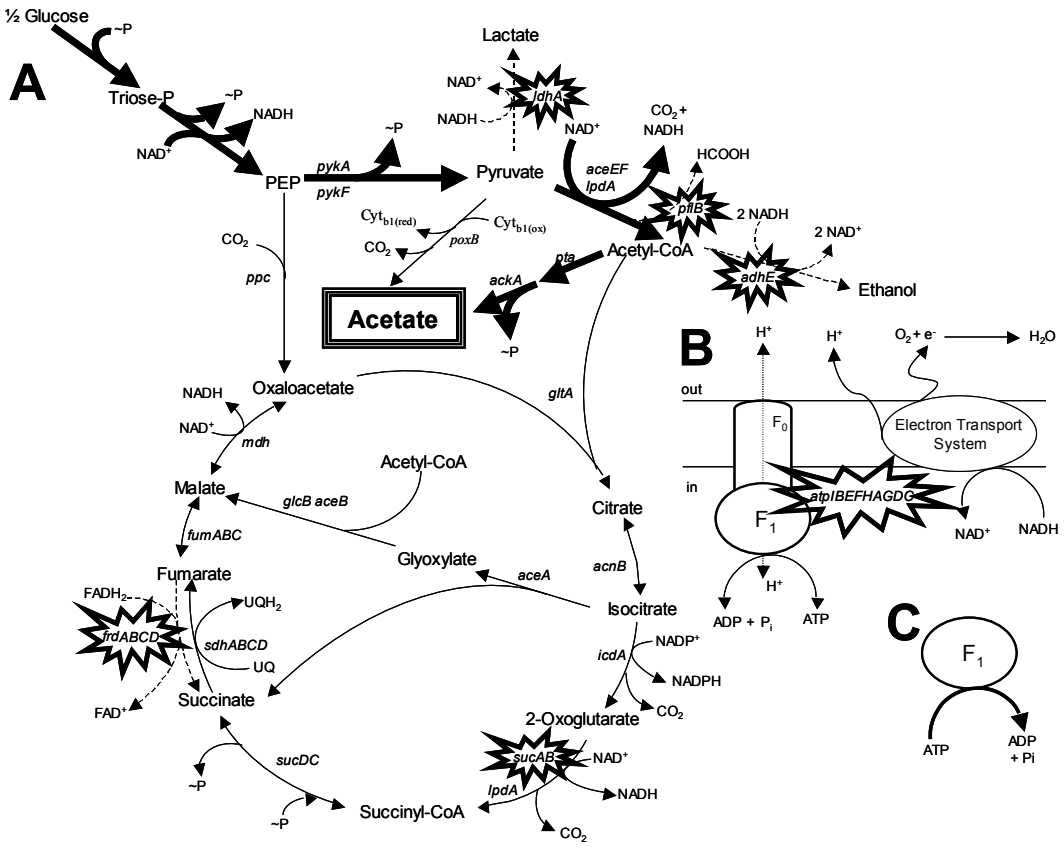

Figure 2-2. Diagram summarizing genetic modifications used to redirect glucose metabolism to acetate. Bold arrows mark principal metabolic routes in TC36. Dashed arrows represent fermentative pathways. Reactions that have been blocked are highlighted. A) Central carbon metabolism. Acetate is the principal product from sugar metabolism with the net production of four ATP equivalents per glucose molecule. B) Oxidative phosphorylation. The $(F_1F_0)H^+$-ATP synthase is inactive in TC36, although the electron transport system remains functional as the primary route for NADH oxidation. C) F_1-ATPase. TC36 lacks the essential subunits coupling the ATP synthase to the membrane and thus defective in ATP synthesis but retains a functional cytoplasmic F_1-ATPase. Genes: *aceA*, isocitrate lyase; *aceB*, malate synthase B; *aceEF,* E1p and lipoate acetyltransferase/dihydrolipoamide acetyltransferase component of the PDHc; *ackA*, acetate kinase; *acnB*, aconitase; *adhE*, alcohol/aldehydrogenase pyruvate formate lyase deactivase; *atpIBEFHAGDC*, $(F_1F_0)H^+$-ATP synthase; *frdABCD*, fumarate reductase; *glcB*, malate synthase G; *gltA*, citrate synthase; *icdA*, isocitrate dehydrogenase; *ldhA*, lactate dehydrogenase; *lpdA*, dihydrolipoate dehydrogenase/dihydrolipoamide dehydrogenase component of the PDHc and 2-oxoglutarate dehydrogenase; *pflB*, pyruvate formate-lyase; *poxB*, pyruvate oxidase; *pta*, phosphotransacetylase; *pykA*, pyruvate kinase II; *pykF*, pyruvate kinase I; *sdhABCD*, succinate dehydrogenase; *sucAB*, 2-oxoglutarate dehydrogenase; *sucDC*, succinyl-CoA synthetase.

Results

Elimination of Fermentation Products (Strain SZ47)

Inspection of native pathways in *E. coli* (Fig. 2-2A) indicated that the production of acetate and CO_2 as sole metabolic products from glucose would require an external electron acceptor such as oxygen. Due to low oxygen solubility, however, it is difficult to satisfy the oxygen demand from active *E. coli* metabolism. Typically a portion of substrate is converted into fermentation products (Tsai et al. 2002; Varma et al. 1993). This problem was eliminated by combining deletions in genes encoding lactate dehydrogenase, pyruvate formatelyase, and fumarate reductase as follows. The *ldhA*::Tn10 mutation in *E. coli* SE2279 was transduced into *E. coli* W3110 using phage P1 to produce strain SZ33. P1 phage grown on SZ33 was used to transfer this mutation into SZ40(Δ(*focA-pflB*)::*FRT* Δ*frdBC*) to produce SZ46. Tetracycline-sensitive derivatives of SZ46 were selected using fusaric acid medium (Zhou et al. 2003). One clone was designated SZ47 (Δ(*focA-pflB*)::*FRT* Δ*frdBC* Δ*ldhA*). The Δ*ldhA* mutation in SZ47 was confirmed by the absence of lactate in fermentation broth, inability to grow anaerobically in glucose-minimal medium, and by PCR analysis using *ldhA* ORFmers (1.0 kbp for the wild type *ldhA* as compared to 1.1 kbp for SZ47). The slightly larger size of the amplified product from SZ47 is attributed to remnants of Tn10.

Disruption of Oxidative Phosphorylation (Strains TC24 and TC57).

Growth under oxidative conditions is characterized by conversion of up to 50% of substrate carbon to cell mass (Neidhardt et al. 1990). To reduce the potential drain of substrate into cell mass, a mutation was introduced into SZ47 that deleted portions of two subunits in $(F_1F_0)H^+$-ATP synthase required for assembly to the plasma membrane (Sorgen et al. 1998), disrupting oxidative phosphorylation while preserving the hydrolytic

activity of F_1-ATPase in the cytoplasm. To construct this deletion, the *atpEFH* coding region of the *atpIBEFHAGDC* operon was amplified by PCR using primers (ORFmers) complementary to the 5'- end of the *atpE* gene and the 3'- end of the *atpH* . The amplified fragment (1.3 kbp) was cloned into pCR2.1-TOPO and one clone was selected in which the *atpEFH* genes were oriented to permit expression from the *lac* promoter (pLOI2805; Fig. 2-3). The *atpF* gene and 117 nucleotides at the 5' end of *atpH* gene were removed from pLOI2805 by digestion with *Hpa*I and *Bst*EII (Klenow-treated). This region was replaced with a 1.7 kbp *Sma*I fragment from pLOI2065 containing the *FRT-tet-FRT* cassette to produce pLOI2807 (Fig.2-3). After digestion with *Sca*I, pLOI2807 served as a template for amplification of the *atpE*Δ(*FH*)::*FRT-tet-FRT* region (2.4 kbp) using the 5' *atpE* and 3' *atpH* primers. Amplified DNA was precipitated, digested again with *Sca*I to disrupt any residual plasmid, and purified by phenol extraction. This DNA was introduced into *E. coli* W3110(pKD46) by electroporation while expressing Red recombinase. Plasmid pKD46 was eliminated by growth at 42^{o}C. Recombinants (double cross-over) were identified using antibiotic markers (tetracycline resistant; sensitive to ampicillin and kanamycin) and by the inability to grow on succinate-minimal plates in the absence of glucose (fermentable carbon source). Integration was further confirmed by PCR analysis using the 5' *atpE* primer and the 3' *atpH* primer (1.3 kbp fragment for W3110; 2.3 kbp fragment for mutants). One clone was selected and designated TC21(Δ*atp*(*FH*)::*FRT-tet-FRT*).

Phage P1 was used to transduce the Δ*atp*(*FH*)::*FRT-tet-FRT* mutation in TC21 to SZ47 and produce TC23. The *tet* gene was removed from TC23 by the FLP recombinase (pFT-A). After elimination of pFT-A by growth at 42^{o}C, the Δ*atp*(*FH*)::*FRT* mutation

was further confirmed by PCR analysis using the 5' *atpE* primer and the 3' *atpH* primer (0.8 kbp for deletion and 1.3 kbp for SZ47). The resulting strain was designated TC24 (Δ(*focA-pflB*)::*FRT* Δ*frdBC* Δ*ldhA* Δatp(*FH*)::*FRT*). TC57 was unable to grow in minimal medium without a fermentable carbon source (substrate level phosphorylation) but retained the ability to oxidize NADH by the electron transport system.

In order to demonstrate the effectiveness of preserving the hydrolytic activity of the F_1 subunit, a deletion was inserted into the *atpA* gene which codes for the alpha subunit of the $(F_1F_0)H^+$-ATP synthase. To construct this deletion, the *atpA* coding region of the *atpIBEFHAGDC* operon was amplified by PCR using primers (ORFmers) complementary to *atpA* . The amplified fragment (1.5 kbp) was cloned into pCR2.1-TOPO and one clone was selected in which the *atpA* gene was oriented to permit expression from the *lac* promoter (pLOI2820). A 249 bp section was removed from the central region of *atpA* by digestion with *SmaI* (blunt). This region was replaced with a 1.7 kbp *Sma*I fragment from pLOI2065 containing the *FRT-tet-FRT* cassette to produce pLOI2821. After digestion with *Hin*dIII and *PvuII*, pLOI2821 served as a template for amplification of the *atpA*::*FRT-tet-FRT* region (2.9 kbp) using the *atpA* primers. Amplified DNA was gel-purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). This DNA was introduced into *E. coli* W3110(pKD46) by electroporation while expressing Red recombinase. Plasmid pKD46 was eliminated by growth at 42°C. Recombinants (double cross-over) were identified using antibiotic markers (tetracycline resistant; sensitive to ampicillin and kanamycin) and by the inability to grow on succinate-minimal plates in the absence of glucose (fermentable carbon source). Integration was further confirmed by PCR analysis using *atpA* primers (1.5 kbp fragment

for W3110; 2.9 kbp fragment for mutants). One clone was selected and designated TC45 (Δ*atpA*::*FRT-tet-FRT*).

Phage P1 was used to transduce the Δ*atpA*::*FRT-tet-FRT* mutation in TC45 to TC36 and produce TC55. The *tet* gene was removed from TC45 by the FLP recombinase (pFT-A). After elimination of pFT-A by growth at 42°C, the Δ*atpA*::*FRT* mutation was further confirmed by PCR analysis using the *atpA* primers (1.2 kbp for deletion and 1.5 kbp for TC36). The resulting strain was designated TC57 (Δ(*focA-pflB*)::*FRT* Δ*frdBC* Δ*ldhA* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*atpFH*::*FRT* Δ*atpA*::*FRT*).

Inactivation of Alcohol Dehydrogenase E (Strain TC31)

The native bifunctional alcohol/aldehyde dehydrogenase (*adhE*) in *E. coli* catalyzes the reduction of acetyl~CoA to ethanol, a reaction that directly competes with the conversion of acetyl~CoA to acetate by phosphotransacetylase and acetate kinase (Fig. 2-2A). To eliminate this activity, phage P1 was used to transduce the Δ*adhE*::*FRT-tet-FRT* mutation in TC20 to TC24 and produce TC30. Chromosomal integration was confirmed by PCR analysis using *adhE* primers (2.7 kbp for TC24 and 3.2 kbp for the Δ*adhE*::*FRT-tet-FRT* mutant). The *tet* gene was deleted from TC30 by FLP recombinase using pFT-A. After elimination of pFT-A by growth at 42°C, a clone was selected and designated TC31(Δ(*focA-pflB*)::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT*).

Interruption of the Tricarboxylic Acid Cycle (strain TC32).

During oxidative growth, up to 50% of substrate carbon can be released as CO_2 (Neidhardt et al. 1990). This loss of carbon can be attributed in large measure to the high efficiency of the tricarboxylic acid cycle and the electron transport system (NADH oxidation). During fermentative metabolism, the production of CO_2 and NADH are

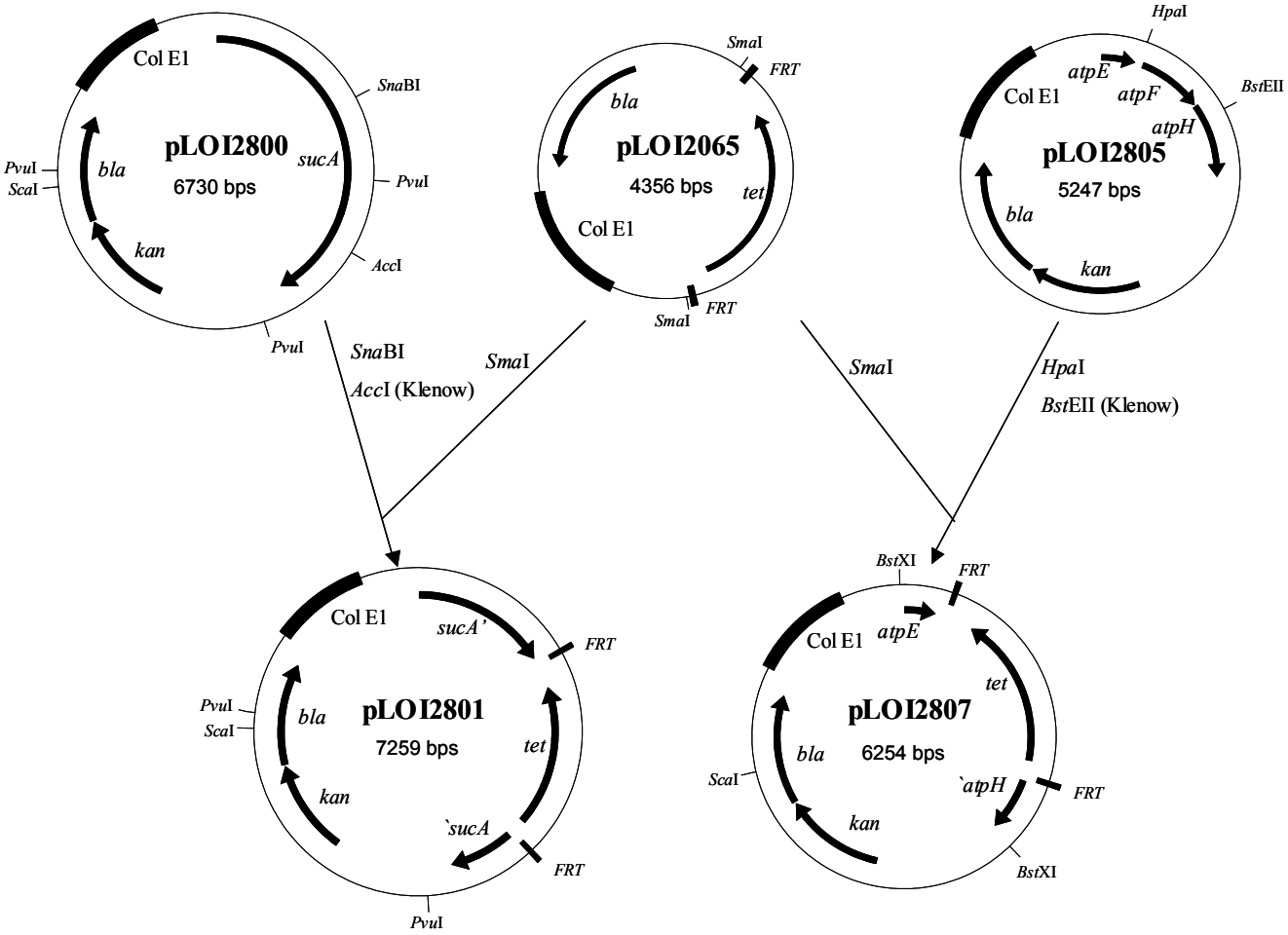

Figure 2-3. Diagram summarizing plasmid construction

reduced primarily by repression of *sucAB* encoding 2-oxoglutarate dehydrogenase (Cunnungham and Guest 1998; Park and Gunsalus 1997), disrupting the cyclic function of the tricarboxylic acid cycle. We have genetically imposed a similar restriction in carbon flow during oxidative metabolism by deleting part of the *sucA* gene.

The *sucA* coding region was amplified using ORFmers. The resulting 2.8 kbp PCR product was cloned into pCR2.1-TOPO to produce pLOI2800 (Fig. 2-3) in which the *sucA* coding region was oriented to permit expression from the *lac* promoter. A 1.1 kbp fragment was removed from the central region of *sucA* by digestion of pLOI2800 with *Sna*BI and *Acc*I (Klenow-treated). This region was replaced with a 1.7 kbp *Sma*I fragment containing the *FRT-tet-FRT* cassette from pLOI2065 to produce pLOI2801 (Fig. 2-3). Plasmid pLOI2801 was digested with *Pvu*I and *Sca*I and used as a template to amplify the 3.3 kbp region containing *sucA*::*FRT-tet-FRT* using *sucA* ORFmers. Amplified DNA was precipitated, digested with *Pvu*I and *Sca*I to disrupt any residual circular plasmid, and extracted with phenol. Purified DNA was electroporated into *E. coli* W3110(pKD46) while expressing Red recombinase. Plasmid pKD46 was eliminated by growth at 42°C. Disruption of *sucA* was confirmed by PCR analysis using *sucA* ORFmers (2.8 kbp fragment for wild type and 3.3 kbp for *sucA*::*FRT-tet-FRT* mutants) and designated TC25.

Phage P1 was used to transduce the *sucA*::*FRT-tet-FRT* mutation from TC25 into TC31. Transfer of this mutation was verified by PCR analysis (2.8 kbp for wild type *sucA* and 3.3 kbp for *sucA*::*FRT-tet-FRT* mutants) and phenotype ($Succ^-$). Inactivation of 2-oxoglutarate dehydrogenase (Δ*sucA*) in this Δ*frdBC* background resulted in an undesirable auxotrophic requirement for succinate ($Succ^-$) during growth on glucose-

minimal medium. The resulting strain was designated TC32(Succ$^-$, Δ(*focA-pflB*)::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT-tet-FRT*).

