IMPROVING THE PERFORMANCE OF *Escherichia coli* KO11 DURING THE FERMENTATION OF XYLOSE TO ETHANOL

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2003

This work is dedicated to my wife, Beverly, and my family. The years of their endless love and support made this work possible.

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Lonnie O. Ingram, for his insight and guidance in my academic development. His inspiration and general enthusiasm for science will have lasting effects on the development of my career.

My deepest appreciation is extended to the other members of my graduate committee: Dr. K. T. Shanmugam, Dr. Julie A. Maupin-Furlow, Dr. Gregory W. Luli and Dr. Jon D. Stewart. Without their guidance and sagacity, this work would not have been possible.

I am grateful to Lorraine Yamano, Dr. Shengde Zhou and Dr. Fernando Martinez-Morales for their help in learning the intricacies of molecular biology. Many thanks to Sean York and Alfredo Martinez for their help in learning our fermentation processes. I would also like to thank Dr. Marian L. Buszko for his guidance with NMR experiments.

My warmest thanks to the other members of Dr. Ingram's laboratory and the biomass research group for the many insightful discussions about scientific matters.

The text and figures in Chapter 2 and Chapter 3, in part or in full, are reprints of the material as it appears in *Applied and Environmental Microbiology* (vol. 68, pp. 1071-1081 and pp. 6263-6272, respectively).

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August 2003

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The large-scale conversion of lignocellulose to fuel ethanol would greatly reduce the U.S. dependence on imported oil. To facilitate this need, *Escherichia coli* has been genetically engineered for the homofermentative production of ethanol from all constituent sugars of lignocellulose. However, high levels of complex nutrients are required for rapid fermentation of xylose, the second most abundant sugar in lignocellulose. With low levels of complex nutrients, the rate of xylose fermentation was limited by the growth of the biocatalyst. In a mineral salts medium containing 1% corn steep liquor as a nutrient source (90 g liter^{-1} xylose), growth was limited by an imbalance in the partitioning of carbon between ethanol production and biosynthetic pathways. Citrate synthase was shown to catalyze the specific growth-limiting reaction. The allosteric controls of citrate synthase regulate carbon flow through the oxidizing arm of the TCA pathway, ultimately producing 2-ketoglutarate and glutamate. Functionally

expressing citrate synthase II (*citZ*) from *Bacillus subtilis* stimulated growth due to its different allosteric and kinetic properties. Acetyl-CoA server as an antagonist to the NADH-mediated allosteric inhibition of the *E. coli* citrate synthase. Supplementing the medium with pyruvate, acetate, acetaldehyde, 2-ketoglutarate or glutamate increased growth and ethanol production by activating, relieving or bypassing the allosteric regulation of the *E. coli* citrate synthase. Conservation of acetyl-CoA by mutating acetate kinase (Δ*ackA*) also increased growth and ethanol production, presumably by increasing the availability of acetyl-CoA (activating citrate synthase). In addition to biosynthetic needs, large intracellular pools of glutamate (>20 mM) function as a protective osmolyte. During growth in the high osmotic environment of the corn steep liquor medium containing 0.6 M xylose, intracellular glutamate was low (< 10 mM) and cells grew poorly, consistent with a glutamate deficiency. The addition of glutamate to the medium and all approaches that stimulated citrate synthase increased the high intracellular pool of glutamate during growth in this medium. Supplementing with other protective osmolytes, such as betaine and dimethylsulfoniopropionate, restored growth without affecting the intracellular pool of glutamate and appear to act directly as alternative osmolytes. These results indicate that the poor growth and ethanol production in 1% corn steep liquor medium (0.6 M xylose), the apparent requirement for high levels of nutrients without a specific auxotrophic requirement and the beneficial effects of increased intracellular glutamate all result from the requirement for high levels of protective osmolytes. Under these conditions, the growth of the biocatalyst (*E. coli*) and ethanol production are limited by insufficient levels of intracellular osmoprotectants rather than the synthesis of glutamate, per se.

CHAPTER 1
INTRODUCTION

The production of fuel ethanol from renewable feedstocks could potentially decrease the U.S. dependance on imported oil as well as decrease the release of fossilized carbon into the atmosphere as carbon dioxide (CO_2), a greenhouse gas. Blends of 95% ethanol with gasoline are effective motor fuels, as demonstrated by Brazil's use of such blends for more than 20 years prior to securing inexpensive sources of fossil fuels. In the year 2002, approximately 140 billion gallons of gasoline were consumed in the United States, most of which was derived from foreign oil. Approximately 2.9 billion gallons of ethanol are produced annually in the U.S., slightly more than 2% of the gasoline consumed. While the volume of ethanol produced increases each year, demands for ethanol and energy also increase. For example, the phasing out of the gasoline oxygenate methyl tertiary-butyl ether (MTBE) over the next several years will further increase the demand for fuel ethanol, an alternate oxygenate. A substantial increase in ethanol production must be achieved to replace MTBE with 10% ethanol.

Today, most of the ethanol derived from fermentation uses cornstarch as the feedstock with yeast as the biocatalyst. Competing demands for cornstarch and variable crop yields cause price volatility. Feedstock is the major contributor to the cost of current ethanol processes. The cost of ethanol production must remain low in order for it to be an economically competitive automobile fuel. The necessity for a less expensive, lower demand feedstock is obvious. Agricultural wastes (corn stover, sugarcane bagasse, wheat

straw, etc.) are relatively inexpensive sources of carbohydrates that can be converted to ethanol (Arntzen and Dale 1999; Ingram and Doran 1995; Ingram et al. 1999; Zaldivar et al. 2002). More than 200 billion gallons of ethanol could be produced using these lignocellulosic materials, sufficient to replace all of the gasoline burned by automobiles in the United States (Arntzen and Dale 1999). As these agricultural wastes have little or no competing uses, they offer long-term solutions to the necessity for inexpensive carbohydrate sources.

However, there is no known organism in nature capable of fermenting all of the various hexose and pentose components of biomass to ethanol. This difficulty is further compounded by the complex, polymeric and somewhat variable structure of the lignocellulosic biopolymers (Clarke 1997). Harsh treatments are required to breakdown these sugar polymers into suitable substrates for fermentation. During these processes, furfural, hydroxymethylfurfural, acetate, and many other cytotoxic byproducts are released into the resulting solutions. An organism must tolerate the environmental conditions created by these treatments to be an effective biocatalyst. With advances in molecular biology, genetically engineering a desirable microorganism to produce ethanol should be possible.

There are essentially two approaches to engineering an organism for the production of ethanol from lignocellulosic residues. Either an ethanol producing microorganism could be engineered to use all of the various sugars or a microorganism already capable of fermenting all of these sugars could be engineered to produce exclusively ethanol. The former approach has been pursued by many groups through the engineering of *Saccharomyces cerevisiae* or *Zymomonas mobilis* (deficient in pentose

metabolism) to utilize these carbohydrates by expressing heterologous transport and metabolic pathways (Aristidou and Penttila 2000; Chotani et al. 2000; Gong et al. 1999). While high productivities have been reported for both organisms in optimal conditions, yeasts capable of fermenting both xylose and arabinose have not been reported in the literature. *Z. mobilis*, a very fastidious organism, is not environmentally hardy, and the harsh conditions resulting from the pretreatment of the lignocellulose severely hinders its productivity.

One of the most studied and characterized organisms, *Escherichia coli* is an excellent candidate for genetic engineering. The complete genetic sequence has been published (Blattner et al. 1997), and much is known about its physiology (Neidhardt et al. 1990). The utility of this organism in industrial processes is second only to yeast. Typical of enteric organsism, *E. coli* is capable of fermenting both the pentoses and hexoses present in lignocellulose. However, *E. coli* is a mixed acid fermenter, producing lactate, acetate, ethanol, formate and succinate as its major fermentation products. Previous work in our laboratory engineered the metabolism of *E. coli* to produce exclusively ethanol (Ohta et al. 1991).

The sugars of hemicellulose hydrolysates, containing mostly xylose, were fermented by the engineered *E. coli* strain, with yields approaching 100% (0.51 g ethanol / g sugar) (Asghari et al. 1996; Lawford and Rouseau 1996; Martinez et al. 1999; York and Ingram 1996a; York and Ingram 1996b). However complex, expensive nutrients (Luria broth) are required to obtain these high yields. High levels of inexpensive nutrients are required to replace these rich nutrients (Asghari et al. 1996; Lawford and Rouseau 1996; Martinez et al. 1999; York and Ingram 1996a; York and Ingram 1996b), but this

creates waste management problems and increases cost. Fermentations which use low levels of complex nutrients or no nutritional supplements would be most desirable for industrial fermentations.

Corn steep liquor (CSL), a by-product from the wet milling of corn, is an inexpensive nutrient source with demonstrated utility in industrial processes. Fermentations of hemicellulose hydrolysate with CSL as the nutrient source exhibited dose-dependent change in ethanol productivities (Martinez et al. 1999). To equal the ethanol productivity achieved with Difco nutrients (5 g $liter^{-1}$ yeast extract and 10 g $liter^{-1}$ tryptone), 50 g $liter^{-1}$ CSL (wet weight; 50% solids) were required. The goal of this present study is to understand the basis of the need for complex nutrients and develop physiological and genetic solutions to circumvent this requirement.

Lignocellulose as a Carbohydrate Source

Most of the dry weight biomass is lignocellulose, composed of cellulose, hemicellulose, pectin and lignin (Clarke 1997). Cellulose, the most abundant polymer on the planet, is a homopolymer of cellobiose (β-1,4-glucose) and represents 20-50% of the dry weight of plant matter. Lignin is a polymer of aromatic alcohols, comprising 10-20% of the dry weight of plant biomass. Representing only 1-10% of the dry weight, pectin is a methylated homopolymer of galacturonic acid. Hemicellulose is a complex, branched polymer of hexoses (glucose, galactose, mannose, rhamnose, and fucose) and pentoses (xylose and arabinose). This polymer represents 20-40% of the plant dry weight and is the most easily solubilized component of lignocellulose.