Elimination of Succinate Requirement (Strain TC36)

Spontaneous mutants of TC32, that did not require succinate in the growth medium (Succ$^+$), were readily obtained after serial transfers in glucose-minimal broth containing decreasing amounts of succinate (4 mM to 0.4 mM) followed by selection on glucose minimal plates without succinate. Over 170 clones were recovered per mL of culture after enrichment (approximately 3% of viable cells). Ten clones were tested and all grew in glucose minimal broth without succinate and produced acetate as the dominant product. One was selected (TC35) for deletion of the *tet* gene using the FLP recombinase. This deletion was confirmed by analysis of PCR products using *sucA* primers (3.3 kbp for TC35 and 1.8 kbp after *tet* deletion). The resulting strain was designated TC36 (Succ$^+$; Δ(*focA-pflB*)::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT*). Although the genetic change that eliminated the succinate requirement was not identified, likely candidates include mutations which provide a low constitutive expression of *aceAB* (glyoxylate shunt) or *citC* (citrate lyase).

Total ATPase activity was examined in disrupted cell extracts of TC36 and W3110 (wild type). The activity in TC36 (0.355 U [mg protein]$^{-1}$) was equivalent to 71% of the unmodified parent (0.502 U [mg protein]$^{-1}$), confirming that F_1-ATPase was not inactivated by the Δ*atpFH*::*FRT* mutation. This is similar to the levels of ATPase reported for an *atpH* mutant of *E. coli* which blocked membrane assembly and coupling to oxidative phosphorylation (Sorgen et al. 1998).

Disruption of Pyruvate Oxidase (Strain TC42)

A single derivative of pCR2.1-TOPO was selected in which the amplified *poxB* gene was oriented in the same direction as the *lac* promoter (pLOI2075). To eliminate extraneous *Bsa*BI sites in the vector, the small *Eco*RI fragment containing *poxB* was ligated into the corresponding site of pLOI2403 to produce pLOI2078. The *Sma*I fragment from pLOI2065 containing a *tet* gene flanked by *FRT* sites was ligated into the unique *Bsa*BI site in *poxB* to produce pLOI2080. Plasmid pLOI2080 served as a template for the amplification of *poxB*::*FRT-tet-FRT* (3.4 kbp) using *poxB* primers. Amplified DNA was integrated into *E. coli* W3110(pKD46) as previously described (Causey et al. 2003) to produce LY74. The *poxB*::*FRT-tet-FRT* mutation in LY74 was transduced into TC36 to produce TC41. The *tet* gene was removed using FLP recombinase and the resulting strain designated TC42 (Δ*focA-pflB*::*FRT* Δ*frdBC*::*FRT* Δ *ldhA* Δ*atpFH*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT poxB*::*FRT*).

Restoration of Pyruvate Formate-lyase Activity (Strain TC51)

Strain LY117 was constructed for restoring pyruvate formate-lyase activity by a colleague, Lorraine Yomano, in which an apramycin marker flanked by *FRT* sites was inserted downstream of the *focA-pflB* operon as follows. The *ycaC* and *ycaD* region (2.1 kbp), located 2.6 kbp downstream from *pflA*, was PCR amplified and cloned into TOPO-pCR2.1 using the 3' *ycaC* primer (ORFmer) and the 3' *ycaD* primer (ORFmer) to generate plasmid pLOI3430. In order to limit the background after electroporation the *EcoRI* fragment from pLOI3430 containing *ycaC ycaD* was ligated into the *EcoRI* site of pLOI2745 generating a new plasmid, pLOI3439, which contains a temperature conditional replicon. The *EcoRI* fragment, containing an apramycin resistance marker flanked by *FRT* sites (1.9kbp), was treated with the Klenow fragment of DNA

polymerase I to generate blunt ends and ligated into the *XmnI* site of pLOI3439. The new plasmid contained a *ycaC*::*FRT-aap-FRT*::*ycaD EcoRI* fragment (3.5 kbp) and a temperature conditional replicon.

Plasmid pLOI3447 was digested with *EcoRI* and the 3.5 kbp *ycaC*::*FRT-aap-FRT*::*ycaD* fragment was purified by gel extraction (Qiagen, Valencia, CA). The DNA was integrated into *E. coli* W3110(pKD46) as previously described (Causey et al. 2003) to produce LY117 (*ycaC*::*FRT-aap-FRT*::*ycaD*). The *ycaC*::*FRT-aap-FRT*::*ycaD* mutation in LY117 was transduced into TC36 to produce TC49. The *aap* gene was was removed using FLP recombinase and the resulting strain designated TC51 ($focA^+$ $pflB^+$ Δ*frdBC*::*FRT* Δ *ldhA* Δ*atpFH*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT poxB*::*FRT*). The $focA^+$ $pflB^+$ genotype was verified by PCR and pyruvate formate-lyase activity was verified by observing formate in fermentation broths.

Effects of Gene Disruptions on Growth and Glycolytic Flux

Strain TC36 was genetically engineered for the production of acetate from carbohydrates such as glucose (Fig. 2-2A, B and C). Batch fermentations with pH control were used to compare the performance of this strain with W3110 (wild type) and two intermediate strains used in the construction, SZ47(Δ(*focA-pflB*) Δ*frdBC* Δ*ldhA*) and TC24(Δ(*focA-pflB*) Δ*frdBC* Δ*ldhA* Δ*atpFH*). Based on preliminary experiments, 5% oxygen saturation and 3% glucose (37°C) were selected as test conditions. Broth pH was maintained at 7.0 to minimize toxicity from undissociated acids.

Disruption of oxidative phosphorylation and the cyclic function of the tricarboxylic acid cycle, elimination of the primary fermentation pathways, and the production of acetate as the primary endproduct from glycolysis had relatively little effect on the growth of *E. coli*. The maximum growth rates for strains W3110 (wild type) and SZ47

(lacking the three native fermentation pathways) were similar although the cell yield for SZ47 was higher (Fig. 2-4A; Table 2 and Table 3). Inactivation of oxidative phosphorylation (Δ*atpFH*) in SZ47 resulted in a modest reduction (12%) in growth rate

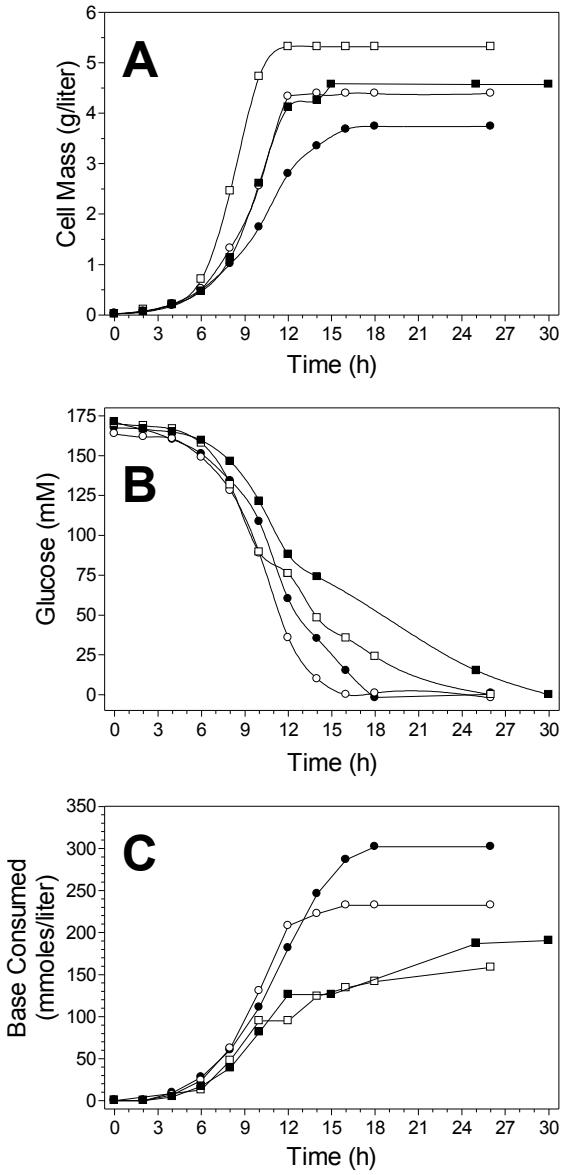

Figure 2-4. Effect of selected mutations on *E. coli* metabolism. A) growth B) glucose utilization C) KOH consumption. Solid squares, W3110 (wild type); Open squares, SZ47 (Δ[*focA-pflB*]::*FRT* Δ*frdBC* Δ*ldhA*); Open circles, TC24 (Δ[*focA-pflB*]::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*[FH]::*FRT*); Solid circles, TC36 (Δ[*focA-pflB*]::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*[FH]::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT*).

(TC24). Cell yield for strain TC24 was equivalent to that of W3110. Strain TC36, containing additional mutations in 2-oxoglutarate dehydrogenase (Δ*sucA*) and alcohol dehydrogenase (Δ*adhE*), had a lower cell yield and growth rate; approximately 80% of the unmodified parent W3110.

Rates for glucose utilization (maximum specific and average volumetric) were higher for TC36 and TC24 than for W3110 and SZ47 (Table 2). This increase in metabolic activity can be primarily attributed to the Δ*atpFH* mutation. ATP levels serve as an allosteric regulator of several key glycolytic enzymes (Neidhardt et al. 1990), and acetate kinase (Suzuki 1969). In strains containing the Δ*atpFH* mutation, increased glycolytic flux and substrate level phosphorylation could partially compensate for the loss of ATP production from respiration-coupled phosphorylation. Differences between strains were particularly evident when comparing incubation times required to complete sugar metabolism (Fig. 2-4B). With TC36 and TC24, glucose was exhausted in 16-18 h as compared to 26 h for SZ47 and 30 h for W3110. The maximum specific rate of glucose utilization (glycolytic flux) was 9 mmole h^{-1} (g dry cell weight)$^{-1}$ in the unmodified parent (W3110), 20 mmole h^{-1} (g dry cell weight)$^{-1}$ in TC24, and 18 mmole h^{-1} (g dry cell weight)$^{-1}$ in TC36.

Table 2-3. Comparison of metabolic rates

		Glucose consumption rate[a]		Pyruvate production rate[a]	
Strain	μ (h^{-1})	Average Vol.[b] (mmol L^{-1} h^{-1})	Max. Spec.[b] (mmol L^{-1} h^{-1} g^{-1})	Average Vol.[b] (mmol L^{-1} h^{-1})	Max. Spec.[b] (mmol L^{-1} h^{-1} g^{-1})
W3110	0.60	7	9	4	10
SZ47	0.55	9	11	4	10
TC24	0.53	10	20	10	16
TC36	0.49	11	18	12	16

[a] Data are derived from averages of two fermentations.
[b] Average volumetric rates for glucose consumption and acetate production or maximum specific rates (dry cell weight basis) for glucose consumption and acetate production.

Table 2-4. Summary of fermentation products

Strain	Conditions	Cell yield ($g\ L^{-1}$)	Fermentation Products[a] ($mmoles\ L^{-1}$)						Yield (%[b])	Carbon recovery (% substrate C[c])
			Acetate	2-Oxoglutarate	Fumarate	Lactate	Pyruvate	Succinate		
W3110	3% glucose 5% DO	4.5	30	39	0.8	33	< 1	5	9	60
SZ47	3% glucose 5% DO	5.3	6	11	0.9	< 1	1	3	2	20
TC24	3% glucose 5% DO	4.4	156	1	1.0	< 1	< 1	2	47	66
TC36	3% glucose 5% DO	3.5±0.2	224±14	16±6	0.4±0.1	< 1	0±0.5	4±1	68	89
TC36	3% glucose 15% DO	3.2	190	24	< 1	< 1	< 1	3	57	88
TC36	3% glucose 5% DO N-limited	2.5	220	31	< 1	< 1	< 1	10	66	95

DO, dissolved oxygen.

[a] Concentrations in broth after all glucose had been depleted, except as noted.

[b] Yield expressed as a percentage of the maximal theoretical yield (0.67 g acetate per g glucose).

[c] Carbon recovery represents the percentage of substrate carbon recovered. Recovered carbon was calculated as the sum of carbon in cell mass, fermentation products, and CO_2 (CO_2 calculated as the difference of [acetate + 2-oxoglutarate]-[succinate + fumarate]).

[d] In the final sample, 44 mM glucose was present.

[e] Excess glucose (9.5%) was added to maintain levels above 100 mM; 107 mM glucose was present in the final sample.

Table 2-4. Continued

Strain	Conditions	Cell yield (g L^{-1})	Fermentation Products[a] (mmoles L^{-1})						Yield (%[b])	Carbon recovery (% substrate C[c])
			Acetate	2-Oxoglutarate	Fumarate	Lactate	Pyruvate	Succinate		
TC36	3+3% glucose 5% DO	3.8	523	21	< 1	3	14	2	78	95
TC36	3+3% glucose 5% DO N-limited	3.0	572	33	< 1	< 1	< 1	6	86	102
TC36	6% glucose 5% DO	4.2	415	47	0.3	< 1	46	7	62	92
TC36	6+4% glucose 5% DO[d]	4.5	767	37	0.5	< 1	72	5	72	97
TC36	Fed batch 5% DO[e]	4.1	878	33	3.4	< 1	< 1	25	75	88

DO, dissolved oxygen.

[a] Concentrations in broth after all glucose had been depleted, except as noted.

[b] Yield expressed as a percentage of the maximal theoretical yield (0.67 g acetate per g glucose).

[c] Carbon recovery represents the percentage of substrate carbon recovered. Recovered carbon was calculated as the sum of carbon in cell mass, fermentation products, and CO_2 (CO_2 calculated as the difference of [acetate + 2-oxoglutarate]-[succinate + fumarate]).

[d] In the final sample, 44 mM glucose was present.

[e] Excess glucose (9.5%) was added to maintain levels above 100 mM; 107 mM glucose was present in the final sample.

The slightly lower glycolytic flux in TC36 as compared to TC24 may be related to the increase in ATP yield resulting from improvements in acetate yield (1 ATP per acetate). Assuming protein represents 55% of dry cell weight, maximal glycolytic flux in TC36 is approximately 0.55 μmoles glucose min^{-1} (mg protein)$^{-1}$.

Production of Acetate and Other Organic Acids

A substantial portion of glucose carbon was not recovered in the carbon balance (Table 3) for W3110 (40%) and SZ47 (80%). This loss is attributed to production of volatile products by high flux through the tricarboxylic acid cycle (CO_2) but may also include the reduction of acetyl~CoA to acetaldehyde and ethanol (Fig. 2-2A). Although ethanol was absent in broth samples from all pH-controlled fermentations (sparged at 1 L min^{-1}), a small amount of ethanol (6 mM) was found in seed cultures of W3110 (shaken flasks). No ethanol was present in seed cultures of TC36, which contains a mutation in alcohol dehydrogenase E. The native electron transport system (5% dissolved oxygen) and fermentation pathways in W3110 (Table 3) serve as complementary routes for NADH oxidation. Eliminating the fermentation pathways (SZ47) doubled the loss of carbon as volatile products (Table 3) and increased cell yield but decreased the rate of acetate production in comparison to W3110 (Table 2 and Table 3).

Inactivation of oxidative phosphorylation (Δ*atpFH*) in SZ47 to produce TC24 resulted in a 26-fold increase in acetate yield and a 3-fold improvement in carbon recovery (Table 3, Fig. 2-5A). Acetate yield and carbon recovery increased by another 30% with the introduction of the *sucA* and *adhE* mutations (TC36). TC36 produced an average of 224 mM acetate in 16 h with small amounts of other products. This represents 68% of the maximum theoretical yield using native pathways (2 acetates per glucose), remaining carbon being divided between cell mass, dicarboxylic acids, and CO_2. The

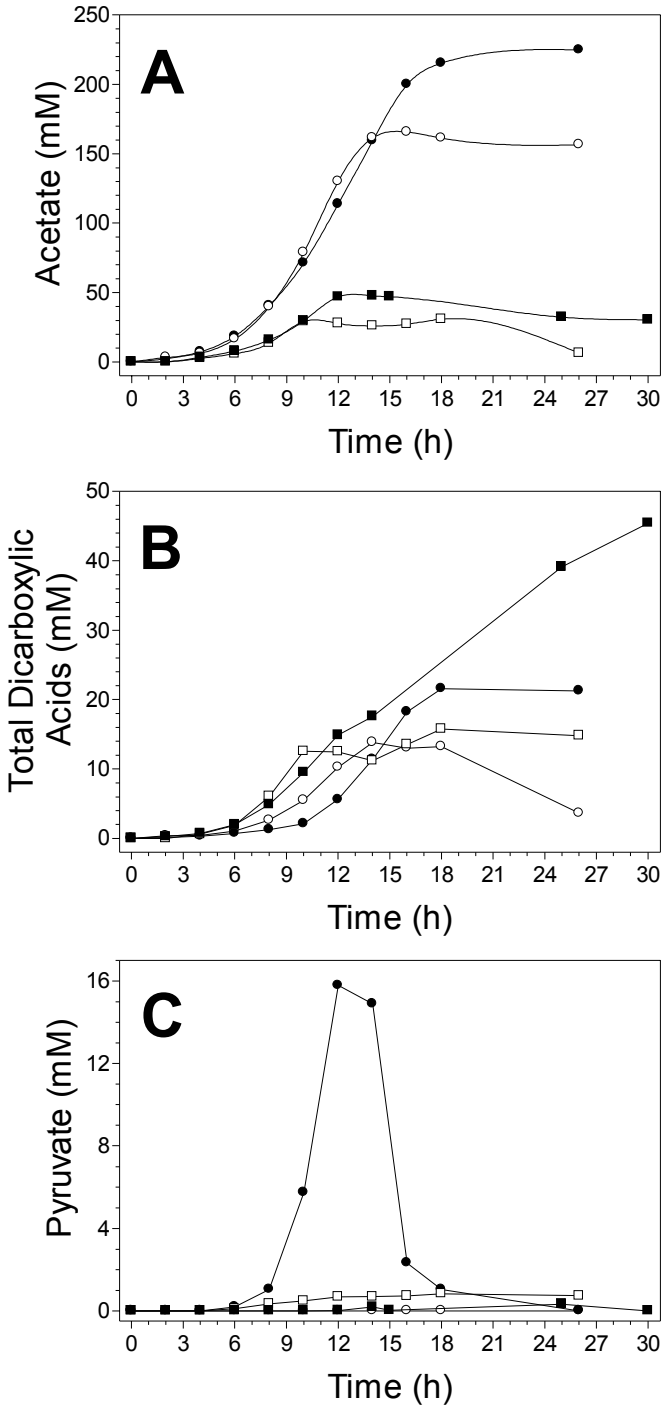

Figure 2-5. Effect of selected mutations on the production of acetate. A) Acetate B) dicarboxylic acids (2-oxoglutarate, succinate and fumarate) C) pyruvate. Symbols: Solid sqaures, W3110 (wild type); Open squares, SZ47 (Δ(*focA--plfB*)::*FRT frdBC* Δ*ldhA*); Open circles, TC24 (Δ(*focA--plfB*)::*FRT frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT*) Solid circles, TC36 ((Δ(*focA--plfB*)::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*sucA*::*FRT* Δ*adhE*::*FRT*).

maximum specific and average volumetric rates of acetate production were approximately 1.6-fold and 2.5-fold higher, respectively, for TC24 and TC36 than for SZ47 and W3110 (Table 2), a difference which can be attributed solely to the mutation in the $(F_1F_0)H^+$-ATP synthase. This mutation eliminated ATP production by oxidative phosphorylation while retaining cytoplasmic F_1-ATPase for the gratuitous consumption of ATP.Strain W3110 accumulated the highest levels of dicarboxylic acids (primarily succinate and 2-oxoglutarate) during glucose metabolism, approximately 3-fold that of the engineered strains (Fig. 2-5B). The order of appearance of dicarboxylic acids in the broth correlated with growth rate and the order of entry into stationary phase for the four strains examined. Dicarboxylic acids were partially consumed as glucose levels declined, and may represent spillover products from excessive glycolysis during the transition from exponential to stationary phase. It is interesting to note that pyruvate levels in the broth of TC36 also increased (16 mM at 12 h) during this transition (Fig. 2-5C). Although this pyruvate was subsequently metabolized, the excretion of pyruvate indicates that glucose uptake and glycolysis per se may not be limiting for acetate production. No significant accumulation of pyruvate was observed for the three other strains (W3110, SZ47 or TC24).