The sugars of hemicellulose are released as monomers through a variety of hydrolysis procedures, but dilute acid hydrolysis is currently the preferred method

(Ingram et al. 1999). This procedure uses moderate heat and low pH to release the sugars of hemicellulose into solution as monomers (Grohmann et al. 1985). The exact ratio of sugars in these hydrolysates can vary considerably depending on the feedstock, but xylose is the most prevalent sugar in hydrolysates of hard woods and grasses (sugarcane, wheat straw, etc.). Generating a concentrated sugar solution during hydrolysis is a formidable challenge, but a goal of 100 g liter^{-1} total sugar monomers in hemicellulose hydrolysates should be achievable. Most of the studies presented here have used 90 g liter^{-1} xylose as the fermentation substrate.

Adaptation to High Sugar Environments

Many industrial fermentation processes operate as either batch fermentations (with all required nutrients and substrates supplied initially) or fed-batch fermentations (multiple additions of nutrients; requires concentrated feed solutions). As the hydrolysis of hemicellulose produces sugar streams up to 100 g liter^{-1} (Ingram et al. 1999), their fermentation to ethanol favors a batch fermentation process to avoid the additional cost of concentrating these sugar streams and potentially concentrating growth inhibitory compounds. However, this relatively high sugar concentration requires *E. coli* to adapt to this higher osmolarity.

The rapid accumulation of potassium is the first response of *E. coli* and related organisms to an increase in the osmotic strength of the medium. Within a minute after an increase in osmotic pressure, glutamate (a negatively-charged amino acid) synthesis is increased to provide charge balance for the accumulated potassium (McLaggan et al. 1994). The short time between the accumulation of potassium and the biosynthesis of glutamate, suggests that the onset of glutamate biosynthesis is a result of allosteric

regulation (<5 min) rather than genetic induction (10-20 min). Additionally, the accumulation of glutamate in response to osmotic stress was found to be dependent on the presence of K^+ in the medium (McLaggan et al. 1994).

Escherichia coli has two biosynthetic pathways for glutamate. Under a nitrogen limitation (0.1 mM ammonium), glutamate synthase-glutamine synthetase has been shown to be the predominant glutamate biosynthetic pathway (Pahel et al. 1978). During growth in excess nitrogen, glutamate dehydrogenase (GDH), a pathway that does not consume ATP, is the primary glutamate biosynthetic pathway (Helling 1994). Additionally, GDH is activated by K^+(Measures 1975). This allosteric regulation of GDH has been proposed to be responsible for osmotically activated glutamate biosynthesis (Helling 1994).

The intracellular concentration of K^+ can be as high as 800 mM in *E. coli* during growth in media of high osmolarity (Cayley et al. 1991; Cayley et al. 1992). Cells deficient in glutamate accumulation have demonstrated growth defects during osmotic challenge (Csonka 1988; McLaggan et al. 1991; Yan et al 1996) due to an inability to maintain sufficient K^+ (Yan et al. 1996). The large increases in intracellular potassium and glutamate are transient, and their levels begin to decrease to 20-50 mM as trehalose or other protective osmolytes accumulate in the cytoplasm (Dinnbier et al.1988; Giaever et al. 1988). However, glutamate pools remain elevated during growth the higher osmotic conditions (Yan et al. 1996).

For the long-term adaptation to media of high osmolarity, *E. coli* synthesizes trehalose (Boos et al. 1990; Dinnbier et al. 1988; Giaever et al. 1988) or accumulates other charge-neutral (zwitterionic) compatible solutes (betaine, proline , ectoine,

dimethylsulfoniopropionate, etc.) (Csonka and Hanson 1991). *E. coli* has a limited capacity for biosynthesis of these compounds. Although *E. coli* is incapable of de novo betaine biosynthesis, choline can be oxidized to betaine. However, this process is restricted to aerobic growth (Landfald and Strøm 1986). Some organisms synthesize proline for long-term osmoadaptation (Kawahara et al. 1989). However, the γ-glutamyl kinase step in proline biosynthesis is subject to strong feedback inhibition in *E. coli*, preventing the biosynthesis of this protective osmolyte (Csonka 1988; Smith 1985; Smith et al. 1984). Thus, many of the protective osmolytes accumulated by *E. coli* must be taken from their environment.

E. coli and related organisms have two primary transport systems for protective osmolytes during osmotic stress, ProP and ProU (Randall et al. 1995). The ProP system uses the proton gradient maintained by the cell to drive the uptake of osmoprotectants. This low affinity system (K_m for proline is 0.3 mM) also transports many other osmoprotectants (Lucht and Bremer 1994). The ProU transport system consists of a periplasmic binding protein with a high affinity for betaine (K_m 1.3 μM), a membrane-spanning component and a membrane bound enzyme which hydrolyzes ATP for the active transport of betaine (Lucht and Bremer 1994).

A hierarchy for osmoprotectants has been empirically established for *E. coli*, primarily for salt-mediated osmotic stress (Randall et al. 1995). Although there have been conflicting reports concerning the validity of this hierarchy for sugar-mediated osmotic stress (Glaasker et al. 1998), betaine is generally regarded as the most effective protective osmolyte for *E. coli.* In at least one report, the ability of betaine to restore growth during osmotic challenge with different carbon sources was dependent on the particular sugar

(Dulaney et al. 1968). Thus, the sugar-mediated osmotic stress anticipated for fermentations of hemicellulose hydrolysates (100 g liter^{-1} sugar) may require the accumulation of different osmolytes.

Xylose versus Glucose Metabolism

The reactions involved in the transport and metabolism of glucose are well understood and outlined in Figures 1-1 and 1-2. Transport of glucose into the *E. coli* cytoplasm is mediated by a phospho-transferase system (PTS). The energy and phosphate required for translocation and phosphorylation of PTS sugars comes from phosphoenolpyruvate (PEP). An additional ATP is required for the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate. Thus, to metabolize glucose, an initial investment of 2 ATP equivalents (1 ATP and 1 PEP) is required.

Fructose-1,6-bisphosphate is cleaved into dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate. These two molecules are interconverted via triose-phosphate isomerase. For the production of pyruvate, the terminal product of glycolysis, glyceraldehyde-3-phosphate is oxidized and phosphorylated to form 1,3-bisphosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase. During this step, nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH. The high group-transfer potential of the phosphate bond on carbon 1 is used in the production of ATP from ADP in the proceeding reaction catalyzed by phosphoglycerate kinase. The reactions leading to the formation of phosphoenolpyruvate do not result in any further energy yield or reducing equivalents.

In converting glyceraldehyde-3-phosphate to PEP, 1 ATP and 1 NADH are produced. The conversion of PEP to pyruvate is either carried out via the

phosphotransferase system or through an ATP yielding reaction catalyzed by a pyruvate kinase. The net reaction of glycolysis can be written as follows:

$$\text{glucose} + 2NAD^+ + 2ADP + 2\ P_i \rightarrow 2\ \text{pyruvate} + 2NADH + 2H^+ + 2\ ATP$$

The production of pyruvate from ½ molecule glucose yields a net of 1 ATP and 1 reducing equivalent (NADH). Reducing equivalents are often considered as pools of both their reduced and oxidized forms, and their ratio is indicative of the metabolic state of the cell (respiration or fermentation) (de Graef et al. 1999; Snoep et al. 1990). Though the ratio of the reduced to oxidized form ($NADH/NAD^+$ ratio) varies widely with different growth conditions, the absolute concentration of the two forms remains relatively constant (de Graef et al. 1999).

In contrast to glucose, xylose is transported into the cell by either a proton symport pathway (*xylE*) or an ATP dependant transporter (*xylFGH*) (Song and Park 1997; Tao et al. 2001; Fig. 1-3). During fermentation, the cellular proton gradient is maintained presumably by energy-consuming reactions (F_1/F_0 ATPase, for example). Thus, a proton symport pathway is fueled indirectly by the hydrolysis of ATP. The transport of each xylose is energized by the hydrolysis of 1 ATP. Once inside the cell, xylose is converted into xylulose by xylose isomerase. Xylulose is then phosphorylated by xylulokinase, utilizing the hydrolysis of a second ATP. Regardless of the pathway, xylose uptake and activation (phosphorylation) require energy derived from the hydrolysis of 2 ATP molecules. In contrast, glucose transport uses a single ATP equivalent (PEP) for both transport and activation.

Intracellular xylulose-5-phosphate is metabolized by the pentose-phosphate pathway (Fig 1-4). Through a series of reactions catalyzed by transketolase and

transaldolase, xylose is converted into intermediates of glycolysis (fructose-6-phosphate and glyceraldehyde-3-phosphate). For every 6 xyloses consumed (30 carbon atoms), 4 fructose-6-phosphates and 2 glyceraldehyde-3-phosphates are produced. These molecules are further metabolized by glycolysis to ultimately yield 10 molecules of pyruvate. Thus, all 30 carbon atoms which began in xylose are converted into pyruvate.