The consumption of base to maintain pH 7.0 provides an overall measure of total organic acid production (Fig. 2-4C). Higher rates and maxima for TC24 and TC36 are consistent with more rapid glucose metabolism. In general, variations in glucose utilization were accompanied by corresponding changes in base utilization. The exponential nature of the early time points reflects growth of the biocatalysts.

Pyruvate Oxidase Contributes to Acetate Production during Stationary Phase

Pyruvate can be metabolized to acetate by the membrane-bound protein pyruvate oxidase using the electron transport system to couple oxygen as a terminal electron acceptor. The *poxB* gene is typically repressed during exponential growth but is induced by stress or entry into stationary phase (Chang and Cronan 1983; Chang et al. 1994). An isogenic Pyruvate oxidase mutant of TC36 was constructed by introducing stop codons in the central region of the gene to produce strain TC42. Introducing the *poxB* mutation had no effect on glucose utilization (Fig. 2-6). No difference was observed in the rate of acetate production for the *poxB* mutant during logarithmic growth (Fig. 2-6). Upon entry into late logarithmic/stationary phase the rate of acetate production by the *poxB* mutant declined resulting in a 20% decrease in final acetate concentration (Fig. 2-6).

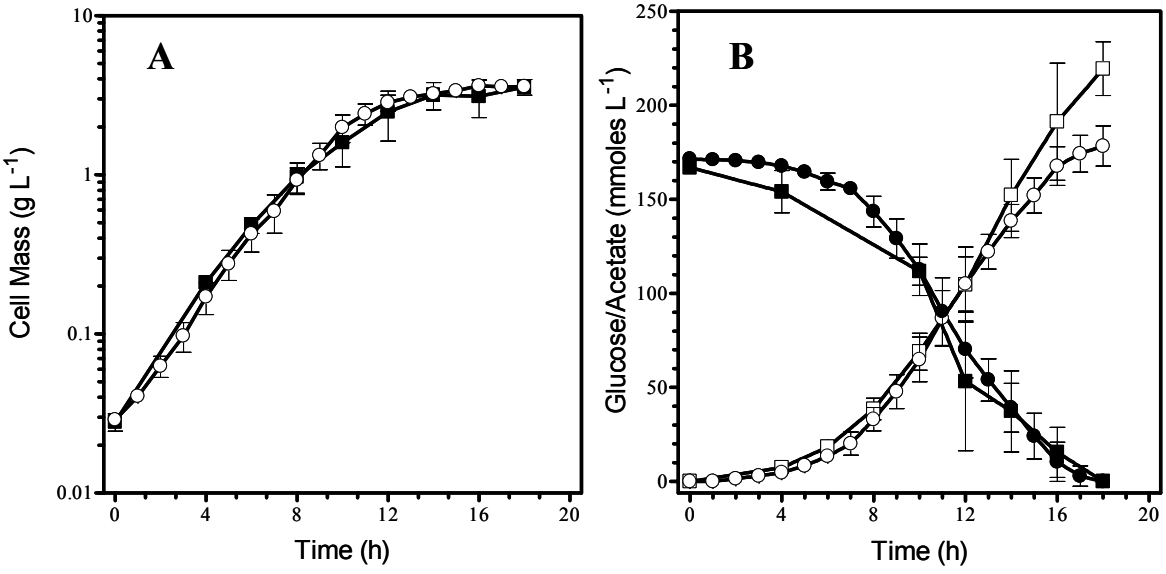

Figure 2-6. Comparison of acetate production between TC36 (*poxB*$^+$) and TC42 (*poxB*$^-$). Data from triplicate fermentations. A) Growth. Symbols: Solid squares, TC36; Open circles, TC42. B) Glucose consumption and acetate production. Symbols: Solid squares, TC36 glucose; Solid circles, TC42 glucose; Open squares, TC36 acetate; Open circles, TC42 acetate.

Restoration of *focA pflB* Had No Effect On Acetate Production from Glucose

Pyruvate formate-lyase catalyzes the non-oxidative decarboxylatation of pyruvate to formate and acetyl-CoA under fermentative conditions. Under the 5% dissolved oxygen *E. coli* W3110 produced 33 mM lactate (Table 2-4) suggesting the cells experience fermentative conditions once the cell density is greater than 2 g L^{-1} dry weight (data not shown). Therefore PFL activity was restored to provide more acetyl-CoA for the phosphotransacetylase reaction in an attempt to increase acetate production. Pyruvate formate-lyase had no effect on growth rate, final cell mass, glucose consumption, or acetate production (Fig. 2-7A and B).

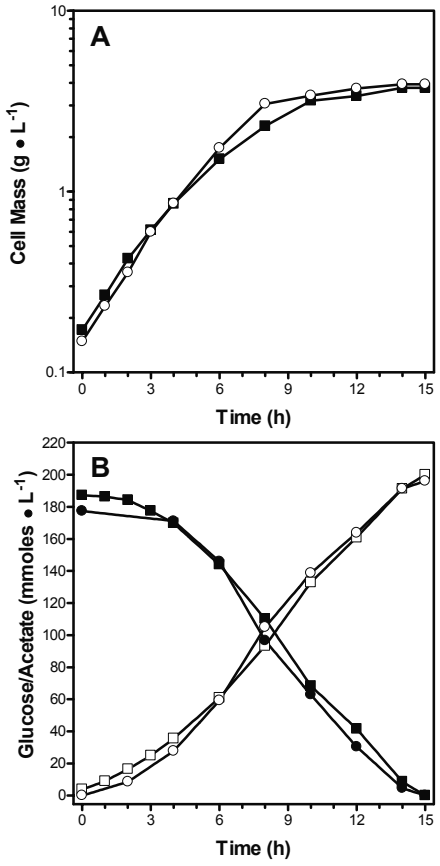

Figure 2-7. Effect of restoring PFL activity on growth and acetate production. A) Growth. B) Glucose consumption and acetate production. Symbols: Circles, TC51; Squares, TC36; Solid symbols represent glucose and open symbols represent acetate in part B.

Elimination of ATPase Activity

The efficacy of the *atpFH* deletion by constructing an isogenic strain containing an additional deletion in the *atpA* gene (TC57). The *atpA* gene codes for the alpha subunit of the $(F_1F_0)H^+$-ATP synthase, an essential protein for catalysis (Boyer 1997). Growth rate and volumetric glucose consumption were reduced by 20% and 25% respectively, for the *atpA* mutant compared to the *atpFH* mutant (Table 2-5). The most striking difference between the two strains was the 62% reduction in the specific glucose consumption rate for TC57. Unfortunately, the elevated glucose consumption rate did not result in an increased rate of acetate production, demonstrated by the modest increase in acetate production rate for TC36 (Table2-5). Where did the rest of the carbon go? Inspection of Figure 2-8 A and B reveals that the increased glucose consumption rateresults in excretion of pyruvate by TC36. The pyruvate is converted to acetate once glucose levels diminish. The increase in pyruvate excretion in TC36 may be due to elevated ADP levels which would allow for increased flow though glycolysis.

Improving Acetate Yields

Dicarboxylic acids and cell mass were the dominant competing co-products from glucose. A limited set of experiments was conducted to evaluate the potential for process

Table 2-5. Effect of *atpA* or *atpFH* deletions on metabolic rates

		Glucose consumption rate[a]		Pyruvate production rate[a]	
Strain	μ (h^{-1})	Average Vol.[b] ($mmol\ L^{-1}\ h^{-1}$)	Max. Spec.[c] ($mmol\ L^{-1}\ h^{-1}\ g^{-1}$)	Average Vol.[b] ($mmol\ L^{-1}\ h^{-1}$)	Max. Spec.[c] ($mmol\ L^{-1}\ h^{-1}\ g^{-1}$)
TC36	0.51± 0.01	10.7±0.9	29.7±3.5	10.7±1.6	31.9±4.7
TC57	0.41	8.0	11.3	9.2	22.1

[a] Five liter fermentations were conducted in 7 L vessels. Dissolved oxygen was allowed to fall from 100% to 5% of air saturation. The data for TC36 represent an average of three fermentations and the data for TC57 represent and average of two fermentations.
[b] Average volumetric rates.
[c] Maximum specific rates per gram of dry cell weight (dcw).

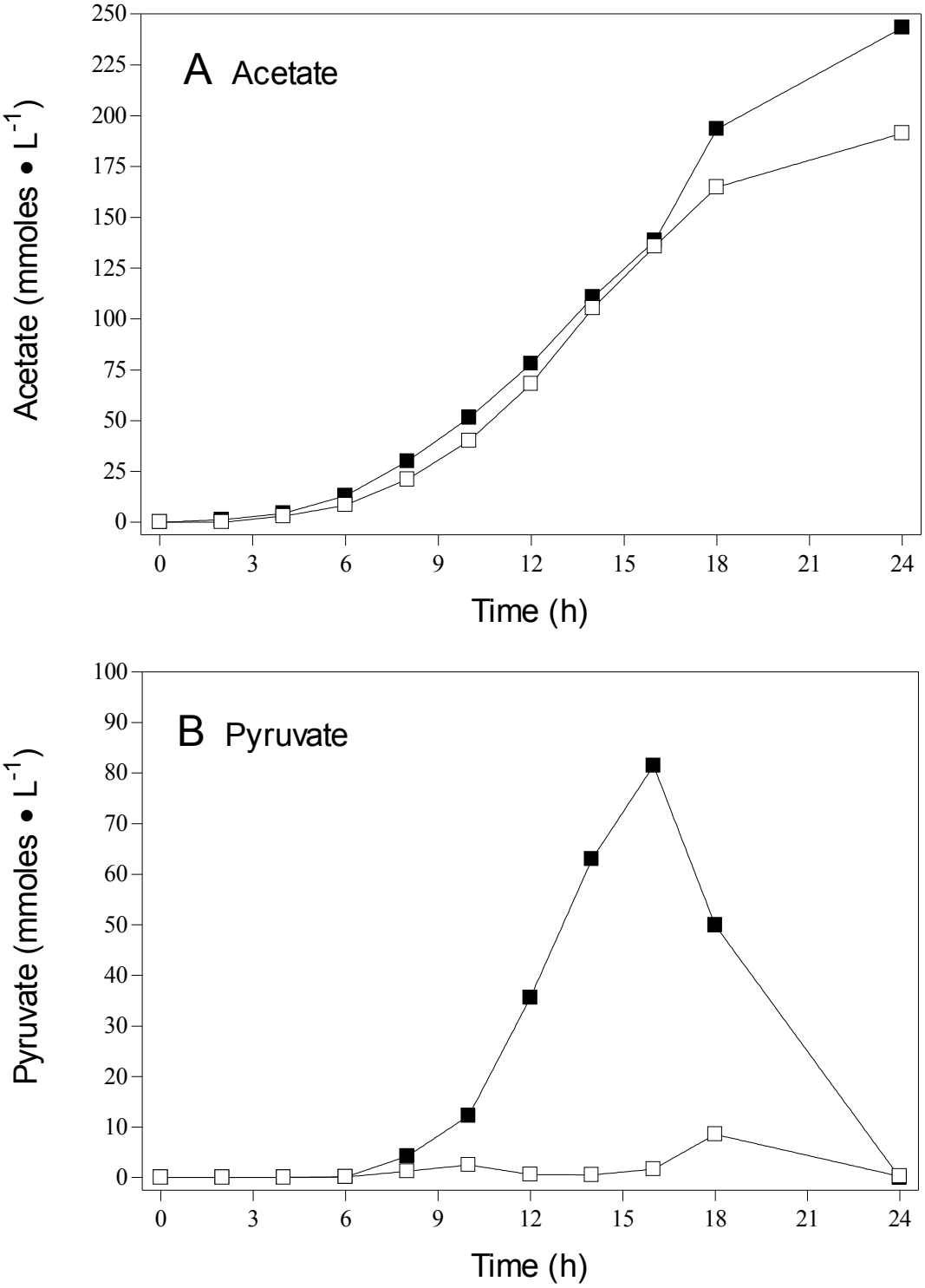

Figure 2-8. Metabolite production in TC36 and TC57. A) Comparison of acetate production between strains. B) Comparison of pyruvate production between strains. Symbols: Solid squares, TC36. Open squares, TC57.

changes to improve acetate yield (Table 2-4). Acetate yield was not improved by increasing the dissolved oxygen level from 5% to 15% of air saturation, reducing ammonia nitrogen (2 g L^{-1} ammonium phosphate) by 40% to limit growth, or increasing the initial concentration of glucose from 3% to 6% (Table 2-4). However, a simple two-step batch feeding strategy was beneficial. A second addition of 3 % glucose at the end of the growth phase (12 h) was metabolized to completion and produced 523 mM acetate with minimal loss to cell mass (Fig. 2-9). Acetate yield for this one-step addition (6% total glucose) was 78 % of the theoretical maximum as compared to 68% for 3% glucose. The highest acetate yield, 86% of the theoretical maximum, was obtained by combining the one-step addition of 3% glucose with the nitrogen limitation to further limit loss of carbon into cell mass (Table 2-4). Additional fed-batch experiments were conducted in which multiple additions were made to glucose levels above 100 mM. With this approach, 878 mM acetate was produced representing 75% of the maximum theoretical yield (Table 2-4).

Discussion

E. coli was genetically engineered to combine the attributes of fermentative metabolism (low cell mass, high product yields) and oxidative metabolism (external electron acceptor) into a single biocatalyst (strain TC36) for the production of oxidized chemicals such as acetate (Fig. 2-2). Chromosomal deletions were used instead of point mutations to maximize stability. All antibiotic resistance genes and auxotrophic requirements were eliminated to permit growth in simple mineral salts medium. During

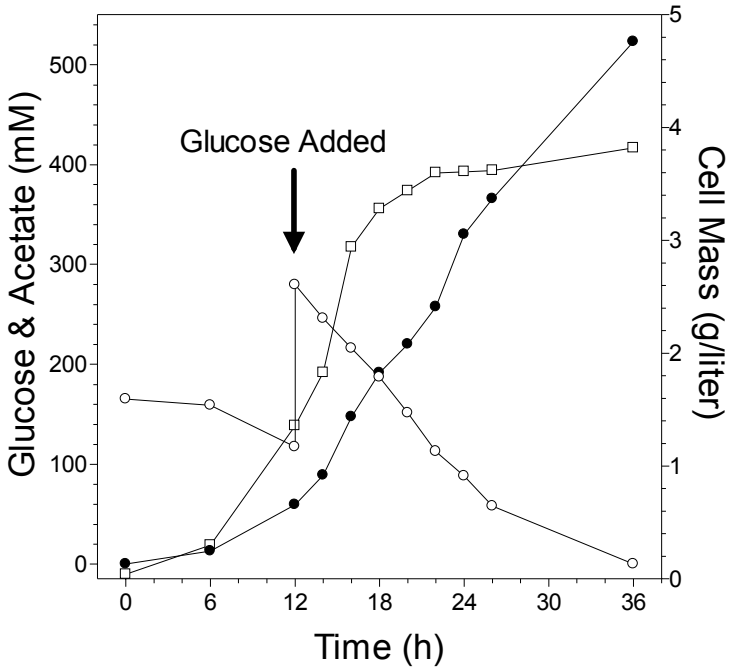

Figure 2-9. Fermentation of 6% gluocse to acetate by TC36 in mineral salts medium. Fermentation was initiated at 3% glucose followed by a second addition of 3% glucose after 12 h. Symbols: Open squares, cell mass; Solid circles, acetate.

oxidative metabolism, up to half of the substrate carbon can be converted to roughly equal amounts of cell mass and CO_2 (Contiero et al. 2000; Neidhardt et al. 1990; Varma et al. 1993) with minimal carbon flow into alternative products. To reduce the opportunity for excessive growth during oxidative metabolism, ATP production from NADH oxidation (oxidative phosphorylation) was eliminated by deleting the portion of $(F_1F_0)H^+$-ATP synthase involved in membrane assembly while preserving a functional cytoplasmic F_1-ATPase to provide gratuitous hydrolysis of ATP. With this mutation, a maximum of 4 ATP molecules (net) can be produced per glucose (assumes all pyruvate is metablized to acetyl~CoA and acetate) as compared to a theoretical maximum of 33 ATP molecules for wild-type strains of *E. coli*. Excessive oxidation of substrate to CO_2 and production of NADH were eliminated by disrupting the cyclic function of the tricarboxylic acid cycle (Δ*sucA*) with the added benefit of reducing oxygen demand for

NADH oxidation. Additional mutations were introduced to eliminate all major fermentation pathways as alternative routes for NADH oxidation, minimizing the formation of alternative products. The resulting strain, TC36, has absolute requirements for a fermentable carbon source (substrate level phosphorylation) and for an external electron acceptor that can couple to the electron transport system during growth in mineral salts medium to maintain redox balance. With genetic blocks in all major fermentation pathways and oxidative phosphorylation, this strain is relatively insensitive to variations in dissolved oxygen.