The energy (ATP) and reducing equivalents (NADH) produced from the reactions common to xylose and glucose metabolism are the same regardless of the original substrate. However, since xylose is a pentose and requires separate energy for transport and activation, growth on xylose results in a relatively low ATP yield. The transport and activation of 6 xylose molecules (30 carbons) requires 12 ATPs, assuming 1 ATP is required for transport regardless of the pathway. In the conversion of this xylulose-5-phosphate to 10 molecules of glyceraldehyde-3-phosphate, an additional 4 ATPs are consumed. The conversion of these 10 molecules of glyceraldehyde-3-phosphate to pyruvate yields 20 ATPs. The net gain of energy in the conversion of 6 molecules of xylose to 10 pyruvate is 4 ATPs. An equal amount of glucose on the basis of moles of carbon (5 molecules; 30 carbon atoms) produces a net of 10 ATPs during conversion to 10 molecules of pyruvate. The net energy gain for glucose catabolism is 1 ATP per pyruvate, 2.5-fold more ATP than from xylose catabolism. While there is a considerable difference in ATP yield between xylose and glucose, only one NADH is produced per pyruvate from either glucose and xylose.

Pyruvate Dissimilation

A facultative anaerobe, *E. coli* accomplishes redox balance either by respiration (Gennis and Stewart 1996) or fermentation (Bock and Sawers 1996). During respiration,

reducing equivalents are oxidized when their electrons are donated to the primary oxido-reductases of the electron transport system. These electrons are passed between the various proteins of the electron transport chain and ultimately used to reduce a terminal electron acceptor (oxygen during aerobic respiration, for example). The energy from these reducing equivalents is preserved in the form of a proton gradient established by the concomitant translocation of H^+ from the cytosol to the periplasm. This proton gradient is used to produce ATP via the F_1/F_0 ATPase.

The pyruvate dehydrogenase complex (PDH) catalyzes the oxidative metabolism of pyruvate to acetyl-Coenzyme A (acetyl-CoA) and CO_2, with the formation of 1 reducing equivalent (NADH). This complex consists of three activities: pyruvate decarboxylase (*aceE*), acetyltransferase (*aceF*), and lipoate dehydrogenase (*lpd*) (Fig. 1-5). The decarboxylation of pyruvate to an enzyme-bound acetyl moiety by the pyruvate decarboxylase of this complex requires a thiamine pyrophosphate (TPP) cofactor, a carrier of the "active" acetaldehyde. The acyl moiety is then transferred to an acyl-carrier through the reduction of a disulfide bond. The resulting thioester has a high group transfer potential and is transferred to Coenzyme A, an acetyltransferase reaction. The disulfide which accepts the acetaldehyde from the TPP must be regenerated through an oxidation/reduction reaction. NAD^+ is reduced to NADH as the sulfhydryl group is oxidized, forming the required disulfide bond. This reaction is subject to strong feedback inhibition by NADH (Hansen and Henning 1966).

During aerobic growth, the tricarboxylic acid (TCA) cycle is responsible for the total oxidation of acetyl-CoA to CO_2 (Cronan, Jr. and LaPort 1996). The first step in this cycle, citrate synthase, is also the rate controlling step (Lee et al. 1994; Walsh and

Koshland, Jr. 1985). This enzyme catalyzes the condensation of acetyl-CoA and oxaloacetate to form citrate (Weitzman 1981). Citrate synthase is primarily regulated by allosteric controls, activated by acetyl-CoA and inhibited either by NADH and 2-ketoglutarate (Gram-negative) or ATP (Gram-positive, archea and eukaryotes) (Weitzman 1981). This provides a link between the energetic needs of the cell and the generation of reducing equivalents (and ultimately ATP) through the TCA cycle.

The TCA cycle is also a source of carbon skeletons for biosynthesis. More than half of the amino acids made by the cell are derived from intermediates of the TCA cycle (Neidhardt et al. 1990). Oxaloacetate must be regenerated for the continued cyclic action as intermediates are drawn into biosynthesis. This anapleurotic reaction is catalyzed by phosphoenolpyruvate carboxylase in *E. coli.* The biosynthetic needs and metabolic state of the cell dictate the activity of this reaction through allosteric control. Acetyl-CoA, fructose-1,6-bisphosphate and GTP are activators of this enzyme (Izui et al. 1981), while malate and aspartate (products of oxaloacetate utilizing reactions) are inhibitors (Izui et al. 1981). Acetyl-CoA and oxaloacetate are co-substrates for citrate synthase. Thus, the allosteric activation of phosphoenolpyruvate carboxylase and citrate synthase by acetyl-CoA links the availability of the two co-substrates for citrate synthase.

During fermentation, no external terminal electron acceptors are available for respiration, resulting in the accumulation of NADH to higher levels than during respiration (de Graef et al. 1999). For redox balance to be maintained, intracellular metabolites serve as electron acceptors. As NAD^+ regeneration becomes difficult, NADH generation is not favored. The formation of acetyl-CoA from the pyruvate dehydrogenase reaction is inhibited by the high NADH/NAD^+ ratio (Hansen and Henning 1966),

necessitating an alternate, non-oxidative route to acetyl-CoA generation. The non-oxidative cleavage of pyruvate to acetyl-CoA and formate is catalyzed by pyruvate formate-lyase (PFL; Knappe and Sawers 1990).

PFL activity is relative to the metabolic state of the cell, similar to PDH and citrate synthase. However, PFL activity is regulated by post-translational modification enzymes which are allosterically controlled. The protein is translated in an inactive form. An oxygen-labile free radical is placed on a glycine residue by a PFL-activase enzyme (*pflA*) forming the active PFL enzyme (Conradt et al. 1984). To protect the enzyme from irreversible inactivation by oxygen, the multi functional alcohol dehydrogenase (*adhE*) also has a PFL-deactivase activity to remove the oxygen-labile free radical (Kessler et al. 1991). The PFL-deactivase activity of *adhE* is inhibited by NADH (Kessler et al. 1992), linking the activation state of PFL to the metabolic state of the cell as described by the $NADH/NAD^+$ ratio. When oxygen supply is limited, NADH accumulates and inhibits PDH and the PFL deactivase activity. This causes a shift in flux to acetyl-CoA from oxidative pyruvate cleavage (PDH) to the non-oxidative cleavage (PFL).

In contrast to respiration, acetyl-CoA is an electron acceptor during fermentation. The two-step reduction of acetyl-CoA to ethanol is catalyzed by alcohol dehydrogenase (*adhE*; Fig. 1-6), regenerating 2 NAD^+. Glycolysis produces only one NADH per pyruvate. Thus, the native alcohol production pathway results in an NADH deficit. This is overcome by converting one of the acetyl-CoA to acetate, producing an additional ATP by substrate-level phosphorylation. In *E. coli* grown under anaerobic fermentation conditions with glucose as the carbon and energy source, equal amounts of acetate and ethanol are produced.

Lactic acid is often produced by *E. coli* during fermentation in addition to acetate and ethanol, primarily as active growth slows and stationary growth. Pyruvate is reduced in a single-step reaction catalyzed by lactate dehydrogenase (LDH, *ldhA* gene product; Bunch et al. 1997), resulting in the re-oxidation of 1 NADH per lactate produced. The pathway for lactate production in *E. coli* is controlled by allosteric regulation, activated by pyruvate (Tarmy and Kaplan 1968). In conditions of surplus supply of pyruvate, the lactate pathway is activated. There is an associated energetic loss as a result of lactate production compared to the co-production of acetate and ethanol, as no ATP is made in the reduction of pyruvate to lactate.

In contrast to respiration, the TCA cycle is interrupted at 2-ketoglutarate dehydrogenase due to transcriptional regulation during fermentation (Iuchi and Lin 1988). The resulting pathway has two sides, the reductive (leading to succinate production) and the oxidative (stopping at 2-ketoglutarate). For succinate production during fermentation, the anapleurotic pathway for oxaloacetate production (PPC) is the first step. As described previously, there are multiple allosteric effectors of this enzyme which control its physiological activity. Oxaloacetate is converted to malic acid through the reverse activity of the malate dehydrogenase, regenerating 1 NAD^+ (Bock and Sawers 1996). Fumarase catalyzes the conversion of malate to fumarate. Fumarate is reduced to succinate by a fermentation specific fumarate reductase (*frdBACD* gene products) with the oxidation of a reduced menaquinone (Cronan, Jr. and LaPort 1996). The oxidative side of the TCA pathway provides carbon skeletons for biosynthesis (Neidhardt et al. 1990).

Engineering *E. coli* for Ethanol Production

The enteric bacterium *E. coli* can use all of the sugar constituents of lignocellulose, while the natural ethanol producing *Saccharomyces cerevisiae* and *Zymomonas mobilis* are limited to growth on hexoses,. Wild-type *E. coli* produces ethanol from the two step reduction of acetyl-CoA , oxidizing two NADH to NAD^+. As a result, acetate (no further reduction required) is made in approximately equal amounts to ethanol. However, *Z. mobilis* and *S. cerevisiae* produce ethanol from pyruvate through a pathway which only re-oxidizes one NADH. The irreversible, non-oxidative cleavage of pyruvate into acetaldehyde and carbon dioxide is catalyzed by pyruvate decarboxylase (PDC). Acetaldehyde is reduced to ethanol, oxidizing 1 NADH. Thus, for each pyruvate that is converted to ethanol via this pathway, one NADH is re-oxidized. With this stoichiometry, all of the pyruvate generated by glycolysis can be converted to ethanol without the necessity of other oxidized products to maintain redox balance.

Previous studies demonstrated that the *Z. mobilis* genes involved in ethanol production are expressed well in *E. coli* (Ingram and Conway 1988). These genes (*pdc* and *adhB*) were used to construct a synthetic operon which was integrated into the chromosome for increased genetic stability of the recombinant strain (Ohta et al. 1991). A deletion was introduced in fumarate reductase to decrease succinate production, problematic in xylose fermentation. The resultant strain, designated KO11, fermented both pentoses and hexoses to ethanol with yields approaching 100% of total sugars present (0.51 g ethanol/g sugar = 100% theoretical yield) during fermentation in laboratory media containing excess complex nutrients. In addition to the alterations to the fermentation profile, there were some notable effects on growth physiology. In broth

cultures, comparatively high cell yields were achieved. On solid media, colonies exhibited a raised morphology, similar to yeast.

Deleterious Effects of Metabolic Engineering

The engineering of metabolic pathways for the production of industrial chemicals as an alternative to chemical synthesis has been performed for a variety of chemicals (Chotani et al. 2000). Metabolic engineering for renewable chemicals such as ethanol (Ingram et al. 1999), acetate (Causey et al, 2003), lactate (Bianchi et al. 2001; Chang et al. 1999a; Dien et al 2001; Kyla-Nikkila et al. 2000; Zhou et al. 2002; Zhou et al. 2003), propanediol (Nakamura et al. 2000; Tong et al. 1991), adipic acid (Niu et al. 2002) and succinate (Donnelly et al 1998a; Donnelly et al. 1998b; Vemuri et al. 2002) have focused primarily on product yields. The metabolic engineering of these new products has often resulted in unexpected changes which increased the need for complex nutrients and decreased potential utility (Bunch et al. 1997; Chang et al. 1999a; Chao and Liao 1994; Chao et al. 1993; Martinez et al. 1999).

Undesirable changes such as reduced growth, decreased glycolytic flux and low volumetric productivity are generally attributed to a lack of ATP (Gokarn et al. 2000; Xie et al. 2001), creation of futile cycles (Chao and Liao 1994; Chao et al. 1993; Patnaik et al. 1992), changes in intracellular metabolite pools or a metabolic imbalance (Aristidou et al. 1992; Bunch et al. 1997; Chang et al. 1999b; Contiero et al. 2000; Liao et al. 1996; Yang et al. 1999a; Yang et al. 1999b; Zhou et al. 2002; Zhou et al. 2003). Often, these detrimental effects are masked by abundant complex nutrients in laboratory media and are only apparent in mineral salts or low-nutrient media (Bunch et al. 1997; Chao and Liao 1994; Chao et al. 1993; Martinez et al. 1999).

Salmonella typhimurium was engineered for succinate production by increasing the expression of *pyc* encoding pyruvate carboxylase (Xie et al. 2001). Although succinate production increased, growth rate declined by 18% and glycolytic flux decreased by 40%. Similar results were reported for an analogous construction in *E. coli* (Gokarn et al. 2000). Donnelly and coworkers (1998a and 1998b) isolated *E. coli* mutants which produced 5-times more succinate than the parent strain, and again growth rate was impaired. Growth rate and cell yield were also decreased by engineering *E. coli* for the production of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) by over-expression of *pps* (phosphoenolpyruvate synthase) (Patnaik and Liao 1994; Patnaik et al. 1992). This inhibition of growth was more pronounced in minimal medium (Chao and Liao 1994).

Acetate production during the aerobic growth of *E. coli* on sugars has been correlated with a decline in metabolic activity and reduced expression of heterologous genes (Aristidou et al. 1995; Bauer et al. 1990; Chang et al. 1999b; Luli and Strohl 1990). Many approaches have been employed to decrease acetate production and increase recombinant products (Aristidou et al. 1995; Barbosa and Ingram 1994; Bauer et al. 1990; Chang et al. 1999b; Contiero et al. 2000; Yang et al. 1999a). Mutations in the primary acetate pathway (*pta,* phosphotransacetylase; *ackA,* acetate kinase) increased the yield of recombinant proteins, but usually reduced cell growth. The detrimental effect on growth was attributed to the accumulation of metabolic intermediates such as acetyl-CoA or acetyl-phosphate. An alternative approach, channeling pyruvate away from acetate by expressing the *Bacillus subtilis alsA* gene encoding acetolactate synthase, also reduced acetate production by 80% and increased product yields, but again reduced cell growth (Yang et al. 1999a). Other attempts to decrease acetate production by increased

expression of *ldhA* (lactate dehydrogenase) were ineffective in rich medium (Yang et al. 1999b). In mineral salts medium, over-expression of *ldhA* was accompanied by a severe growth limitation (Bunch et al. 1997).

Lactate dehydrogenase (*ldhA*) has been expressed to divert carbon away from acetate accumulation. Despite the relatively high K_m for pyruvate, lactate production increased by 50% (Yang et al. 1999b). Interestingly, the amount of acetate produced in these fermentations was not altered. However, these studies were conducted in a rich laboratory medium. In a mineral salts medium (M9), expression of LDH resulted in severe growth defects (Bunch et al. 1997). These growth defects were attributed to a decrease in pyruvate availability necessary for growth.

Project Goals

Though strain KO11 is prototrophic, high levels of complex nutritional supplements are required for the rapid fermentation of sugars to ethanol (Martinez et al. 1999). For example, during the fermentation of 90 g liter^{-1} xylose to ethanol, the addition of CSL as a nutritional supplement (0-50 g liter^{-1}) had a dose dependent effect on final cell concentration. With the increase in biocatalyst concentration, there was a proportional increase in fermentation rate and decrease in required fermentation time. Although the addition of 50 g liter^{-1} CSL is not cost-prohibitive, the handling of this much material on the scale of an industrial fermentation could be problematic and generate excessive cost for waste disposal.

These studies will examine the basis for the high nutrient requirement for KO11 for the rapid conversion of sugar to ethanol. As a starting point, growth and ethanol production will be evaluated in a medium containing 1% CSL, 90 g liter^{-1} xylose, and

mineral salts. Physiological and genetic approaches will be used to characterize the growth limitation. Solutions for solving this limitation will be presented. Further work will demonstrate the basis for a specific biosynthetic pathway under the conditions tested. Knowledge gained in these studies will have applications in the further development of the commercial production of ethanol from plant biomass by metabolically engineered *E. coli* and will significantly contribute to the field of metabolic engineering by emphasizing the importance of metabolic intermediates down-stream of the product forming node.

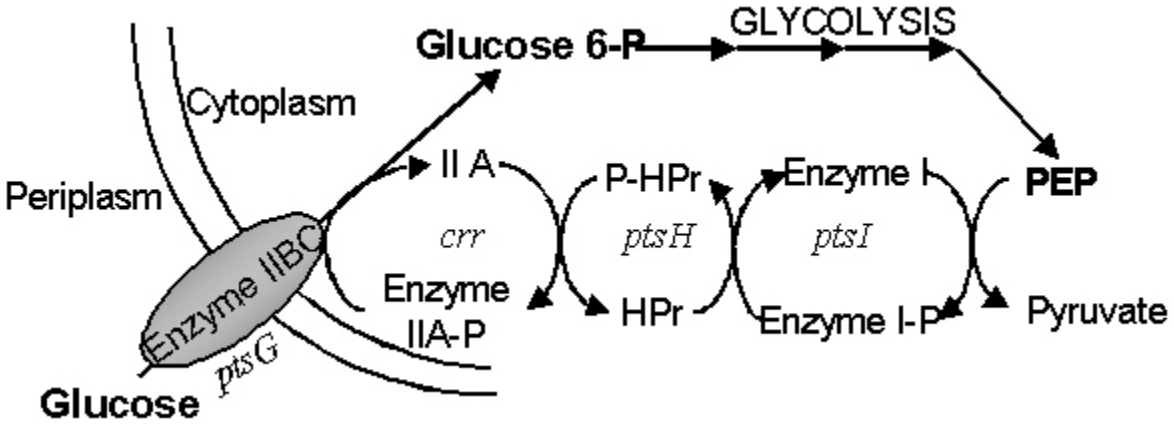

Figure 1-1. Glucose transport by the phosphotransferase system. The phosphate from PEP passes through a cascade of enzymes and ultimately to intracellular glucose. The hydrolysis of 1 ATP equivalent (PEP) is used to energize transport and activate glucose.

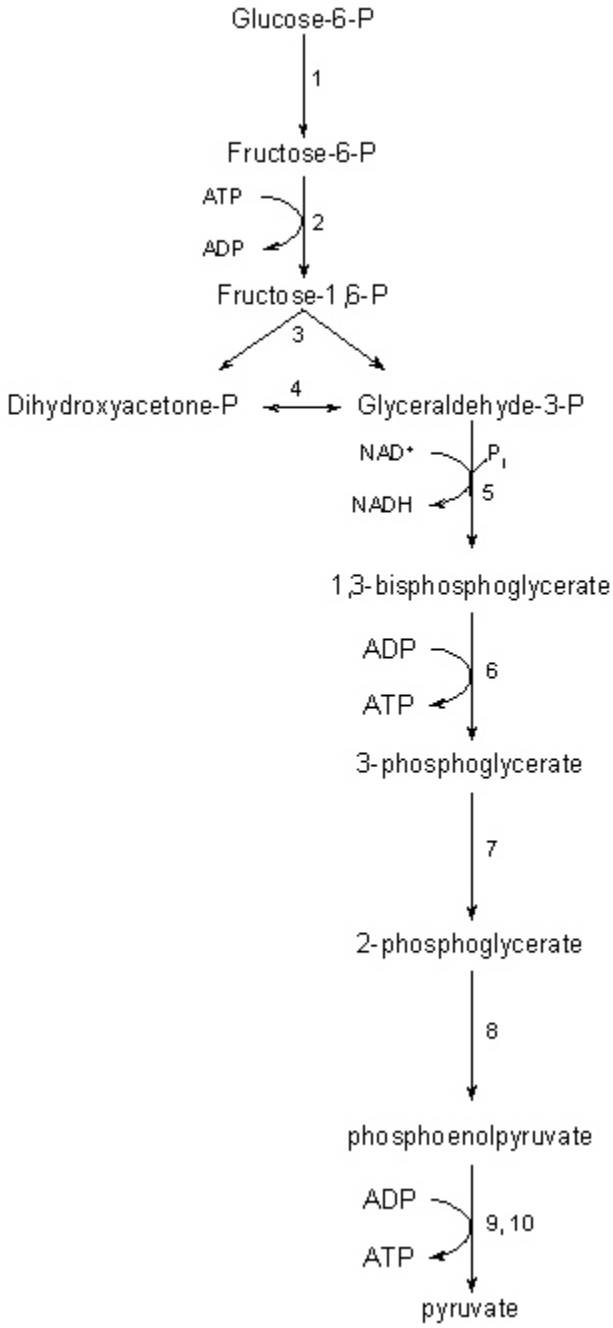

Figure 1-2. Glycolysis. The enzymes and genes that catalyze the conversion of glucose to pyruvate are as follows: (1) phosphoglucose isomerase, *pgi*; (2) phosphofructokinase, *pfkA*; (3) fructose-6-phosphate aldolase, *fba*; (4) triose phosphate isomerase, *tpi*; (5) glyceraldehyde-3-phosphate dehydrogenase, *gapA*; (6) phosphoglycerate kinase, *pgk*; (7) phosphoglycerate mutase, *gpmA* or *pgmI*; (8) enolase, *eno*; (9) phosphotransferase system; (10) pyruvate kinase, *pykA* or *pykF*.