The $(F_1F_0)H^+$-ATP synthase and 2-oxoglutarate dehydrogenase mutations introduced into TC36 to miminize the levels of ATP and NAD(P)H produced from glucose under oxidative conditions would also be expected to promote glycolysis through native allosteric controls (Neidhardt et al. 1990; Underwood et al. 2002a; Underwood et al. 2002b), providing a mechanism for the observed 2-fold increase in glycolytic flux as compared to W3110 (wild type). With additional mutations in fermentation pathways, further metabolism of pyruvate was limited primarily to small biosynthetic needs and conversion to acetyl~CoA by the pyruvate dehydrogenase complex. Although pyruvate dehydrogenase is activated by low NADH, acetyl~CoA production may be limited by the availability of free CoA (note pyruvate accumulation in TC36 broth between 9 h and 15 h; Fig. 5C). Resulting increase in pyruvate pools would serve as an allosteric activator of phosphotransferase (Suzuki 1969), the first committed step for acetate production from acetyl~CoA. Gratuitous ATP hydrolysis by F_1-ATPase should ensure the availability of ADP for the final step in acetate production catalyzed by acetate kinase (Fig. 2-2A). Excess pyruvate can also be directly oxidized to acetate by pyruvate oxidase (*poxB*), an

enzyme that is induced during the later stages of growth and by environmental stress (Chang et al. 1994). This enzyme may also contribute to acetate production by TC36.

Eliminating oxidative phosphorylation while preserving (F_1) H^+ATPase resulted in a 2-fold increase in glycolytic flux (TC24 and TC36). Previously, Chao and Liao (1994) and Patnaik et al. (1992) demonstrated a similar 2-fold stimulation of glycolytic flux in *E. coli* using plasmids to express genes that created futile cycles to consume ATP. Recently, Koebmann et al. (2002) independently concluded that glycolytic flux is limited by ATP utilization during the oxidative metabolism of glucose. In their studies, flux increased in a dose-dependent manner with controlled expression of F_1-ATPase genes from a plasmid. Our results with TC24 and TC36 containing a chromosomal deletion (Δ*atpFH*) provide further support for this hypothesis. Thus, glycolytic flux appears to be regulated by the economy of supply and demand as proposed by Hofmeyer and Cornish-Bowden (2000). During the oxidative metabolism of glucose, glycolytic flux is limited by the metabolic ability to utilize ATP (availability of ADP) rather than by glucose transport or the catalytic capacities of central glycolytic enzymes. With this in mind, similar strategies that delete subunits essential for membrane assembly of the (F_1F_0)H^+-ATP synthase, create futile cycles for ATP consumption, or increase cytoplasmic levels of the ATPase activities may prove useful to decrease cell yield, increase metabolic flux, and increase product yield in many other bioconversion processes.

Strain TC36 can be used as a biocatalyst platform for the efficient production of oxidized products. Under conditions of excess glucose, strain TC36 produced a maximum of 878 mM acetate, 75% of the maximum theoretical yield (Table 2-4) or 0.50 g acetate per g glucose. Only cell mass and small amounts of organic acids were

produced as co-products with acetate. It is likely that 878 mM acetate approaches the upper limit of tolerance for metabolism in TC36. Concentrations as low as 50 mM acetate have been shown to induce a stress response in *E. coli* (Kirkpatrick et al. 2001). The minimal inhibitory concentration for growth has been previously reported as 300 - 400 mM acetate at neutral pH (Lasko et al. 2000; Zaldivar and Ingram 1999). Oxygen transfer often becomes limiting during aerobic bioconversion processes, promoting the accumulation of reduced products (Tsai et al. 2002; Varma et al. 1993). Synthesis of reduced products was eliminated by mutations in genes (Δ*focA-pflB* Δ*frdBC* Δ*ldhA* Δ*adhE*) encoding the four major fermentation pathways. Excessive oxygen demand and NADH production were also reduced by a deletion in 2-oxoglutarate dehydrogenase (Δ*sucA*). The resulting strain, TC36 (Δ*focA-pflB*Δ*frdBC* Δ*ldhA* Δ*atpFH* Δ*sucA* Δ*adhE*) metabolizes sugars to acetate with the efficiency of fermentative metabolism, diverting a minimum of carbon to cell mass (biocatalyst) and CO_2. By replacing the acetate pathway, a variety of alternative oxidized products can be produced using the mutational strategies employed for the construction of TC36.

Commercial production of acetate with biocatalysts involves a 2-step process in which sugar is first fermented to ethanol by yeasts followed by aerobic oxidation to acetate by *Acetobacter* (Berraud 2000; Cheryan et al. 1997). Although titers of around 650 mM are typically produced, higher titers can be readily achieved by the addition of complex nutrients in fed-batch processes requiring 60-120 h. Overall, yields for commercial processes have been estimated to be 76% of the theoretical maximum (2 acetate per glucose; 0.67 g acetic acid per g glucose). Genetically engineered *E. coli* TC36 can produce acetate in a simpler, single step process using glucose with titers and

yields equivalent or higher than current batch processes. Although maximum titers with TC36 were lower than can be achieved by ethanol oxidation using *Acetobacter* (Berraud 2000), acetate production rates from glucose by TC36 were almost two-fold higher than ethanol oxidation and required only mineral salts as nutrients. *E. coli* TC36 offers a unique set of advantages over currently employed biocatalysts for the commercial production of acetate:

1. The fermentation is a single step process using sugars as substrates.
2. Strain TC36 has high rates of acetate production, high acetate yields and a simple nutritional requirement (mineral salts plus carbon source).
3. Robust metabolism permitting the bioconversion of hexoses, pentoses, and many disaccharides and sugar alcohols.

Derivatives of this strain may prove useful as biocatalysts for the production of other compounds that are equivalent to, or more oxidized than the substrate.

CHAPTER 3
CONVERSION OF ARABINOSE, MANNOSE, SORBITOL AND XYLOSE TO ACETATE

Introduction

One advantage to employing *E. coli* as a biocatalyst is its ability to consume hexose and pentose sugars, the carbohydrate constituents of lignocellulosic plant biomass. The plant cell wall is primarily composed of two major carbohydrate polymers: cellulose and hemicellulose (Ingram et al. 1999; McKendry 2002). The carbohydrate composition of the cell wall differs with respect to the type of plant (hardwood, softwood, grass, etc.). Generally, cellulose constitutes 20-55% of agricultural residues and hemicellulose constitutes 20-40% (Sun and Cheng 2002). Pectin, a methylated polymer of galacturonic acid, typically constitutes a small percentage (2-4%) of agricultural residues (Ingram et al 1999). Cellulose is a polymer of glucose (cellobiose) therefore once treated with fungal cellulase the monomers become available for degredation. Hemicellulose is a complex carbohydrate polymer composed of xylose and arabinose in hardwoods and crop residues (Asghari et al. 1996; Ingram et al 1999). Xylose, galactose and mannose are the major sugars in softwoods (Asghari et al. 1996; Ingram et al 1999). The stoichiometry of hemicellulose sugars vary depending on the type of plant.

Our laboratory is interested in the conversion of biomass, such as sugarcane bagasse, into fuels and chemicals. The hemicellulose fraction of sugarcane is composed mostly of xylose (89%) with minor amounts of arabinose and glucose (Asghari et al. 1996). In this chapter the engineered *E. coli* strain TC36 is shown to efficiently convert

all major sugar constituents of plant biomass into acetate. In addition, strain TC36 also metabolized sorbitol, a sugar alcohol, into acetate.

Materials and Methods

Microorganisms and Media

Escherichia coli TC36 and TC51 (described in Chapter 1) were used for our study. The NBS salts described in Chapter 1 were used in minimal salts agar and for fermentations as described in Chapter 1. Arabinose, mannose, sorbitol, or xylose was added (2% in plates and 3% in broth) as the sole carbon and energy source, when appropriate, from a 50% stock solution. When needed for pH control, 3-[N-morpholino]propanesulfonic acid (0.1 M, pH 7.4) was added, but was not included in pH-controlled fermentations.

Fermentations

Fermentations (5 L) were conducted at 37^{o}C (dual Rushton impellers, 350 rpm; pH 7.0) in Bioflow 3000 fermentors (New Brunswick Scientific, Edison, NJ). Dissolved oxygen levels were 100% of air saturation at the time of inoculation and allowed to fall to 5% of air saturation during cell growth with continuous air sparging (0.2 vvm). This level of oxygen was maintained by mixing O_2 with air at a constant flow rate of 1.0 L min^{-1}. Broth pH was controlled with 11.4 M KOH.

Seed cultures were started as described in Chapter 1. Fermentors were inoculated at an initial OD_{550} of 0.05 except for xylose fermentations. For xylose fermentations an initial OD_{550} was not sufficient for logarithmic growth therefore these fermentations were inoculated at an initial OD_{550} of 0.5.

Results

Fermentation of Sugars Derived from Hemicellulose (Arabinose, Mannose and Xylose)

Pentose sugars constitute the majority of sugars found in hemicellulose. Xylose is the most abundant sugar found in sugarcane derived hemicellulose. *Escherichia coli* TC36 converted 180 mM xylose into 205 mM acetate, 69% of the maximum theoretical yield (Table 3-1). All of the xylose carbon was accounted for by HPLC analysis of the fermentation broth (Table 3-1). Minor quantities of glycolytic and TCA cycle intermediates were observed in the fermentation broth, 2-oxoglutarate being the most abundant (Table 3-1). Acetate accounted for 88% of the organic acids in the broth. The maximum specific growth rate of TC36 grown on xylose is 75% of the maximum specific growth rate when grown on glucose under the same conditions (Fig. 3-1). The growth rate reduction may be attributed to decreased ATP production on xylose compared to glucose (see discussion).

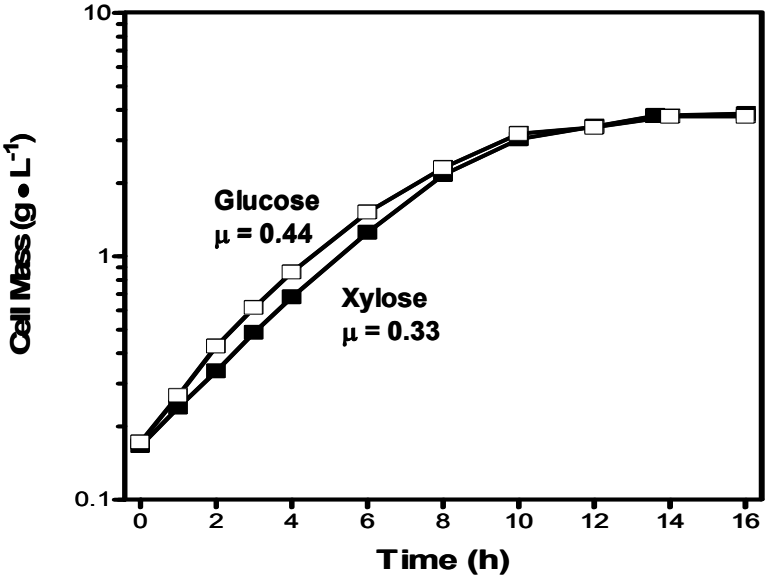

Figure 3-1. Growth comparison of TC36 with xylose or glucose as the sole carbon source. Fermentations were begun at an initial OD_{550} of 0.5. Symbols: Solid rectangles, xylose; Open rectangles, glucose.

Table 3-1. Products formed by TC36 during aerobic catabolism of various sugars

					Concentration mmoles L^{-1}[a]				
Condition[b]	Cell mass ($g\ L^{-1}$)	μ_{max} (h^{-1})[c]	Carbon Balance (%)	Acetate Yield (%)[d]	Acetate	Pyruvate	2-Oxo-glutarate	Succinate	Malate
Arabinose[e]	4.01	0.46	94	63	220	<1	21	3	4
Glucose[f]	3.47	0.51	87	62	250	<1	19	14	<1
Mannose[e]	3.26	0.23	83	59	187	<1	13	2	2
Sorbitol[f]	3.44	0.30	87	61	195	<1	15	2	<1
Xylose[f]	3.85	0.33	102	69	205	7	19	2	<1

[a] Final concentrations at the end of fermentation. Fumarate was not included in Table 3-1 because the concentrations was observed to be less than 1 mM for all fermentations.

[b] All fermentations were conducted with 3% initial sugar concentration. Arabinose, mannose, and sorbitol fermentations were inoculated at an initial OD_{550} of 0.05 and the xylose fermentations were inoculated at an intial OD_{550} of 0.5.

[c] Maximum specific growth rate.

[d] Acetate yield as a percentage of the maximum theoretical yield (0.67 g of acetate per g of glucose).

[e] Results from one fermentation.

[f] Averages of two fermnetations.

Arabinose constitutes the second most abundant sugar in hemicellulose derived from sugarcane. *E. coli* TC36 converted 207 mM arabinose into 220 mM acetate, 63% of the maximum theoretical yield (Table 3-1). Again, acetate was the dominant (89%) organic acid recovered from the fermentation broth. The final cell mass and maximum specific growth rate were equivalent to those of TC36 grown on glucose under the same conditions (Tables 2-3 and 3-1).

Mannose is an optical isomer of glucose and constitutes a small fraction of the sugars found in hemicellulose. *E. coli* TC36 converted mannose to acetate with a maximum theoretical yield of 59% (Table 3-1). The specific growth rate (0.23 h^{-1}) and final cell density on mannose was the lowest of all sugars tested (Table 3-1). Acetate accounted for 92% of all organic acids recoved, although 17% of the substrate carbon was not recovered (Table 3-1).

Oxidation of Sorbitol to Acetate

Sorbitol is a six carbon sugar alcohol which is more reduced than glucose. One more NADH is generated from the oxidation of sorbitol than from glucose. The specific growth rate on sorbitol was 59% of the growth rate on glucose and the final cell density was 99% of glucose grown cultures (Table 3-1). The maximum theoretical acetate yield attained was 61%, which is comprable to that acheived on glucose under the same conditions.

Discussion

Arabinose and xylose are pentose sugar constituents of hemicellulose. Both are transported into the cell by high affinity ABC (ATP binding cassette) transporters specific for the respective sugar (Neidhardt et al. 1996). There are also low affinity H^+-linked symporters involved in transport of the pentoses (Henderson 1990). Once in the

cell, arabinose and xylose are catabolized through the pentose phosphate pathway (PPP). Arabinose enters the PPP at the level of ribulose-5-phosphate and xylose enters at the level of xylulose-5-phosphate. Net ATP yield is much lower for the catabolism of the pentoses (0.67 per xylose or arabinose) than for glucose (2 per glucose) with lactate as the end-product (Tao et al. 2001). The NADH produced during xylose and arabinose catabolism is the same as that produced from glucose catabolism.

Acetate yield and final cell density were equivalent for the pentose fermentations and glucose fermentations. If ATP was to become limiting during pentose fermentations one would expect greater flux to acetate to generate more ATP or a reduction in the final cell mass. These results imply that net ATP is not limiting the growth of TC36 on arabinose and xylose during aerobic growth. One may expect reduced maximum specific growth rate on xylose due to a slower rate of ATP production, although the maximum specific growth rate for arabinose grown cells is 1.4-fold greater than that of xylose grown cells. If the rate of ATP production was limiting growth rate, one would expect arabinose and xylose cultures to exhibit similar rates. Arabinose was demonstrated to be used preferentially by *E. coli* K12 when xylose or ribose is present in minimal medium (Kang et al. 1998). The regulatory mechanism was determined to be at the transcriptional level (Kang et al. 1998). Preferential use of arabinose suggests that *E. coli* derives some thermodynamic or catalytic advantage from catabolism of arabinose over the other pentoses. Unfortunately, the mechanism has not been documented.

Mannose was the poorest carbon source examined for growth and acetate yield. Growth on mannose was characterised by a 53% reduction in specific growth rate, as compared to glucose grown cells, and an acetate yield of 59%. Mannose is transported

into the cell by a PTS-linked mannose permease (Erni et al. 1987) and isomerized to fructose-6-phosphate. The growth rate reduction observed for mannose may reflect a slower rate of transport into the cell or isomerization (decreased V_{max}) to fructose-6-phosphate for mannose than for glucose.

Sorbitol is a polyalcohol commonly found in ripe berries. Commercial production of sorbitol uses high-pressure hydrogenation or electrolytic reduction of glucose. Sorbitol was used as a substrate for TC36 in order to demonstrate the ability of the organism to convert a reduced substrate into an oxidized product, acetate. Strain TC36 was able to converted sorbitol efficiently to acetate and maintain a growth rate 61% that of glucose-grown cultures. Alteration of the process conditions, such as increasing the dissolved oxygen above 5%, providing more electron acceptor for respiration, may improve growth rate and/or acetate yield. Conversion of sorbitol to acetate demonstrates the utility of the mutations constructed in TC36. This biocatylist is not limited to the production of compounds reduced or neutral relative to the substrate.

In this chapter, the ability of *E. coli* TC36 to efficiently convert the major sugars found in hemicellulose into acetate is demonstrated. In addition, strain TC36 is capable of converting of substrate carbon into an oxidized product, such as acetate and CO_2.

Chapter 4
ENGINEERING *Escherichia coli* FOR THE EFFICIENT CONVERSION OF GLUCOSE TO PYRUVATE

Introduction

There is a growing trend in the commodity and specialty chemicals industries towards the use of microorganisms as biocatalysts. Many commodity chemicals (ethanol, lactate, 1,3-propanediol, and adipic acid) and specialty chemicals (polyketides and carotenoids) can be made directly or indirectly from the same central metabolite, pyruvic acid.

The metabolic fate of pyruvate, a key branch point in central carbon metabolism, is regulated in large part by the redox state of the cell. During the fermentative metabolism of glucose, NADH levels are high, and > 95% of pyruvate is used as terminal electron acceptor for NADH oxidation; < 5% is partitioned into biosynthesis (see Figure 1-3 for an overview). During oxidative metabolism, pyruvate is oxidatively decarboxylated by the pyruvate dehydrogenase complex (PDHc) to produce CO_2 and acetyl-CoA. Oxygen or other external compounds serves as the terminal electron acceptor, and NADH levels remain relatively low. Up to half of the glucose carbon is used for biosynthesis, the remainder being fully oxidized by the tricarboxylic acid cycle to CO_2. Construction of an *E. coli* strain that excretes pyruvate as the major product of glucose catabolism requires that pyruvate utilizing pathways be eliminated (Fig. 4-1A) and carbon accumulation in the form of cell mass be minimized.