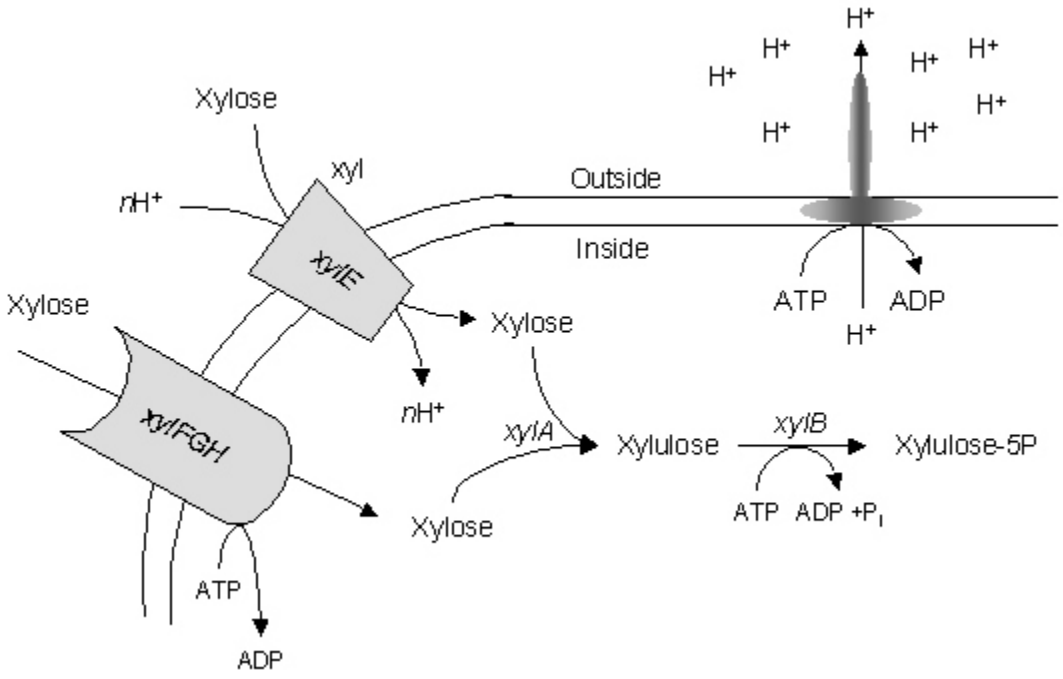

Figure 1-3. Xylose transport in *E. coli*. In contrast to glucose, the transport and activation of xylose is not coupled, each requiring energy.

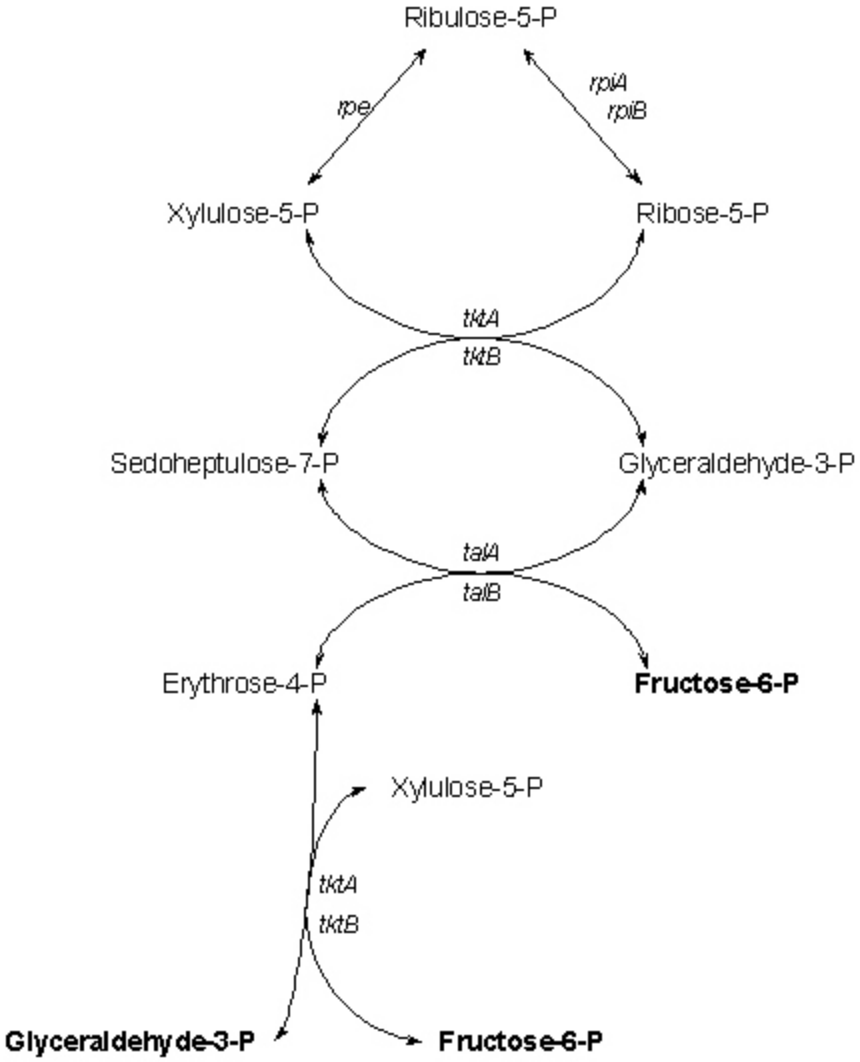

Figure 1-4. Xylose metabolism. Xylose is metabolized to intermediates of glycolysis (in bold) by the pentose-phosphate pathway. For the sake of carbon balance, 6 xylose are converted to 4 fructose-6-phosphate and 2 glyceraldehyde-3-phosphate. Note that neither ATP nor reducing equivalents are produced or consumed in this pathway.

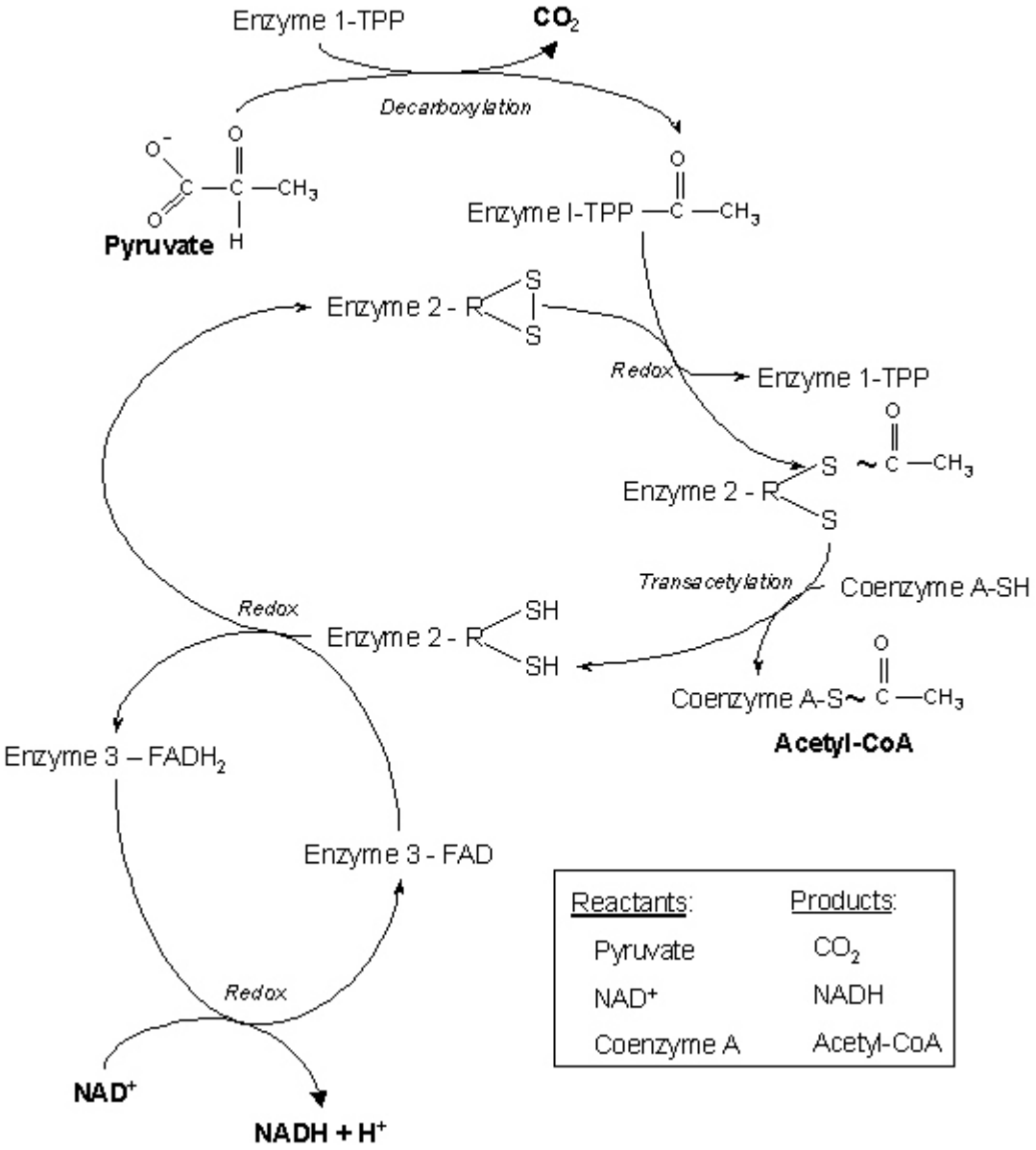

Figure 1-5. Reactions of the pyruvate dehydrogenase complex. Enzyme I (*aceE*) catalyzes the oxidative decarboxylation of pyruvate through a pyruvate dehydrogenase activity. The thiamine pyrophosphate bound activated acetaldehyde is passed to the lipoate transacetylase (Enzyme 2; *aceF*), and ultimately to Coenzyme A. To regenerate the reduced lipoate, the FAD bound to Enzyme 3 (dihydrolipoate dehydrogenase; *lpd*) is reduced. NAD^+ is reduced to NADH by this $FADH_2$, allowing for another cycle.

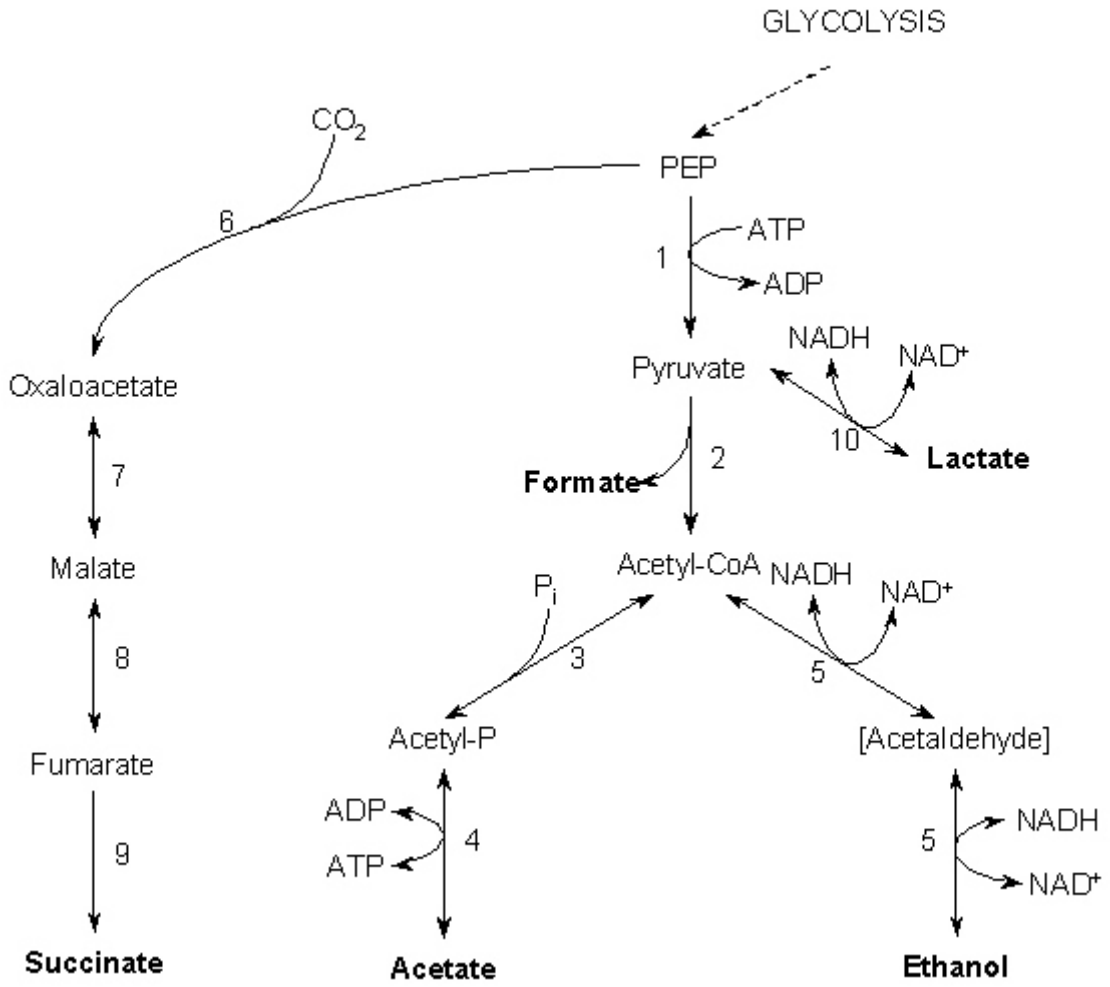

Figure 1-6. Fermentation pathways of *E. coli*. (1) pyruvate kinase, *pykA* or *pykF* or PTS sugar transport; (2) pyruvate formate-lyase, *pflB*; (3) phosphotransacetylase, *pta*; (4) acetate kinase, *ackA*; (5) PFL-deactivase / alcohol dehydrogenase / acetaldehyde dehydrogenase, *adhE*; (6) PEP carboxylase, *ppc*; (7) malate dehydrogenase, *mdh*; (8) fumarase, *fumB*; (9) fumarate reductase, *frdABCD*; (10) lactate dehydrogenase, *ldhA*.

CHAPTER 2
FLUX THROUGH CITRATE SYNTHASE LIMITS THE GROWTH OF ETHANOLOGENIC *Escherichia coli* KO11 DURING XYLOSE FERMENTATION

Introduction

Our laboratory has previously engineered *E. coli* strain B for the production of ethanol from pentose-rich, hemicellulose syrups by expressing high levels of *Zymomonas mobilis pdc* (pyruvate decarboxylase) and *adhB* (alcohol dehydrogenase) (Ingram et al. 1999; Ohta et al. 1991). This strain was chosen for metabolic engineering because of its hardiness, wide substrate range, and ability to grow well in mineral salts medium without organic nutrients (Alterthum et al. 1989; Luli and Strohl 1990). During xylose fermentation, ATP yield in *E. coli* is low (~0.67 ATP per xylose) due to separate energy requirements for uptake and phosphorylation (Tao et al. 2001). Unlike most genetically engineered strains of *E. coli*, KO11 grew to higher densities than the parent in both mineral salts and complex media (Martinez et al. 1999). Initial studies with Luria broth demonstrated rapid and efficient conversion of sugars to ethanol by KO11, with yields approaching 95% of the theoretical maximum. However, volumetric productivity and ethanol yields were considerably lower in mineral salts medium without complex nutrients (Lawford and Rouseau 1996; Martinez et al. 1999; Moniruzzaman and Ingram 1998; York and Ingram 1996a; York and Ingram 1996b).

Supplementing mineral salts medium with complex nutrients significantly increased ethanol production. The least expensive complex nutrient, corn steep liquor, supported growth rates and ethanol productivities near those for Luria broth but only

when provided at high concentrations (5% w/v). Although not prohibitively expensive, the addition of high levels of complex nutrients adds to the cost of ethanol production and increases the requirements for waste treatment.

The lower rate of ethanol production (volumetric productivity) in minimal media (compared to Luria broth) resulted from low cell densities and reduced expression of recombinant *pdc* and *adhB* (lower metabolic activity). Inorganic components did not appear to be limiting and no specific auxotrophic requirements could be identified (Martinez et al. 1999). Reduced expression of heterologous genes was attributed to "biosynthetic burden", the competitive reduction in synthesis of heterologous products due to de-repression of native genes for biosynthetic enzymes (Martinez et al. 1999). In this study, I have used a mineral salts medium containing 1% CSL and investigated the basis of the requirement for higher levels of nutrients during xylose fermentation. Four hypotheses were examined as the basis for the decreased growth in the CSL medium: 1) availability of macro-nutrients; 2) loss of a biosynthetic pathway due to metabolic engineering; 3) insufficient ATP during xylose fermentation; and 4) an imbalance in central metabolism.

Materials and Methods

Microorganisms and Media

E. coli B (ATCC 11303) and an ethanologenic derivative, strain KO11 (Ohta et al. 1991), were used in all fermentation experiments. KO11 contains a deletion in the *frd* region (anaerobic fumarate reductase) which eliminates succinate production. Genes encoding the *Zymomonas mobilis* ethanol pathway (*pdc, adhB*) and chloramphenicol acetyltransferase (*cat*) were integrated into the *pfl* gene (chromosome) by a single

cross-over event resulting in a functional, full length *pfl* gene downstream. Both *E. coli* B and KO11 are prototrophic. Stock cultures were stored in glycerol at -75°C. Working cultures were transferred daily on solid medium containing mineral salts and 1% CSL. Xylose (2%) and chloramphenicol (alternating between 40 and 600 mg liter^{-1}) were included in solid media for K011.