Pyruvic acid is currently manufactured for use as a food additive, nutriceutical, and weight control supplement (Li et al. 2001a; Anonymous 2002). Pyruvic acid can also be used as a starting material for the synthesis of amino acids such as alanine, tyrosine, phenylalanine, and tryptophan (Li et al. 2001a) and for acetaldehyde production (Ingram and Conway 1987). Pyruvate is produced commercially by both chemical and microbial processes (Li et al. 2001a). Chemical synthesis from tartrate involves toxic solvents and is energy-intensive (Ondrey 2001; Blair 1999) with an estimated production cost of $8,650 per ton of pyruvate (Li et al. 2001a). Microbial pyruvate production is based primarily on two microorganisms, a multi-vitamin auxotroph of the yeast *Torulopsis glabrata* (Li et al. 2001b) and a lipoic acid auxotroph of *Escherichia coli* containing a mutation in the F_1 subunit of the $(F_1F_0)H^+$-ATP synthase (Yokota et al. 1994). Both strains require precise regulation of media composition during fermentation and complex supplements. Estimated production costs for pyruvate production with these strains is 14.5% ($1,255 per ton pyruvate) that for chemical synthesis (Li et al. 2001a). Recently, Tomar et al. (2003) have described a new *E. coli* mutant for pyruvate production from glucose and acetate in complex medium. This strain contains mutations in phosphoenolpyruvate carboxylase (*ppc*), pyruvate dehydrogenase (*aceF*), and alcohol dehydrogenase (*adhE*).

Nutrients in culture medium often represent a major cost associated with commercial fermentations. The use of a mineral salts medium and an inexpensive carbon source offer the potential to improve the economics of many biological processes by reducing the costs of materials, product purification, and waste disposal (Zhang and Greasham 1999). A microbial platform that combines attributes of aerobic and

fermentative metabolism for the production of compounds that are redox neutral or oxidized relative to glucose, described in Chapter 2, was developed by our laboratory (Causey, PNAS. 2003). In order to demonstrate further the flexibility of the biocatalyst with regard to the variety of compounds that can be produced, we have engineered *Escherichia coli* W3110 for the efficient production of pyruvic acid as the major end product of glucose catabolism.

Materials and Methods

Microorganisms and Media

E. coli W3110 (ATCC 27325) and derivatives (Table 4-1) were grown on mineral salts medium (Causey et al. 2003) containing, glucose (2% in plates; 3% in broth). When needed for pH control, 3-[N-morpholino]propanesulfonic acid (0.1 M, pH 7.4) was added, but was not included in pH-controlled fermentations. During plasmid and strain construction, cultures were grown in Luria-Bertani (LB) broth or on LB plates (1.5% agar) (Miller 1992). Glucose (2%) was added to LB medium for all strains containing mutations in $(F_1F_0)H^+$-ATP synthase. Antibiotics were included as appropriate (kanamycin, 50 mg L^{-1}; ampicillin, 50 mg L^{-1}; apramycin, 50 mg L^{-1}; and tetracycline, 12.5 or 6.25 mg L^{-1}).

Genetic Methods

Standard methods were used for plasmid construction, phage P1 transduction, electroporation, and polymerase chain reaction (PCR) (Miller 1992; Sambrook and Russell 2001). Coding regions for *ackA* and *poxB* were amplified using ORFmer primers (Sigma-Genosys, Inc., The Woodlands, TX) and initially cloned into pCR2.1-TOPO. Chromosomal integration of mutated genes was facilitated by pKD46 containing an arabinose-inducible Red recombinase (Datsenko and Wanner 2000).

Table 4-1. Sources and characteristics of strains and plasmid

Strains/Plasmids	Relevant Characteristics	Reference
Strains		
W3110	K12 wild type	ATCC 27325
TOP10F'	*lacI*^{q} (episome)	Invitrogen
SZ61	W3110, Δ*ackA*::*FRT-tet-FRT*	Zhou et al. 2003
TC36	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT*	Causey et al. 2003
TC37	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*ackA*::*FRT-tet-FRT*	Causey et al. 2004
TC38	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*ackA*::*FRT*	Causey et al. 2004
TC42	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*poxB*::*FRT*	Causey et al. 2004
TC43	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*ackA*::*FRT* Δ*poxB*::*FRT-tet-FRT*	Causey et al. 2004
TC44	W3110, (Succ$^+$), Δ(*focA-pflB)*::*FRT* Δ*frdBC* Δ*ldhA* Δ*atp*(*FH*)::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*ackA*::*FRT* Δ*poxB*::*FRT*	Causey et al. 2004
Plasmids		
pCR2.1-TOPO	*bla kan,* TOPO^{TM} TA cloning vector	Invitrogen
pFT-A	*bla flp* low-copy vector containing recombinase and temperature-conditional pSC101 replicon	Posfai et al. 1997
pKD46	*bla* γ β *exo* low-copy vector containing red recombinase and temperature-conditional pSC101 replicon	Datsenko and Wanner 2000

Integrants were selected using antibiotic resistance, screened for appropriate antibiotic resistance markers and phenotypic traits, and verified by analyses of PCR products and fermentation profiles. *FRT*-flanked antibiotic resistance genes were deleted using FLP recombinase (Martinez-Morales et al. 1999; Posfai et al. 1997).

Deletion of Acetate Kinase (Strains TC38 and TC44)

The Δ*ackA*::*FRT-tet-FRT* mutation was transduced from SZ61 into TC36 and TC42 to produce strains TC37 (Δ*focA-pflB*::*FRT* Δ*frdBC*::*FRT* Δ *ldhA* Δ*atpFH*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*ackA*::*FRT-tet-FRT*) and TC43(Δ*focA-pflB*::*FRT* Δ*frdBC*::*FRT* Δ *ldhA* Δ*atpFH*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT poxB*::*FRT* Δ*ackA*::*FRT-*

tet-FRT), respectively. After removal of the *tet* gene using FLP recombinase, resulting strains were designated TC38 (Δ(*focA-pflB*::*FRT* Δ*frdBC*::*FRT* Δ*ldhA* Δ*atpFH*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT* Δ*ackA*::*FRT*) and TC44 (Δ*focA-pflB*::*FRT* Δ*frdBC*::*FRT* Δ*ldhA* Δ*atpFH*::*FRT* Δ*adhE*::*FRT* Δ*sucA*::*FRT poxB*::*FRT* Δ*ackA*::*FRT*), respectively.

Fermentation

Fermentations (5 L and 10L) were conducted at 37^{o}C (dual Rushton impellers, 350 - 450 rpm; pH 7.0) in Bioflow 3000 fermentors (New Brunswick Scientific, Edison, NJ). Unless stated otherwise, dissolved oxygen levels were 100% of air saturation at the time of inoculation and allowed to fall to 5% of air saturation during cell growth with continuous air sparging (0.2 vvm). This level of oxygen was maintained by mixing O_2 with air at a constant flow rate of 1.0 L min^{-1}. Broth pH was controlled with 11.4 M KOH. For fed-batch studies, glucose was added from a sterile 4 M stock. Two fed batch regimes were investigated: 1) 3% initial glucose followed the addition of 3% glucose after 15 h (6% total); 2) 3% initial glucose with the addition of 590 mL of 4 M glucose at a constant rate over a 20-h period (9.8% total glucose).

Seed cultures prepared as previously described (Causey et al. 2003) were used to provide an inoculum of 16.5 mg dry cell weight L^{-1}. Broth samples were removed to measure organic acids, residual glucose, and cell mass. Volumetric and specific rates were estimated from measured values for glucose and acetate using GraphPad Prism (GraphPad Software, San Diego, CA). A smooth curve was generated with 10 points per min (Lowess method) to fit measured results. The first derivative (acetate or glucose versus time) of each curve served as an estimate of volumetric rate. Specific rates (mmoles L^{-1} h^{-1} [mg dry cell weight]$^{-1}$) were calculated by dividing volumetric rates by respective values for cell mass.

Analyses

Organic acids and glucose were measured using a Hewlett Packard HPLC (HP 1090 series II) equipped with a UV monitor (210 nm) and refractive index detector (Causey et al. 2003). Cell mass was estimated using a Bausch & Lomb Spectronic 70 spectrophotometer (1.0 OD_{550nm} is equivalent to 0.33 g dry cell weight L^{-1}).

Results

Pyruvate as a Co-Product during Acetate Fermentations

Escherichia coli TC36 (Δ*focA-pflB* Δ*frdBC* Δ*ldhA* Δ*atpFH* Δ*adhE* Δ*sucA*) was previously engineered from W3110 (wild type) for the production of acetate (Fig. 3-1A) by combining chromosomal deletions that minimize cell yield, oxygen consumption, CO_2 evolution, and reduced fermentation products (Causey et al. 2003). In this strain, glycolytic flux was higher than the parent due to a deletion of genes (Δ*atpFH*) encoding two membrane proteins that couple the F_1 and F_0 components of the $F_1F_0(H^+)$ATP synthase complex. This mutation eliminated ATP production by oxidative phosphorylation and created an active, cytoplasmic F_1ATPase (Fig. 3-1B and 3-1C). Glycolytic flux in TC36 exceeded the capacity for acetate production under our initial test conditions (5% air saturation at inoculation and during fermentation), resulting in transient accumulation of approximately 16 mM pyruvate near the end of exponential growth (Fig. 3-2). The peak level of pyruvate was increased to 81 mM (Fig. 4-2) by inoculating the fermentor at an initial dissolved oxygen level of 100% air saturation (rather than 5% of saturation) with continuous air sparging until the oxygen level declined to 5% air saturation, then adding oxygen to maintain 5% of air saturation. Under this condition, pyruvate yield at the peak was 25% of the maximum theoretical yield and 11% of the maximum theoretical yield at the end of fermentation (Fig. 4-2; Table 4-2).

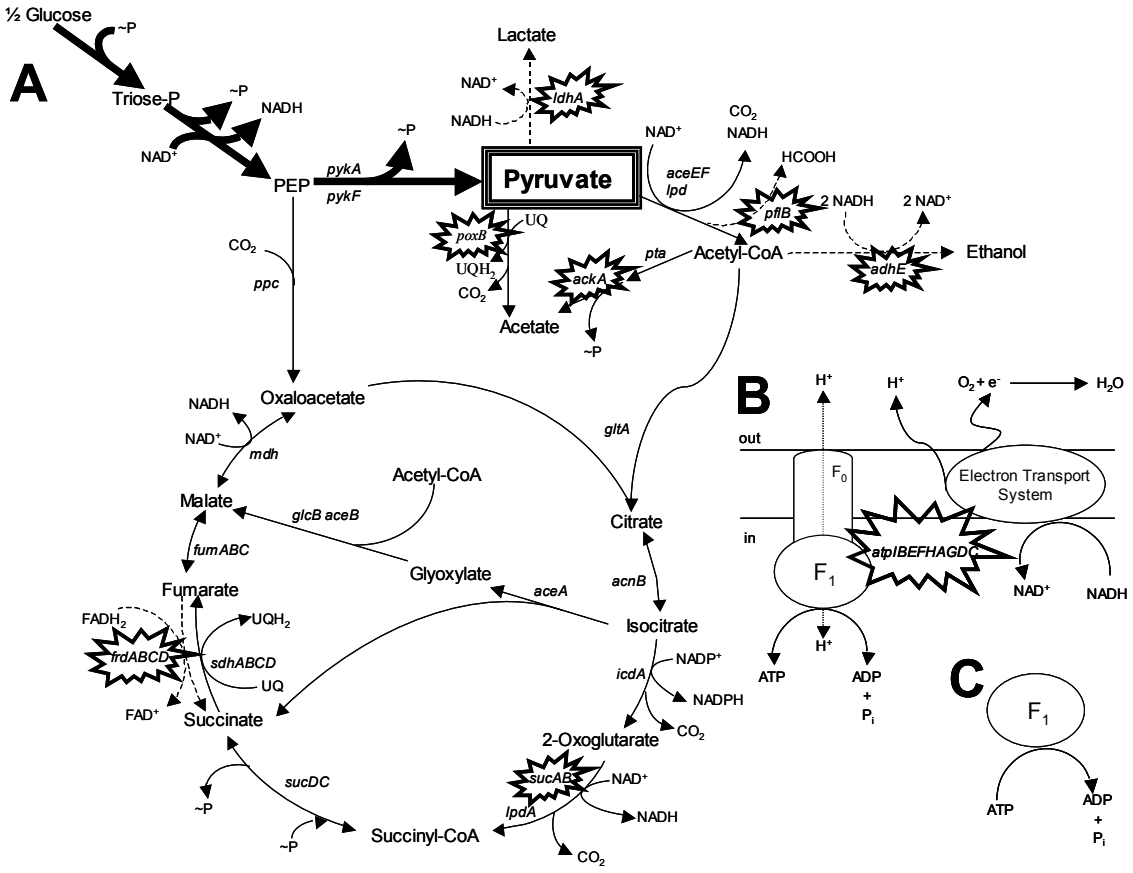

Figure 4-1. Genetic modifications used to redirect glucose metabolism for pyruvate production. Metabolic steps that have been blocked by mutation or deletion of genes are highlighted. A) Central carbon metabolism. B) Oxidative phosphorylation. C) Cytoplasmic F_1 ATPase subunit (active). Genes: *aceA*, isocitrate lyase; *aceB*, malate synthase B; *aceEF,* E1p and lipoate acetyltransferase/dihydrolipoamide acetyltransferase component of the PDHc; *ackA*, acetate kinase; *acnB*, aconitase; *adhE*, alcohol/aldehydrogenase pyruvate formate lyase deactivase; *atpIBEFHAGDC*, $(F_1F_0)H^+$-ATP synthase; *frdABCD*, fumarate reductase; *glcB*, malate synthase G; *gltA*, citrate synthase; *icdA*, isocitrate dehydrogenase; *ldhA*, lactate dehydrogenase; *lpdA*, dihydrolipoate dehydrogenase/dihydrolipoamide dehydrogenase component of the PDHc and 2-oxoglutarate dehydrogenase; *pflB*, pyruvate formate-lyase; *poxB*, pyruvate oxidase; *pta*, phosphotransacetylase; *pykA*, pyruvate kinase II; *pykF*, pyruvate kinase I; *sdhABCD*, succinate dehydrogenase; *sucAB*, 2-oxoglutarate dehydrogenase; *sucDC*, succinyl-CoA synthetase.

We have explored additional genetic modifications of TC36 to increase the efficiency of pyruvate production from glucose. Although many metabolic routes lead to acetate, two primary routes are present in *E. coli* (Fig. 4-1A): 1) conversion of acetyl~CoA to acetate by phosphotransacetylase (*pta*) and acetate kinase (*ackA*); and 2) direct oxidation of pyruvate to acetate by pyruvate oxidase (*poxB*) (Fig. 4-1A). Derivatives of TC36 were constructed with mutations in both pathways.

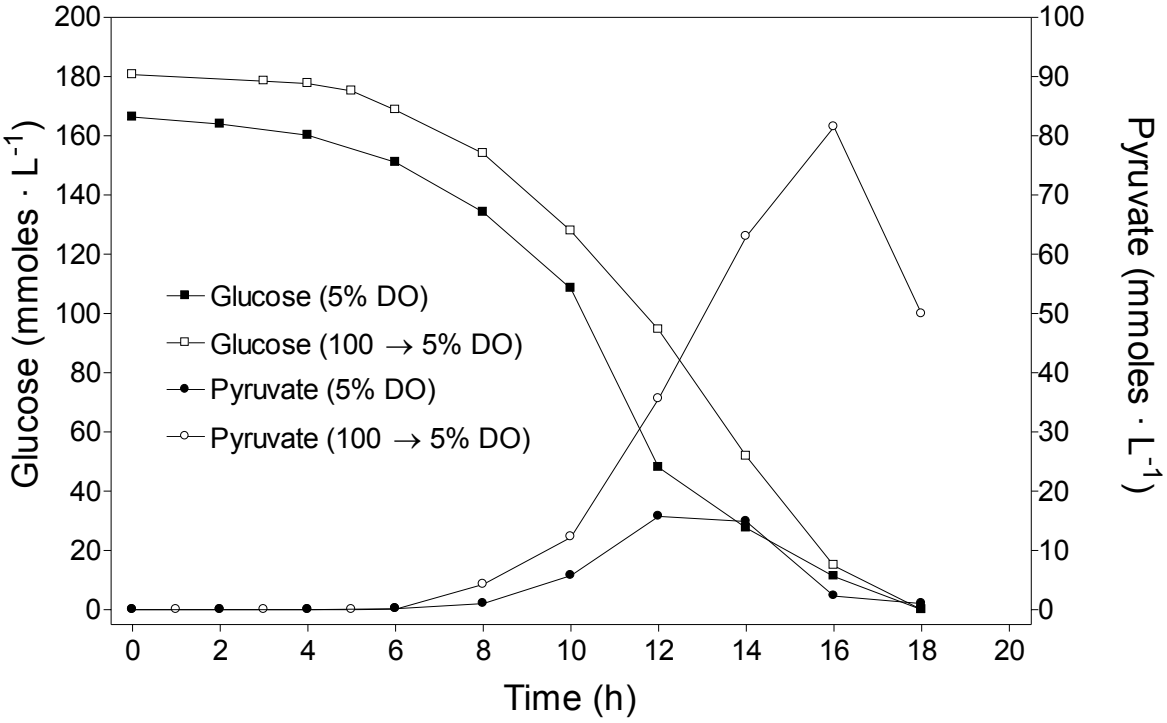

Figure 4-2. Effect of oxygen level on pyruvate production by TC36. Cells were inoculated into fermentation broth at 100% air saturation and continuously sparged with air until the oxygen levels declined to 5% saturation. At this time, oxygen was blended to maintain 5% O_2 saturation during the remaining period of incubation (open symbols). Alternatively, media was sparged with a mixture of air and nitrogen to provide 5% air saturation prior to inoculation. Sparging was switched to air and oxygen as needed to maintain 5% air saturation (closed symbols).