A citrate synthase mutant, *E. coli* W620 (*glnV*44, *gltA6, galK30, pyrD36, spdL129, thi-1*), was obtained from the *E. coli* Genetic Stock Center (CGSC # 4278) and used to test expression of the *B. subtilis citZ* gene (citrate synthase). This strain contains a *gltA*6 mutation (citrate synthase) that prevents growth on M9 medium containing thymine and glucose (Herbert and Guest 1968).

Corn steep liquor medium (CSL+X) contained (per liter in distilled water): 10 g of corn steep liquor (~50% solids), 1 g of KH_2PO_4, 0.5 g of K_2HPO_4, 3.1 g of $(NH_4)_2SO_4$, 0.4 g of $MgCL_2 \bullet 6H_2O$, and 20 mg of $FeCl_3 \bullet 6H_20$. A one-liter stock solution of CSL was prepared by dilution of 200 g with distilled water, adjustment to pH 7.2 with 50% NaOH and steam sterilization. Before use, the sterile stock solution of CSL was aseptically clarified by centrifugation (10,000 x g, 5 minutes). Mineral solutions were prepared as described previously (Martinez et al. 1999). Broth cultures and fermentations contained 9% (w/v) xylose medium, unless indicated otherwise. In some experiments, Luria broth containing xylose was included for comparison.

Fermentation Conditions

Seed cultures (100 ml in 250 ml-flask) were grown 14-16 hours at 35°C with agitation (120 rpm). Cells were harvested by centrifugation (5,000 x g, 5 min) and used as an inoculum to provide an initial concentration of 33 μg ml^{-1} dry weight (0.1 OD_{550nm}).

Fermentation vessels contained a total volume of 350 ml (35°C, 100 rpm). Cultures were maintained at pH 6.5 by the automatic addition of 2N KOH (Moniruzzaman and Ingram 1998). For strain B, 6 N KOH was used to maintain pH after the initial 24 h.

Supplements were added with distilled water as necessary (10 ml total volume). Organic acids and amino acids were neutralized with NaOH, sterilized by filtration and added at a final concentration of 2 mg ml^{-1}. Acetaldehyde was added at a final concentration of 0.25 mg ml^{-1} or 0.5 mg ml^{-1}. Cell mass, ethanol, organic acids and sugars were monitored at 24 h intervals.

Aerobic Growth Studies

Cells were grown with aeration in 250 ml, baffled flask (35°C, 220 rpm) containing 50 ml of CSL+X medium. A range of sugar concentrations was tested (0.5% - 5%) to determine maximal cell density under conditions of sugar excess. Media were inoculated directly using cells grown on solid media (18-24 h). Ethanol and cell mass were measured after 16 h. For comparison, Luria broth containing 5% (w/v) xylose was also included.

Analytical Methods

Cell mass was estimated as OD_{550nm} using a Baush & Lomb Spectronic 70 (1 OD_{550} = 0.33 mg ml^{-1} dry cell weight). Ethanol was measured by gas chromatography using a Varian model 3400 CX as described previously (Moniruzzaman and Ingram 1998). Organic acids and sugars were measured by HPLC using a HP 1090 Series II chromatograph equipped with a BioRad Aminex HPX-87H ion exclusion column (45°C, 4 mM H_2SO_4, 0.5 ml min^{-1}, 10 µl injection) and dual detectors (refractive index monitor and UV detector at 210 nm).

Fermentation products were also analyzed by NMR to confirm the identities of HPLC peaks. Broth samples were centrifuged to remove cells. Supernatants (0.9 ml) were mixed with deuterium oxide (0.1 ml) and sodium 3-(trimethylsilyl)propionate (10 mM internal standard) in 5 mm sample tubes. Proton spectra were obtained using a modified Nicolet NT300 spectrometer in the Fourier transform mode (Buszko et al. 1998) as follows: frequency, 300.065 MHz; excitation pulse width, 5 μs; pulse repetition delay, 3 s; spectral width, 3.6 KHz. A minimum of 100 acquisitions were obtained for each sample.

Genetic Methods

The *citZ* gene encoding *B. subtilis* citrate synthase II has been previously described (Jin and Sonenshein 1994). This gene was amplified by PCR (forward primer, 5'-TGTGCTCTTCCATGTTTTTACAACACTGTTAAAG-3'; reverse primer, 5'-TTGCTCTTCGTTAGGCTCTTTCTTCAATCG-3') using genomic DNA from *B. subtilis* strain YB886 as the template (Barbosa and Ingram 1994). Primers were added to the *Taq* PCR Master mix (Qiagen) as recommended by the manufacturer. Conditions of thermal cycling were as follows: 1) two initial cycles with denaturation at 94°C (60 s), annealing at 50°C (60 s) and elongation at 68°C (90 s); 2) twenty-eight cycles with denaturation at 94°C (10 s), annealing at 70°C (60 s), and elongation at 68°C (90 s); and 3) a final elongation step at 72°C (10 min). The PCR product (1.5 kbp) was cloned into pCR2.1-TOPO (Invitrogen) using ampicillin (50 μg ml^{-1}) for selection. Colonies were screened for size and ability to complement the *gltA* mutation of *E. coli* W620 on glucose-minimal medium (Herbert and Guest 1968). The *citZ* gene was also confirmed by DNA sequencing using a LI-COR model 4000L sequencer (Middendorf et al. 1992).

NAD(P)H/NAD(P)$^+$ ratio

Whole cell fluorescence was used as a relative measure of reduced nucleotides *in situ* (Tartakosvsky et al. 1996; Trivedi and Ju 1994). Since only the reduced form of NAD(P)H fluoresces at 460 nm, an immediate decrease in the fluorescence of fermenting cells is interpreted as a decline in the level of NAD(P)H and the NAD(P)H/NAD(P)+ ratio. Cells were grown for 12 h in CSL+X medium, harvested by centrifugation (5,000 x g, 5 min) and washed 3 times in mineral salts. The pellet was then suspended in mineral salts solution at a concentration of 1.0 OD_{550}. Emission at 460 nm (excitation at 340 nm) was recorded at 5 s intervals using an Aminco-Bowman Series 2 Luminescence Spectrometer. Cells were energized by the addition of 1% xylose resulting in an immediate increase in fluorescence, primarily due to the increase in NADH/NAD+ ratio. Test compounds were added at a final concentration of 2 mg ml^{-1} (organic acids, amino acids) or 0.25 mg ml^{-1} (acetaldehyde) using distilled water as a control. Results for each test compound were expressed relative to the xylose-dependent increase in fluorescence. Control experiments confirmed that quenching of cellular fluorescence did not occur when additives were mixed with energy-deficient cells (without xylose).

Enzyme Assays

Citrate synthase was assayed using a modification of the method described previously (Evans et al. 1993, Faloona and Srere 1969). Cultures were grown in one-liter flasks (250 ml Luria broth) for 16 h at 35°C (150 rpm). Cells were harvested by centrifugation, washed 3 times in buffer containing 50 mM Tris-Cl (pH 8.0) and 20% glycerol and suspended in 2 volumes of same buffer. Cell-free preparations were made by two passages through a French Pressure cell (20,000 psi) followed by treatment with

~100 µg ml^{-1} deoxyribonuclease I. Cell debris was removed by centrifugation (15,000 x g, 1h, 4°C). The supernatant was dialyzed against 20 mM Tris-Cl and 20% glycerol. Each assay (1 ml) contained 20 mM Tris-Cl (pH 8.0), 10 mM KCl, 1 mM 5',5'-dithio-bis-(2-nitrobenzoic acid), 10 mM oxaloacetate and 0.5 mM acetyl-CoA . Reactions were initiated by the addition of cell lysate and monitored for 300 s at 412 nm. Specific activity was reported as µmol of reduced coenzyme A produced per min per mg protein.

Results

Macro-nutrient Limitation.

Previous studies have shown that up to 5% CSL is needed to support anaerobic growth and ethanol production at a rate near that of Luria broth (Martinez et al. 1999). Based on a comparison of *E. coli* elemental composition (Taylor 1946), the macro-nutrient salts in CSL+X medium should provide sufficient nitrogen and phosphorus to support the growth of at least 5 mg ml^{-1} dry weight. During anaerobic growth in pH-stats with CSL+X (Fig. 2-1), the maximal cell density for KO11 was only about 1 mg ml^{-1}, 33% lower than the parent *E. coli* B (1.5 mg ml^{-1}) in CSL+X medium and only 25% of that reached by KO11 (4 mg ml^{-1}) in Luria broth plus sugar (Martinez et al. 1999, York and Ingram 1996b, York and Ingram 1996b). During aerobic growth with the same nutrients, however, KO11 grew to a maximum density of 2.7 mg ml^{-1}. This is almost two-fold higher than *E. coli* B under the same conditions and 2.7-fold higher than KO11 during anaerobic growth. Together, these results indicate that the anaerobic growth of KO11 in 1% CSL with 9% xylose is not limited by the availability of macro-nutrients (ie. N, P, etc.) or by the inactivation of a biosynthetic pathway due to genetic

manipulation. However, metabolic engineering of the ethanol pathway does appear to contribute to the reduced growth of KO11 in this medium under anaerobic conditions.

Energy Limitation.

The separate energy requirement for uptake (ATP-dependent transporter or proton symport) and phosphorylation (xylulokinase) results in a low net yield of ATP from xylose fermentation (0.67 ATP per xylose), 33% of the yield from glucose (2 ATP per glucose) (Tao et al. 2001). An additional ATP can also be produced from pyruvate by the acetate pathway. Since KO11 produced less acetate than strain B (Table 2-1), the growth of KO11 could be limited by the availability of ATP. To test this hypothesis, I compared the growth of KO11 in 1% CSL containing 9% xylose with growth in 1% CSL containing 9% glucose (Fig. 2-2A and Fig. 2-2B). Glucose was fermented to ethanol at a higher rate than xylose. However, the cell yield of KO11 was identical for both sugars, despite the 3-fold difference in net ATP production. Although cell densities were low, cells remained metabolically active for at least 96 h and produced most of the ethanol after growth had ceased.