Acetate Kinase (Δ*ackA*) Deletion Enhances Pyruvate Production and Inhibits Glycolysis (Strain TC38)

To block acetate production by the acetate kinase pathway, the central region of the *ackA* gene was deleted in TC36 to produce TC38. Acetate production was reduced by 85% (Fig. 4-3C; Table 4-2), consistent with the acetate kinase pathway being the dominant route for acetate production in TC36. However, this deletion also reduced net ATP production (estimated) by 30% (Fig. 4-1A), cell yield by 36% (Fig. 4-3A; Table 4-2), and maximum specific growth rate by 45% (Table 4-3). Glycolytic flux was reduced by 45% (Table 4-3) increasing the time required to complete fermentations from 18 h for TC36 to 24 h for TC38 (Fig. 4-3B). Although both volumetric and specific rates of glucose metabolism were lower for TC38 (Table 4-3), pyruvate yield was 5.5-fold higher (Table 4-2; Fig. 4-3D) and the specific rate of pyruvate production was 4-fold higher (Table 4-3) than for TC36. Small amounts of 2-oxoglutarate, succinate, and fumarate were produced by both strains. From 10% to 15% of the carbon was not recovered as cell mass or acidic fermentation products and is presumed to be lost as CO_2 due to metabolite cycling. With strain TC38, the pyruvate yield (195 mM) was 58% of the theoretical maximum. Acetate (28.9 mM) remained as the second most abundant product.

Pyruvate Oxidase (*poxB*) Mutation Causes a Modest Reduction in Acetate Accumulation

Pyruvate can be metabolized to acetate by the membrane-bound protein pyruvate oxidase using the electron transport system to couple oxygen as a terminal electron acceptor. The *poxB* gene is typically repressed during exponential growth but is induced by stress or entry into stationary phase (Chang and Cronan 1983; Chang et al. 1994). Pyruvate oxidase was inactivated in TC36 by constructing three stop codons, one in each reading frame, in the central region to produce TC42.

Table 4-2. Products formed from glucose catabolism by *E. coli* strains used in our study

						Product Concentrations (mmoles L^{-1})[a]				
Strain	Condition	Replicates	Cell mass (g L^{-1})	Carbon Balance (%)	Pyruvate Yield (%)[b]	Pyruvate	Acetate	2-Oxo-glutarate	Succinate	Fumarate
W3110	3% glucose	2	4.13	80.6	5.9	20.8	180.0	8.3	13.7	0.9
TC36	3% glucose	3	3.47±0.23	89.0±2.7	10.5±7.9	38.1±27.2[c]	197.7±21.1	16.6±16.2	13.7±13.2	1.4±0.2
TC38	3% glucose	3	2.21±0.09	84.3±5.2	57.5±2.6	194.5±9.1	28.9±16.7	10.5±1.9	8.1±9.1	0.8±0.7
TC38	3% glucose[d]	2	2.40	84.7	68.8	241.9	7.0	7.9	<2.0	<0.2
TC42	3% glucose	2	3.40	86.8	29.1	79.0	178.4	76.2	24.3	1.7

[a] Unless stated otherwise the concentrations represent measurements at the time of glucose exhaustion.
[b] Maximum theoretical yield is 2 moles pyruvate per mol glucose (0.978 g pyruvate per g glucose)
[c] The pyruvate concentrations during glucose fermentations ranged from 14.9 to 111.9 mM. Pyruvate production was very sensitive to dissolved oxygen flucuations.
[d] Dissolved oxygen was allowed to fall from 100% to 50% of air saturaiton.
[e] Three percent initial glucose followed by the addition of 208 mL of a 4 M glucose solution.
[f] Three percent initial glucose followed by the addition of 590 mL of a 4 M glucose solution over a period of 20 h.

Table 4-2. Continued

Strain	Condition	Replicates	Cell mass (g L^{-1})	Carbon Balance (%)	Pyruvate Yield (%)[b]	Product Concentrations (mmoles L^{-1})[a]				
						Pyruvate	Acetate	2-Oxo-glutarate	Succinate	Fumarate
TC44	3% glucose	3	2.36±0.10	88.5±0.6	69.3±1.5	252.5±6.2	11.6±1.2	3.6±1.2	16.8±0.7	1.1±0.2
TC44	3% glucose, ½ Nitrogen	2	2.02	73.6	38.8	125.2	50.3	30.0	7.7	2.9
TC44	3%+3% glucose[e]	2	2.63	86.7	72.3	479.8	39.8	31.7	10.9	0.7
TC44	6% glucose	2	1.95	94.8		588.9	46.0	26.1	<2.0	0.7
TC44	excess glucose[f]	2	2.51	--	--	749.0	62.4	45.3	14.7	3.3

[a] Unless stated otherwise the concentrations represent measurements at the time of glucose exhaustion.
[b] Maximum theoretical yield is 2 moles pyruvate per mol glucose (0.978 g pyruvate per g glucose)
[c] The pyruvate concentrations during glucose fermentations ranged from 14.9 to 111.9 mM. Pyruvate production was very sensitive to dissolved oxygen flucuations.
[d] Dissolved oxygen was allowed to fall from 100% to 50% of air saturaiton.
[e] Three percent initial glucose followed by the addition of 208 mL of a 4 M glucose solution.
[f] Three percent initial glucose followed by the addition of 590 mL of a 4 M glucose solution over a period of 20 h.

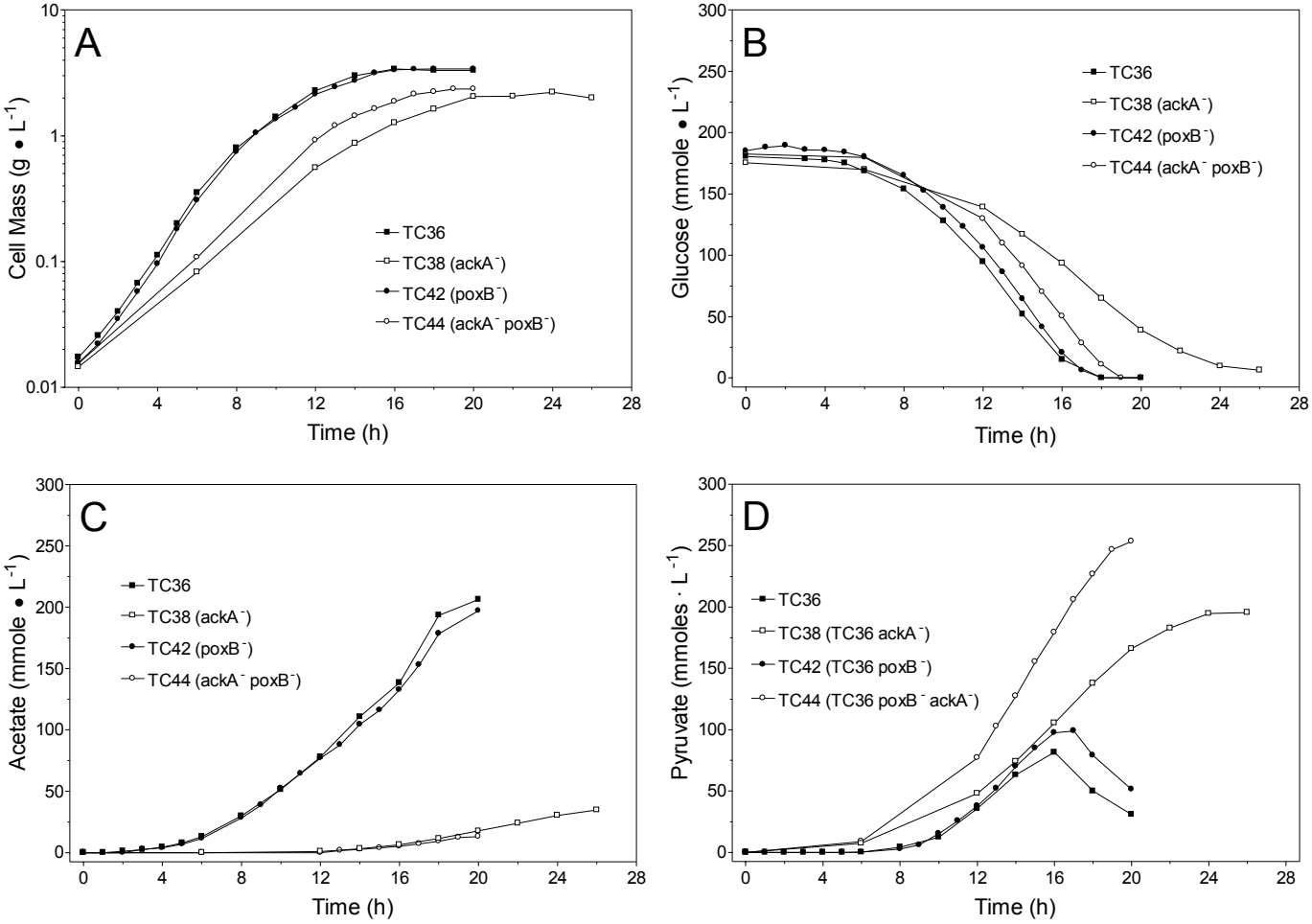

Figure 4-3. Batch fermentation of glucose by mutant strains of *E. coli*. A) Cell Growth B) Glucose utilization C) Acetate production D) Pyruvate Production.

In contrast to the *ackA* deletion (TC38), the *poxB* mutation (TC42) caused a relatively small decease in acetate and pyruvate (Table 4-2; Fig. 4-3C and 4-3D) consistent with a minor role for the POX pathway. Pyruvate yield with TC42 was less than 30% of the theoretical maximum. Unlike the mutation in *ackA* (TC38), inactivation of *poxB* (TC42) did not reduce the rate of growth or glucose metabolism (Table 4-3).

Combining Mutations for Pyruvate Oxidase (*poxB*) and Acetate Kinase (*ackA*) Improved Pyruvate Production (Strain TC44)

Strain TC44 (Δ*focA-pflB* Δ*frdBC* Δ*ldhA* Δ*atpFH* Δ*adhE* Δ*sucA poxB*::*FRT* Δ*ackA*) was constructed in which both the acetate kinase and pyruvate oxidase mutations were combined in the TC36 background. Inactivation of *poxB* was beneficial for growth and pyruvate production (Fig. 4-3A; Table 4-2 and Table 4-3) in comparison to TC38 (isogenic strain containing functional *poxB*). Both volumetric and specific rates of glucose metabolism were higher for TC44 than for TC36 or TC38 (Table 4-3). Acetate production by TC44 was half that of TC38 and pyruvate yield was 17% higher.

Table 4-3. Comparison of metabolic rates

		Glucose consumption rate[a]		Pyruvate production rate[a]	
Strain	μ (h^{-1})	Average Vol[b] (mmol L^{-1} h^{-1})	Max. Spec.[c] (mmol L^{-1} h^{-1} g^{-1})	Average Vol.[b] (mmol L^{-1} h^{-1})	Max. Spec.[c] (mmol L^{-1} h^{-1} g^{-1})
W3110	0.69	11.9	17.4	nd	nd
TC36[d]	0.49±0.03	10.1±2.6	17.6±1.5	nd	nd
TC36[e]	0.51±0.01	10.7±0.9	29.7±3.5	3.8±3.0	5.3±3.1
TC38[e]	0.28±0.01	6.7±0.6	16.3±2.2	8.3±0.7	21.1±3.7
TC38[f]	0.21	6	28	8	28
TC42[e]	0.55	10	17	6	10
TC44[e]	0.34±0.02	9.7±0.7	27.2±4.1	13.1±0.3	40.4±7.4

[a] Standard deviations are included for data from three or more fermentations; others represent an average of two fermentations.
[b] Average volumetric rates.
[c] Maximum specific rates. dcw, dry cell weight.
[d] Dissolved oxygen controlled at 5% of air saturation by adjusting the ratio of O_2 to N_2.
[e] Dissolved oxygen allowed to fall from 100% to 5% of air saturation during growth with air sparging; O_2 added to maintain 5% air saturation.
[f] Dissolved oxygen allowed to fall from 100% to 50% of air saturation during growth with air sparging; O_2 added to maintain 50% air saturation.

The specific rate of pyruvate production by TC44 was 8-fold that of TC36 and twice that of TC38 (Table 4-3). The time required to complete fermentation with TC44 was 30% shorter than with TC38 (Fig. 4-3B). Broth containing 3% glucose (167 mM) was metabolized to 2.2% pyruvate (252 mM) after 18 h in mineral salts medium (Fig. 4-3D). Although acetate levels were substantially reduced by combining the *poxB* and *ackA* mutations (Fig. 4-3C), acetate (12 mM) and dicarboxylic acids (20 mM) remained as minor products.

The Beneficial Role of a *poxB* Mutation for Pyruvate Production

Although eliminating the primary route for acetyl~CoA dissimilation (Δ*ackA*) in TC38 increased pyruvate production, this was accompanied by detrimental effects on growth and fermentation rates (Fig. 4-2; Table 4-2 and Table 4-3) that were substantially reduced by inactivation of PoxB (TC44). Pyruvate oxidase competes with NADH oxidation for oxygen as a terminal electron acceptor and represents a potential mechanism for this action. Oxygen transfer rates are frequently limiting during aerobic fermentations at relatively high levels of saturation (Varma et al. 1993), and may be even more problematic under our fermentation conditions (5% of air saturation). Increased PoxB activity resulting from larger pyruvate pools in the acetate kinase mutant (TC38) would be expected to decrease the availability of NAD^+ for glycolysis and to increase the levels of NADH, an allosteric inhibitor of key biosynthetic enzymes such as citrate synthase (Weitzman 1966) and phosphoenolpyruvate carboxylase (Krebs and Bridger 1976). This hypothesis was tested in part by examining the effect of increasing the oxygen level during fermentation with TC38.

Increasing oxygen saturation from 5% to 50% during TC38 fermentations (Table 4-2 and Table 4-3) was beneficial, consistent with our hypothesis. Cell yield, pyruvate

yield, and the specific rate of glucose metabolism were 8% to 41% higher for TC38 at 50% air saturation than at 5% air saturation. These results were very similar to those observed for the isogenic *poxB* mutant, TC44, during fermentation at 5% air saturation. Increasing the oxygen saturation during TC38 fermentations also decreased the final concentrations of acetate to a level equivalent to TC44 at 5% air saturation and decreased the production of dicarboxylic acids (Table 4-2).

Improving Pyruvate Yields and Titers of TC44 by Altering Fermentation Conditions

Prior experience with the acetate-producing strain, TC36, demonstrated that yields could be improved by simple changes in fermentation conditions (Causey et al. 2003). Unlike TC36, decreasing the ammonia level by half did not increase product yields for TC44 (Table 4-2). However, doubling the initial concentration of glucose or providing a second addition of glucose (3% plus 3%) increased pyruvate yields by 11% and doubled the final pyruvate titer. With excess glucose, 749 mM pyruvate was produced. This may represent the limit for pyruvate tolerance. Addition of 600 mM pyruvate to mineral salts medium inhibited the growth of wild type W3110 (data not included).

Discussion

Pyruvate occupies a key branch point in central carbon metabolism, its fate dependent on the redox state of the cell. During respiration, pyruvate is oxidatively decarboxylated by the pyruvate dehydrogenase complex (PDHc) to form NADH, CO_2 and acetyl-CoA. During fermentative metabolism, pyruvate acts as a final electron acceptor for NADH oxidation and is converted to reduced fermentation products such as lactic acid. During fermentation, approximately 5% of the metabolized pyruvate is converted to acetyl-CoA and formate (pyruvate formate-lyase; *pflB* gene) for

biosynthesis. Construction of an *E. coli* strain that excretes pyruvate at high yield during glucose catabolism required the elimination of non-essential pathways for pyruvate utilization and mutations that reduce the utilization of pyruvate for cell growth. Previously, we described a derivative of *Escherichia coli* W3110 (strain TC36) that combines many of the attributes of aerobic and fermentative metabolism and efficiently converts glucose to acetate, a redox neutral product (Causey et al. 2003). This strain was used as a microbial platform to engineer TC44 for the conversion of glucose to pyruvate, a more oxidized product, by mutating genes encoding acetate kinase (*ackA*) and pyruvate oxidase (*poxB*). Strain TC44 converted glucose to pyruvate with a yield of 0.75 g pyruvate g^{-1} glucose (77.9% of theoretical yield) under optimal conditions in minimal medium. Under conditions of glucose excess, a maximum of 749 mM pyruvate was produced.

Strain TC44 contains multiple mutations that ensure the rapid and efficient conversion of sugars into pyruvate. The minimal effect of these multiple mutations on growth rate was surprising. Indeed, glycolytic flux and maximal growth rate for TC44 were only 20% lower than the unmodified W3110 under similar growth conditions (unpublished results). The pyruvate oxidase mutation was surprisingly beneficial for growth and pyruvate production. Introduction of this mutation into the acetate-producing strain (TC36) resulted in a small improvement in growth rate in contrast to the mutation of acetate kinase that decreased growth rate by half (Table 4-3). The decreased growth rate of the acetate kinase mutant, TC36, cannot be explained by the predicted 30% reduction in ATP yield since growth rate was substantially restored by adding a subsequent mutation in pyruvate oxidase. These results suggested that the reduction in

growth rate by the acetate kinase mutation may be due to increased PoxB activity associated with a larger pyruvate pool.

Pyruvate oxidase transfers electrons from pyruvate to ubiquinone upon the decarboxylation of pyruvate (Cunningham and Hager 1975). An increase in pyruvate metabolized by pyruvate oxidase would increase oxygen demand and reduce the oxygen available for the oxidation of NADH. Since the total nicotinamide adenine dinucleotide pool is relatively constant (Wimpenny and Firth 1972) and the NAD^+/NADH ratio is responsive to changes in oxygen availability (Wimpenny and Firth 1972), increased pyruvate oxidase activity would also reduce the level of NAD^+ available for glycolysis. High NADH levels also serve as an important allosteric regulator of key enzymes such as citrate synthase (Weitzman 1966) and phosphoenolpyruvate carboxykinase (Krebs and Bridger 1976). Allosteric inhibition of these enzymes in minimal medium would limit both glycolytic flux and the partitioning of carbon into biosynthesis. Oxidation of NADH is also essential to provide the stochiometric levels of NAD^+ required for glycolysis. Thus, inactivating pyruvate oxidase may be expected to improve growth by eliminating the increase in oxygen demand, restoring the NAD^+ pools for glycolysis, and decreasing the level of NADH. This hypothesis was supported by further experiments in which the availability of oxygen was increased from 5% of air saturation to 50% of air saturation during fermentation with the acetate kinase mutant, TC38. The increase in oxygen (50% air saturation) substantially reversed the negative impact of the acetate kinase mutation on growth rate and metabolism, analogous to the effect of adding a pyruvate oxidase mutation. A similar increase in oxygen was of no benefit for TC44 in which both acetate pathways had been mutated (data not included).

During the construction of TC44 from the acetate-producing strain TC36, mutations in acetate kinase and pyruvate oxidase were tested individually prior to constructing the combination. These intermediate strains allowed an assessment of the relative roles of each pathway for acetate production in TC36. The acetate kinase pathway represents the dominant route for acetate production since deletion of the *ackA* gene decreased acetate production by 85% (Table 4-2). The pyruvate oxidase mutation decreased acetate production by 10% while the combination of both mutations decreased acetate production by 94%. The low level of acetate produced by TC44 containing both mutations is presumed to reflect a combination of multiple minor pathways and remains as a potential target for future improvement in pyruvate yield.

Pyruvate can be produced by a variety of microorganisms including mutants of yeasts and bacteria (Table 4-4). Typical production rates for these biocatalysts are around 1 g L^{-1} h^{-1} with yields exceeding half the weight of substrate. *Torulopsis glabrata*, a yeast strain currently used for the commercial production, can achieve pyruvate titers of 69 g L^{-1} although yields are somewhat lower than for TC44 and more elaborate process controls are required. *T. glabrata* strains used in the commercial process are multivitamin auxotrophs requiring tight regulation of vitamin concentrations which result in complex vitamin feeding strategies during fermentation (Li et al. 2001a). Previous *E. coli* strains constructed for pyruvate production were cultured in complex media and have been plagued by low titers and yields (Tomar et al. 2003; Yokota et al. 1994). In contrast to previous biocatalysts where vitamins and other complex nutrients are required for effective production of pyruvate by fermentation, *E. coli* TC44 requires only mineral salts, glucose and very simple process controls. The lack of a requirement for vitamin

Table 4-4. Comparison of biocatalysts for pyruvate production

Strain	Relevant genotype/ phenotype	Carbon Source	Nitrogen Source	Ferm-entation Time (h)	[Pyruvate] (g L^{-1})	Vol. Prod. (g L^{-1} h^{-1})	Pyruvate yield (g g^{-1})	Ref.
Candida lipolytica AJ 14353	B_1^- Met^-	glucose	NH_4NO_3	72	44	0.61	0.44	Li et al. 2001a
Debaryomyces hansenii Y-256	B_1^- Bio^-	glucose	Peptone	96	42	0.44	0.42	Li et al. 2001a
Torulopsis glabrata ACII-3	B_1^- Bio^- B_6^- NA^- acetate leaky	glucose	Soy hydrolysate $(NH_4)_2SO_4$	47	60	1.28	0.68	Li et al. 2001b
Torulopsis glabrata WSH-IP 303	B_1^- Bio^- B_6^- NA^-	glucose	NH_4Cl	56	69	1.23	0.62	Li et al. 2001b
Escherichia coli TBLA-1	*lipA2* bgl^+ *atpA401*	glucose	Poly-peptone	24	30	1.25	0.60	Yokota et al. 1994
Escherichia coli CGSC7916	*aceF fadR adhE ppc*	glucose acetate	Tryptone $(NH_4)_2HPO_4$	36	35	0.97	0.65	Tomar et al. 2003
Escherichia coli TC44	*pflB frdBC ldhA atpFH adhE sucA ackA poxB*	glucose	$(NH_4)_2HPO_4$	43	52	1.21	0.76	Causey et al. 2004

supplements, complex nutrients or complicated process controls for TC44 should provide a substantial savings in production costs. In addition, the lack of complex nutrients in the fermentation broth should also reduce costs associated with product purification and waste disposal. Based on this comparison, *E. coli* TC44 appears to represent a competitive alternative to the current pyruvate-producing biocatalysts due to high yields, high product titers, simple fermentation conditions, and the ability to grow well in mineral salts medium with glucose as the sole carbon source.

The remarkable tolerance of *E. coli* to drastic changes in metabolic flow that allows the production of acetate or pyruvate as alternative products of metabolism implies considerable elasticity in permitted pool sizes for key metabolic intermediates such as pyruvate and acetyl~CoA. It is interesting to note that pyruvate transiently accumulated in broth during fermentations with TC36, but was rapidly co-metabolized with glucose during the latter stages of fermentation. Similar transient accumulation and re-utilization was previously observed in minimal medium for other central metabolites (carboxylic acids and acetate) in other derivatives of W3110 (Causey et al. 2003). Exogenously added pyruvate was shown to be rapidly mixed with intracellular pyruvate followed by dissimilation (Yang et al. 2001). Carboxylic acids (Lutgens and Gottschalk 1980; Underwood et al. 2004) and acetate (Causey et al. 2003; Underwood et al. 2002) are also co-metabolized with sugars. Active transport systems have been identified in *E. coli* for pyruvate (Lang et al. 1987), acetate (Gimenez et al. 2003), and dicarboxylic acids (Lo et al. 1972; Pos et al. 1998; Seol and Shatkin 1991). Thus for pyruvate and perhaps any other compound, the extracellular milieu may be reasonably regarded as a reservoir for the temporary expansion of metabolic pools. A similar argument can be made for

CO_2(Merlin et al. 2003) and for acetaldehyde during ethanol production (Dombek and Ingram 1984; Stanley and Pamment 1993; Stanley et al.1993). The transient accumulation of metabolites in the aqueous milieu may be of evolutionary advantage. This accumulation could arguably regarded as a form of metabolic conditioning of the environment, as a storage reservoir, or as an expanded metabolic pool to relieve a temporary imbalance in metabolic flux. Enzymes are relatively stable in *E. coli.* Substantial remodeling of metabolism is primarily regulated at the level of transcription with dilution during subsequent growth. Transient storage of products from imbalanced metabolism in the extracellular milieu could increase the metabolic flexibility of *E. coli* during adaptation to a changing environment

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CHAPTER 5
EFFECT OF BETAINE ON THE FERMENTATION PERFORMANCE OF TC44 (PYRUVATE PRODUCTION) AND TC36 (ACETATE PRODUCTION)

Intoduction

The membranes that encompass bacterial cells are permeable to water but provide a barrier against most solutes. High osmolarity of the extracellular environment would result in dehydration unless the cell is able to increase the intracellular solute concentration (compatable solutes). Compatable solutes may be taken up from the environment (K^+, proline, betaine, etc.) or synthesized by the organism (glutamate and trehalose). *Escherichia coli* compensates for hyperosmotic stress, first, by increasing intracellular K^+ and glutamate concentrations and, second, synthesis of trehalose (Csonka and Hanson 1991). In minimal medium, trehalose is synthesized and replaces K^+ and glutamate as the major compatable solute in the cytoplasm (Csonka and Hanson 1991). Other osmoprotectant compounds, such as betaine, are also used for osmoprotection when they are available (Perroud and Le Rudulier 1985).

Betaine (and glycine betaine) is an effective osmoprotectant not synthesized by *E. coli.* Betaine has been demonstrated to protect bacteria against hyperosmotic stress as well as heat (reviewed in Csonka and Hanson 1991; Holtmann and Bremer 2004). In addition, the growth of ethanologenic *E. coli* KO11 was shown to be improved by the presence of betaine or choline in the growth medium (Underwood et al. 2004). Transport of betaine into the cytoplasm is through an energy dependent proton symport system (Perroud and Le Rudulier 1985; Styrvold et al. 1986). The transport protein, ProU, is

membrane bound and is transcribed from the *proU* promoter (Csonka and Hanson 1991). The *proV* and *proW* gene products are the membrane-associated proteins and the *proX* gene product is the periplasmic binding protein (Csonka and Hanson 1991). Transport does not depend on the solute causing osmotic stress but on the magnitude of the osmotic shock (Perroud and Le Rudulier 1985). *E. coli* can also transport choline into the cell and oxidize the choline to glycine betaine (Landfald and Strøm 1986). Betaine cannot be used as a carbon or nitrogen source (Perroud and Le Rudulier 1985). Betaine is effective as an osmoprotectant under both aerobic and anaerobic conditions but choline is only effective under aerobic conditions (Landfald and Strøm 1986).

Cayley et al. (1991) measured the cytoplasmic osmolytes of *E. coli* grown in MOPS-buffered glucose minimal medium, similar to the NBS medium used in our studies, with or without betaine. In the absense of betaine, K^+ accounted for 53% of the total osmolytes measured in the cytoplasm (Cayley et al. 1991; Cayley et al. 1992). Glutamate, trehalose and MOPS were present in minor quantities. Addition of betaine resulted in a modest increase in total cytoplasmic osmolyte pool (~ 10%), betaine accounted for 58% of the measured osmolytes (Cayley et al. 1991; Cayley et al. 1992). Potassium and glutamate were the only other osmolytes in the cytoplasm at concentrations above the limits of detection.

In this chapter the effects of betaine on the growth and product formation of TC36 and TC44 are described. Growth of *E. coli* W3110 in glucose minimal medium supplemented with increasing concentrations of acetate or pyruvate in the presence or absence of betaine will also be discussed.

Materials and Methods

Microorganisms and Media

Escherichia coli W3110 (wild type) was used to determine the minimum inhibitory concentration of acetate and pyruvate in the presence and absence of 1 mM betaine. The acetate producing strain TC36 (Table 2-1) and the pyruvate producing strain TC44 (Table 4-1) were used in 5 L fermentations. Strains were carried on NBS 2% glucose minimal plates as described in Chapter 2. Acid tolerance experiments were conducted with NBS 2% glucose minimal broth containing 0-1 M acetate or pyruvate. Seed culture and Batch fermentations were conducted as described in Chapter 4 (5 L working volume, 37^{o}C, 5% dissolved oxygen, 0.5 vvm gas flow and 350 rpm agitation) except that 10 % final glucose concentration was used. Duplicate fermentations were conducted.

Acetate and Pyruvate Tolerance

Acetate and pyruvate tolerance studies were conducted using NBS 2% glucose medium at pH 7 or pH 8 containing 0-1 M acetate or pyruvate. Betaine HCl was added to a final concentration of 1 mM or excluded from the broth. One millimolar betaine was previously determined to be the optimum concentration for protection against osmotic shock (Perroud and Le Rudulier 1985). Ten microliters of a logarimically growing culture of *E. coli* W3110 (~ 1 OD_{550}) was inoculated into 3 mL of the appropriate medium in a 13 x 100 mm test tube (triplicate cultures). The cultures were grown in a 37^{o}C water bath with shaking at 120 rpm. After incubation for 24 h the OD_{550} was read using a Bausch & Lomb Spectronic 70 spectrophotometer.

Results

Effect of Betaine on Growth and Acetate Production for *E. coli* TC36

Betaine has previously been shown to protect *E. coli* from osmotic shock (reviewed in Csonka 1991; Underwood et al. 2004).

Acetate fermentations with TC36, conducted using excess glucose, have acetate concentrations greater than 800 mM resulting in a 1.6 osmolar increase of the broth after neutralization with KOH. It was expected that addition of 1 mM betaine to the growth medium would improve growth rate by protecting TC36 from the osmotic stress imposed on the organism by 10% glucose. In addition, betaine was expected to increase acetate productivity by reducing the effect of the rising potassium acetate concentration throughout the fermentation.

E. coli TC36 fermentations were conducted in minimal medium containing 10% glucose with or without 1 mM betaine. The maximum specific growth rate was increased 2.8-fold by the addition of 1 mM betaine to the culture medium (Table 5-1) although both cultures entered stationary phase at approximately 24 h (Fig. 5-1A). Betaine had no effect on volumetric consumption of glucose or acetate production rates (Table 5-1; Fig. 5-1B,C). The maximum specific glucose consumption rate was reduced by 19% when betaine was incorporated into the medium (Table 5-1). Initially, the specific rate of acetate production was higher in the betaine-grown cultures (Fig. 5-1C) resulting in an increase in the maximum specific acetate production rate of 17% (Table 5-1). The rate of acetate production was lower during stationary phase with betaine in the medium (Fig. 5-1C). Overall, betaine did not improve the performance of TC36.

Table 5-1. Comparison of metabolic rates for TC36 in the presence or absence of 1 mM betaine

		Glucose consumption rate[a]		Acetate production rate[a]	
E. coli TC36	μ (h^{-1})	Average Vol.[b] (mmol L^{-1} h^{-1})	Max. Spec.[c] (mmol L^{-1} h^{-1} g^{-1})	Average Vol.[b] (mmol L^{-1} h^{-1})	Max. Spec.[c] (mmol L^{-1} h^{-1} g^{-1})
- Betaine	0.15	10	16	10	20
+Betaine	0.42	11	13	11	24

[a] The data represent an average of two fermentations.
[b] Average volumetric rates.
[c] Maximum specific rates per gram of dry cell weight.

Effect of Betaine on Growth and Pyruvate Production for *E. coli* TC44

Similar to acetate fermentations with TC36, strain TC44 experiences hyperosmotic environments when glucose and pyruvate concentrations are high. It was expected that addition of 1 mM betaine would at least partially alleviate the osmotic stress imposed on TC44. The maximum specific growth rate was increased 3.2-fold by the addition of betaine (Table 5-2). Cell mass reached 2 g L^{-1} by 24 h, more than double the cell mass at 60 h when betaine was not present (Fig. 5-2A). Betaine had a dramatic positive effect on both glucose consumption and pyruvate production (Fig. 5-2B,C). All of the glucose was consumed in 60 h when betaine was present, yet in the absence of betaine 90% of the glucose remained after 60 h (Fig. 5-2B). The average volumetric rate of glucose consumption was increased 10-fold and the specific rate of glucose consumption was increased 5.8-fold by the incorporation of betaine in the medium (Table 5-2).

Table 5-2. Comparison of metabolic rates for TC44 in the presence or absence of 1 mM betaine

		Glucose consumption rate[a]		Pyruvate production rate[a]	
E. coli TC44	μ (h^{-1})	Average Vol.[b] (mmol L^{-1} h^{-1})	Max. Spec.[c] (mmol L^{-1} h^{-1} g^{-1})	Average Vol.[b] (mmol L^{-1} h^{-1})	Max. Spec.[c] (mmol L^{-1} h^{-1} g^{-1})
- Betaine	0.09	1	6	2	13
+Betaine	0.29	10	35	16	32

[a] The data represent an average of two fermentations.
[b] Average volumetric rates.
c Maximum specific rates per gram of dry cell weight.

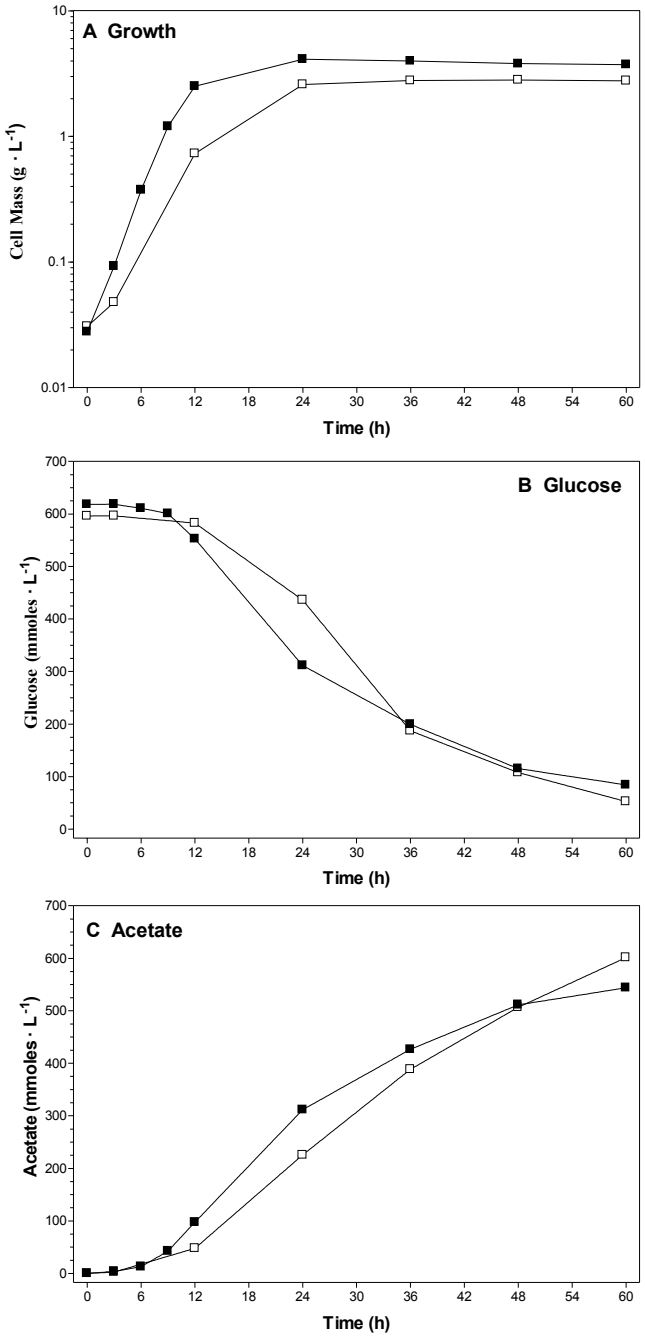

Figure 5-1. Effect of 1 mM Betaine on growth and acetate production for TC36. Fermentations were conducted in NBS medium with 10% glucose as the carbon source. Betaine was added to a final concentration of 1 mM when indicated. Fermentations were performed in duplicate. A) Growth. B) Glucose consumption. C) Acetate production. Symbols: Solid squares, + betaine; Open squares, -- betaine.

Volumetric and specific pyruvate production rates were dramatically increased 8-fold and 2.5-fold, respectively. In contrast to TC36, betaine improved both growth rate and production rates for TC44.

Determination of the Minimal Inhibitory Concentration of Acetate and Pyruvate on *E. coli* W3110 in NBS Medium

There were two possibilities that could explain drastic difference between the fermentation performance of the acetate producing strain, TC36, and the pyruvate producing strain, TC44. One possibility was that TC36 lost the ability to transport betaine by mutation(s) in the *proU* operon. Although possible, it is unlikely that TC36 lost the ability to transport betaine considering that TC44 was constructed from TC36 (Chapter 4). The more likely conclusion is that betaine was unable to provide the same level of protection for acetate as it did for pyruvate. Therefore, we determined the minimum inhibitory concentration for acetate and pyruvate in *E. coli* W3110.

E. coli W3110 was grown in NBS salts containing 2% glucose and 0-1M acetate or pyruvate as described in the Materials and Methods section. In the absence of betaine the minimum inhibitory concentration for acetate was 300 mM (Fig. 5-3A). The minimum pyruvate concentration necessary to stop growth was about 300 mM, 100 mM greater than that for acetate (Fig. 5-3A). Growth was partially inhibited by both acetate and pyruvate at the lowest concentration (100 mM) tested in the absence of betaine (Fig. 5-3A). Addition of 1 mM betaine did not increase the minimum concentration of acetate necessary to stop growth of W3110 (Fig 5-3B). Betaine may increase the acetate tolerance slightly, of W3110 at low (< 300 mM) concentrations. The minimum pyruvate concentration necessary to completely inhibit growth of W3110 was increased 29% to 700 mM with the addition of 1 mM betaine (Fig. 5-3B). In addition, up to

200 mMpyruvate had a positive effect on the growth of W3110 when betaine was present in the medium.

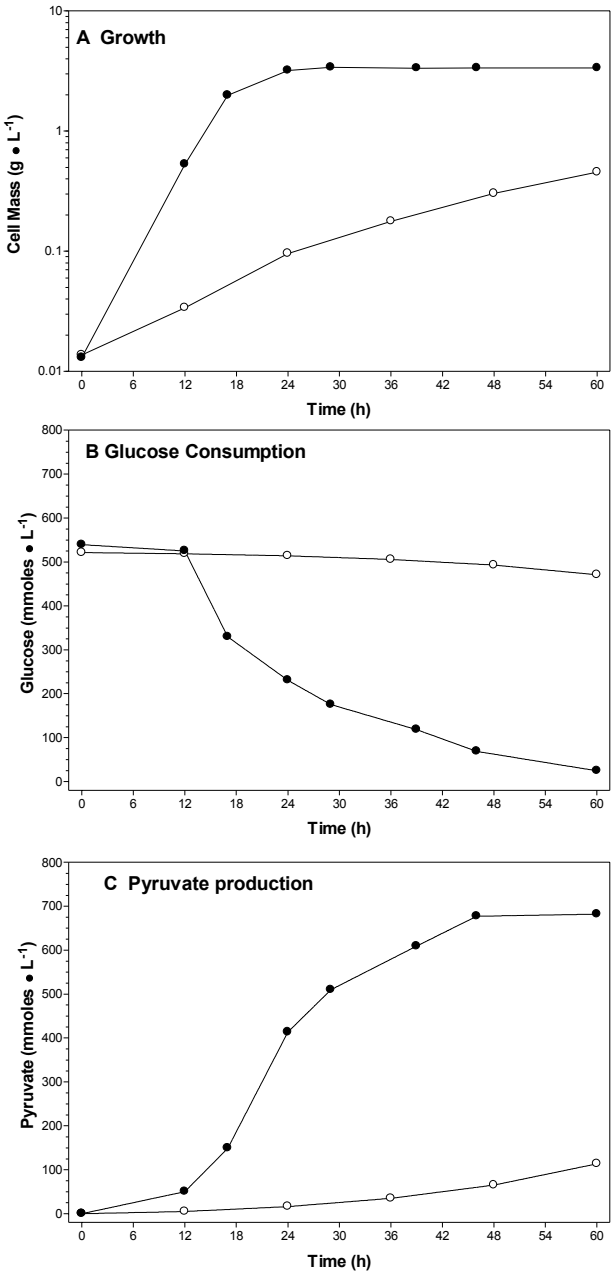

Figure 5-2. Effect of 1 mM Betaine on growth and pyruvate production for TC44. Fermentations were conducted in NBS medium with 10% glucose as the carbon source. Fermentations were performed in duplicate. A) Growth. B) Glucose consumption. C) Pyruvate production. Symbols: Solid circles, 1 mM betaine added to the medium; Open circles, no betaine added to the medium.

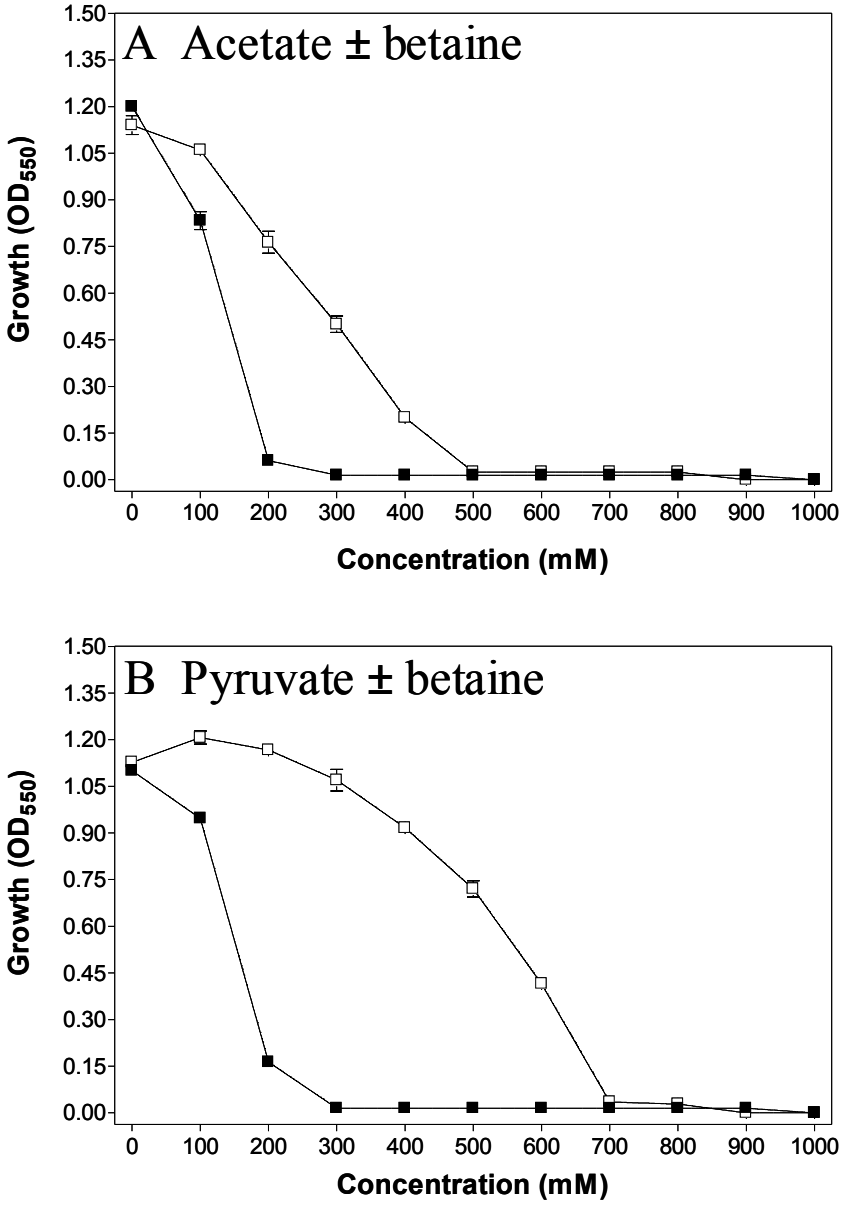

Figure 5-3. Effect of 1 mM betaine on the minimum inhibitory concentration of acetate and pyruvate for *E. coli* W3110. W3110 was grown in 3 mL of NBS salts containing 2% glucose, 0-1 M A) acetate or B) pyruvate. Results plotted from triplicate experiments. Symbols: Solid squares, no betaine; Open squares, 1 mM betaine added to the medium.

Discussion

Betaine did not increase acetate productivity by strain TC36. Early in the fermentation, when glucose levels were high and acetate levels were low, incorporating betaine into the medium resulted in a 17% increase in the growth rate. By 24 h the acetate concentration was 300 mM and TC36 was in stationary phase (Fig 5-1A,C). Acetate production was higher for fermentations that included betaine in the medium during the first 24 h. These data are consistant with the studies performed to determine the minimum inhibitory concentration of acetate necessary to stop the growth of *E. coli* W3110. Acetate was found to be growth inhibitory at 300 mM regardless of the presence of betaine. Growth was slightly elevated at lower acetate concentrations when betaine was present in the medium. This would explain the increased acetate production early during fermentations containing betaine and no benefit once acetate reaches 300 mM.

Pyruvate fermentations with TC44 were greatly enhanced when betaine was incorporated into the medium. All of the metabolic parameters measured were increased in fermentations containing 1 mM betaine. Pyruvate production continues until all of the glucose is consumed. These data are consistant with growth inhibition studies that showed 2.3-fold increase in tolerance to pyruvate when 1 mM betaine was in the medium.

Why would betaine be beneficial for pyruvate and not for acetate? One explaination could be the difference in pKa values resulting in different concentrations of the protonated species. The protonated, or uncharged, form of weak acids are considered to be toxic because the uncharged form of the acid is able to traverse the cytoplasmic membrane. The charged form cannot freely diffuse across the membrane. Acetate has a pKa of 4.76 and pyruvate has a pKa of 2.49. Applying the Henderson-Hasselbach

equation the ratio of the conjugate acid (HA) to the conjugate base (A^-) can be calculated. Although the conjugate acid concentration is very low for both acids at pH 7, the [A^-]/[HA] is 187-fold higher for pyruvate than for acetate. Lactic acid, which has a pKa value intermediate (3.86) between pyruvate and acetate, also has a minimum inhibitory concentration (500 mM) between that of pyruvate and acetate (personal communication with S. Zhou). To date there has been no published study comparing the tolerance of *E. coli* growing in the presence of weak organic acids when betaine is present or not present in the culture medium.

CHAPTER 6
CONCLUSIONS

E. coli is a facultative anaerobe producing a mixture of organic acids and ethanol from glucose for NAD^+ regeneration under low oxygen tension. Energy for biosynthesis is derived by oxidative substrate-level phosphorylation (glycolysis and acetate kinase) which results in 2-4 moles ATP produced per mole of glucose consumed. When oxygen tension is high, NADH produced during catabolism is oxidized by shuttling electrons down the electron transport chain. The transfer of electrons is coupled to the extrusion of protons generating a proton motive force. Protons are transported down the concentration gradient by the $(F_1F_0)H^+$-ATP synthase with the energy released stored in ATP. A calculated maximum of 25-33 ATP equivalents per glucose (depending on electron fluxes through NADH dehydrogenase I or II and cytochrome *bo* or cytochrome *bd* oxidases) are produced under optimal aerobic conditions (Calhoun et al. 1993). When oxygen tension is low, *E. coli* derives all of the energy for biosynthesis from oxidative substrate level phosphorylation. NADH oxidation is coupled to the reduction of internal metabolites, such as pyruvate and acetyl-CoA. Therefore, fermentative growth of *E. coli* results in conversion of glucose carbon into reduced products with minimal carbon loss to cell mass and CO_2. In contrast to fermentative growth, aerobic metabolism results in the production of cell mass and CO_2 in an approximate fifty-fifty ratio under optimal conditions (Ingraham et al. 1990).

The goal of the work described in this document was to combine aspects of anaerobic and aerobic metabolism in *E. coli* for the production of chemicals, not limited

to reduced compounds, from carbohydrate. The biocatalyst was constructed by making deletions in genes (*frdBC, pflB, ldhA, adhE*) coding for enzymes that produce the fermentations products (lactate, formate, ethanol, and succinate). Substrate utilization rate was increased by limiting ATP production through oxidative phosphorylation while maintaining cytoplasmic ATPase activity (deletion of *atpFH*). Substrate loss to CO_2 was limited by blocking the cyclic function of the TCA cycle (deletion of *sucA*). The strain incorporating all of the mutations mentioned above, efficiently converted glucose to acetate, a compound that is redox neutral relative to glucose. Figure 6-1 outlines the benefits derived each set of mutations.

Chromosomal deletions that eliminated the major pathways for organic acid production did not increase acetate production and fermentation time was not significantly increased (Fig. 6-1A,B). Cell mass increased slightly due to the low accumulation of organic acid in the medium (Fig. 6-1A,B). Construction of the *atpFH* mutation alone resulted in a 4.5-fold increase in acetate production and a reduction in the fermentation time by half compared with W3110 (Fig. 6-1A,C). Combining the mutations that limited organic acid production with the *atpFH* deletion resulted in a further increase in acetate production over TC40 (Fig. 6-1C,D). Incorporating mutations limiting carbon loss to CO_2 and disruption of the TCA cycle into TC24 (strain TC36) resulted in acetate produced at 70% of the theoretical yield with minimal carbon lost to cell mass or CO_2 (Fig. 6-1E).

During glucose fermentations to acetate with TC36, pyruvic acid would accumulate during logarithmic growth and subsequently be consumed when glucose became limiting. The transient accumulation of pyruvate indicated that TC36 could be engineered for

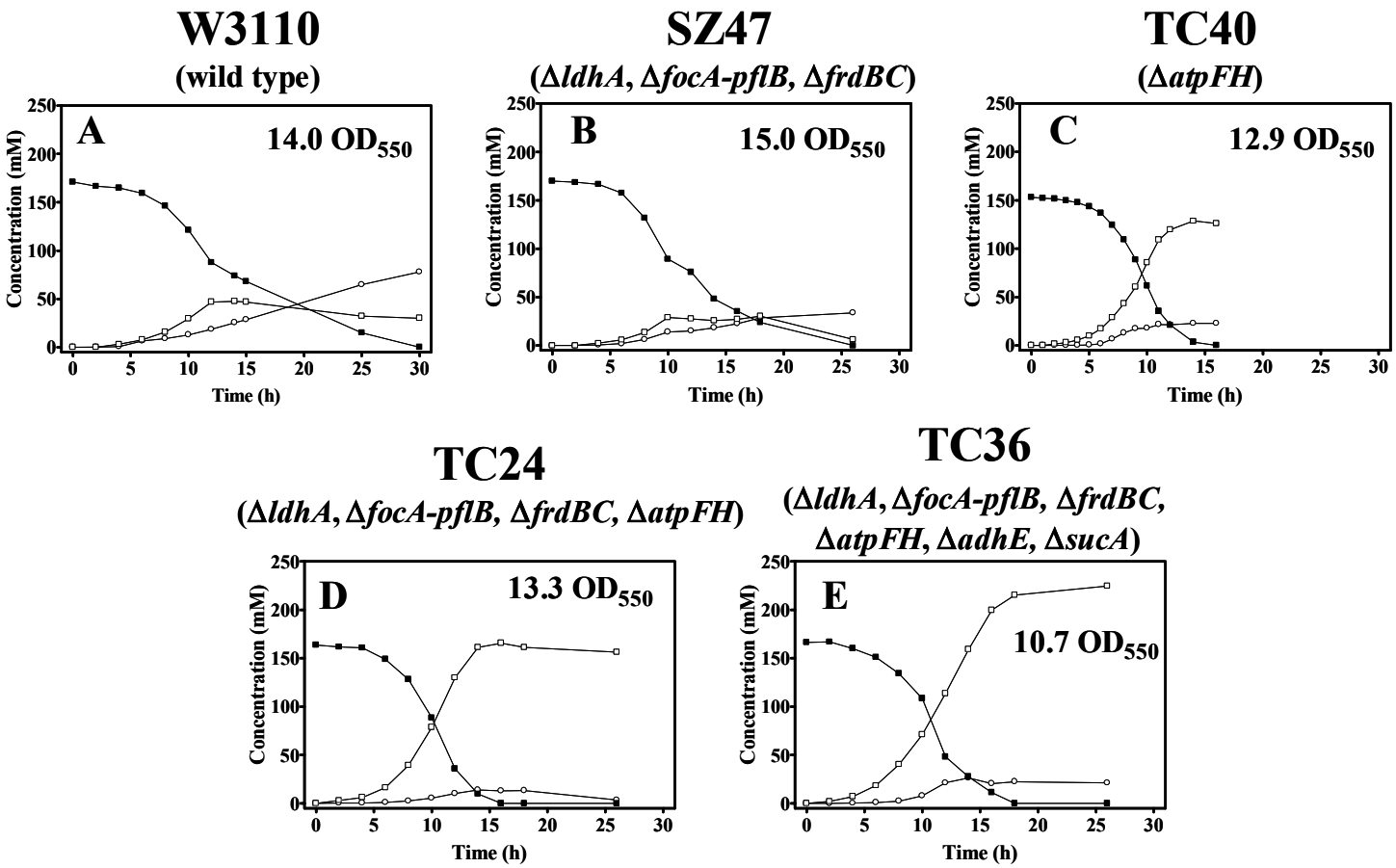

Figure 6-1. Fermentation profiles for selected strains during the construction of *E. coli* TC36. Optical densities represent the maximum cell mass attained. A) Wild type fermentation profile. B) Elimination of fermentation products. C) Elimination of oxidative phosphorylation. D) Elimination of fermentation pathways and oxidative phosphorylation. E) Elimination of fermentation pathways, oxidative phosphorylation and limiting CO_2 production. Symbols: Solid squares, glucose; Open squares, acetate; Open circles, total dicarboxylic acids.

pyruvate production by eliminating acetate production pathways, AckA-Pta and PoxB. The major acetate-producing pathway in *E. coli* is encoded by the *ackA* (acetate kinase) and *pta* (phosphotransacetylase) genes. Acetate is produced from acetyl-CoA through the phosphorylated intermediate acetyl-phosphate. Pyruvate oxidase (*poxB* gene product) produces acetate directly from the decarboxylation of pyruvate. Chromosomal deletions were constructed in *ackA* and *poxB* to eliminate acetate production (strain TC44). Strain TC44 was able to convert glucose to pyruvate at 78% of the maximum theoretical yield.

Both biocatalysts (TC36 and TC44) experience osmotic stress due to high sugar and salt concentrations during fermentations. Betaine added to the fermentation medium resulted in improved growth and pyruvate production with strain TC44. Betaine had no positive effect on TC36 fermentations. We believe the lack of improvement observed for TC36 fermentations was not due to differences between the strains, TC36 and TC44, but to the different effects of acetate and pyruvate on the cells. At pH 7, 1 mM betaine did not improve the tolerance of *E. coli* W3110 to acetate but greatly increased the growth when pyruvate was used instead.

E.coli TC36 and TC44 are versatile biocatalysts that can be used to convert the carbohydrate fraction of agricultural waste into useful compounds. The biocatalysts are not limited to the production of reduced compounds due to an intact electron transport chain. Tolerance to product may also be improved in medium containing low concentrations of betaine or choline. These biocatalysts will prove useful for the production of chemicals from plant biomass.

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BIOGRAPHICAL SKETCH

Bryce was born in Forrest City, Arkansas on October 3, 1973. He lived in Arkansas until 1989, at which time he moved with his family to Destin, Florida. Bryce graduated from Fort Walton Beach High School in 1992. Shortly after graduation, he joined the United States Army. He served in the field artillary for a little more than two years. After he left the Army Bryce worked for his father and attended Okaloosa-Walton Community College where he obtained an Associate of Arts degree. Bryce then attended the University of Florida where he graduated with honors in 1999 with a BS in microbiology. In August of 1999, Bryce began graduate school in the Department of Microbiology and Cell Science at the University of Florida. Bryce worked on engineering the metabolism of *Escherichia coli* for the production of fuels and chemicals while studing under the giudence of Dr. Lonnie Ingram. During graduate school, Bryce met his wife, Celeste, also pursuing graduate studies in microbiology.