Metabolic Imbalance Relieved by Addition of Pyruvate or Acetaldehyde.

Pyruvate serves a dual role during fermentation, as a source of carbon skeletons for biosynthesis and as a source of electron acceptors (acetaldehyde) to allow continued ATP production by glycolysis. During sugar fermentation to ethanol, one NADH is produced per pyruvate. Each NADH must be oxidized by reducing an electron acceptor such as acetaldehyde or by biosynthetic reactions (Mat-Jan et al. 1989). In a growing wild type *E. coli*, partitioning of pyruvate between biosynthesis and redox needs is presumed to be balanced for optimal growth. Metabolic engineering of the ethanol pathway

contributed to the reduced growth of KO11 only under fermentative conditions, consistent with a metabolic imbalance resulting from uncontrolled utilization of pyruvate for ethanol production. This possibility was confirmed by the addition of pyruvate to CSL+X medium. Pyruvate addition resulted in a dose-dependent increase in cell growth and ethanol production that was particularly evident after 24 h (Fig. 2-3A and Fig. 2-3B). With 2 mg ml^{-1} of added pyruvate, growth and ethanol production were twice that of the control without pyruvate addition (Table 2-2). Supplementing with pyruvate did not cause a buildup of TCA intermediates or acidic fermentation products (Table 2-1). Note that formate was produced in all fermentations, confirming the that the *pfl* gene encoding pyruvate formate-lyase remains functional in KO11.

The addition of pyruvate to media has been shown to increase the intracellular pyruvate pool in *E. coli* (Yang et al. 2001), increasing the ratio of potential electron acceptors for the oxidation of NADH (from glycolysis). When added at a level of 2 mg ml^{-1}, pyruvate was metabolized concurrently with cell growth during the first 24 h after inoculation (Table 2-1). The pyruvate-dependent increase in cell mass (~1 mg ml^{-1}) was roughly equivalent to half of the added pyruvate (Table 2-2, Fig. 2-3A). Remaining pyruvate is presumed to be metabolized to acetaldehyde by recombinant *Z. mobilis* pyruvate decarboxylase. Since acetaldehyde has been previously shown to stimulate growth and ethanol production by yeasts (Walker-Caprioglio et al. 1985) and *Z. mobilis* (Stanley et al. 1997), it seemed possible that the stimulation of cell growth by pyruvate could be mediated in part by an increase in acetaldehyde from pyruvate (Table 2-2, Fig. 2-3C and Fig. 2-3D). Concentrations of acetaldehyde above 0.50 mg ml^{-1} were toxic. With lower concentrations of acetaldehyde (0.25 and 0.50 mg ml^{-1}), cell growth and

ethanol production were increased. Like pyruvate, added acetaldehyde was fully metabolized during the initial 24 h after inoculation (Table 2-1). A near optimal level of acetaldehyde was provided by 2 additions of 0.25 mg ml^{-1} each to CSL+X medium (initially and after 12 h). This was almost as effective as pyruvate (2 mg ml^{-1}) in stimulating ethanol production and also caused a 65% increase in cell mass. The basis for the increase in cell growth is not readily explained by the limited routes for acetaldehyde metabolism in *E. coli* as compared to those for pyruvate, a key central metabolite. These results provide evidence that the beneficial effect of added pyruvate results primarily from an increase in electron acceptors.

Pyruvate as a Source of Carbon Skeletons for Biosynthesis.

The pyruvate-stimulated increase in cell growth reflects a two-fold increase in the flow of carbon into biosynthesis. Pyruvate and upstream metabolites in glycolysis are used for the biosynthesis of approximately half of cellular constituents. Pools for these upstream intermediates may increase when pyruvate is added, increasing availability for biosynthesis. Pyruvate (and phosphoenolpyruvate) is also converted to a series of biosynthetic intermediates by the TCA pathway and linking reactions. The TCA pathway provides half of the carbon skeletons for cell protein. None of the TCA intermediates can be produced readily from acetaldehyde by biosynthetic reactions. Note that the TCA pathway is not cyclic during fermentation. This pathway is interrupted between 2-ketoglutarate and succinate by ArcAB-mediated repression of genes (*sucAB*) encoding 2-ketoglutarate dehydrogenase (Iuchi and Lin 1988). One side of the TCA pathway produces 2-ketoglutarate, the precursor for the glutamic acid family of amino acids, polyamines, among others. Precursors such as oxaloacetate on the other side of the TCA

pathway are derived from phosphoenolpyruvate. Oxaloacetate is used for synthesis of the aspartic acid family of amino acids, etc. The addition of pyruvate could potentially increase the flow of carbon into both sides.

TCA pathway intermediates were tested as additives to CSL+X medium. Utilization of these additives was investigated using HPLC and NMR (Table 2-2, Fig. 2-4). All except two, succinate (100% remaining) and isocitrate (78% remaining), were metabolized efficiently during the initial 24 h of fermentation (Table 2-1). Additions of malate and fumarate resulted in a similar small increase in fumarate, but did not stimulate growth or ethanol production. Despite the potential interconversion of these intermediates, fumarate did not accumulate when oxaloacetate was added. Addition of aspartate, the transamination product of oxaloacetate, was similarly ineffective. Indeed, addition of oxaloacetate, malate, fumarate and aspartic acid reduced growth and ethanol production. In contrast, 2-ketoglutarate was almost as effective as pyruvate in stimulating growth and ethanol production by KO11. A similar stimulation was also observed for glutamate, the transamination product of 2-ketoglutarate.

TCA intermediates that are immediate precursors of 2-ketoglutarate were not beneficial. Isocitrate was not readily metabolized. Citrate was metabolized but had no effect on growth and ethanol production. Growth with added citrate was accompanied by an accumulation of fumarate and a high acetate/formate ratio similar to that with pyruvate (Table 2-1). Note that this ratio is near unity for other fermentations with TCA intermediates, providing a clue to the ineffectiveness of citrate. The addition of citrate may induce citrate lyase (Furlong 1987; Lutgens and Gottschalk 1980; Schneider et al. 2000), an enzyme that cleaves citrate into an equimolar mixture of oxaloacetate and

acetate. Oxaloacetate is readily metabolized to furmarate. Both fumarate and acetate were higher in fermentations with added citrate than with 2-ketoglutarate and other TCA intermediates, consistent with the induction of citrate lyase. Induction of this enzyme is presumed to block the beneficial effects of this TCA intermediate for biosynthesis.

When considered together, studies with added TCA intermediates provide evidence that the beneficial effect of pyruvate for growth and ethanol production by KO11 in CSL+X medium results in large part from an increase in the flow of carbon skeletons into 2-ketoglutarate and subsequent products of biosynthesis. However, investigations with added pyruvate and acetaldehyde provided evidence that an increase in electron acceptors was arguably of primary importance for the beneficial effect of pyruvate. For both to be possible, both must be mediated by a common mechanism.

Whole-cell Fluorescence.

The ratio of NAD(P)H/NAD(P)+ has been shown to alter cellular patterns of metabolic flux (de Graef et al. 1999). NAD(P)H is an allosteric inhibitor of many enzymes including pyruvate dehydrogenase (Graham et al. 1989), phosphotransacetylase (Suzuki 1969), malate dehydrogenase (Sanwal 1969) and citrate synthase (Faloona and Srere 1969; Weitzman 1966). In KO11, the addition of acetaldehyde or pyruvate (metabolized to acetaldehyde by recombinant pyruvate decarboxylase) would be expected to decrease the level of NAD(P)H and the NAD(P)H/NAD(P)+ ratio by increasing the pool of acetaldehyde available for reduction to ethanol. This has been investigated in non-growing cells by examining the effects of these additives on whole-cell fluorescence. (Fig. 2-5A and Fig. 2-5B).

Fluorescence changes in responses to additives were immediate and stable as shown for acetaldehyde (Fig. 2-5A). Relative fluorescence increased when fermentation was initiated by the addition of xylose, and decreased immediately upon the addition of acetaldehyde (alcohol dehydrogenase) and pyruvate (pyruvate decarboxylase plus alcohol dehydrogenase), consistent with expected changes in the oxidation of NADH. The fluorescence of energized cells also decreased immediately upon the addition of 2-ketoglutarate and oxaloacetate (Fig. 2-5B). The apparent decline in NAD(P)H in response to these two TCA pathway intermediates may be due to reductive amination (oxaloacetate and 2-ketoglutarate). Addition of the respective amino acid products, glutamic acid and aspartic acid, did not cause a similar change. With added oxaloacetate, malate dehydrogenase provides an additional opportunity for NADH oxidation.

Additions of malate, fumarate, succinate, citrate and isocitrate did not significantly alter whole cell fluorescence. Together, these data demonstrate that three compounds which increased the growth and fermentation of KO11 in CSL+X medium (acetaldehyde, pyruvate and 2-ketoglutarate) also decreased the NAD(P)H/NAD(P)+ ratio in cells. Compounds which did not decrease this ratio were not beneficial. Oxaloacetate was an exception. Although this compound decreased the NAD(P)H/NAD(P)+, growth and fermentation were retarded. The negative effects of added oxaloacetate may be attributed to the induction of pyruvate carboxykinase. Together with phosphoenolpyruvate carboxylase, this enzyme creates a futile cycle for ATP (Chao et al. 1994; Chotani et al. 2000). ATP yields are low for xylose and ATP wasted by this futile cycle may offset any potential benefits from increased oxidation of NADH.

Citrate Synthase, a Link Between NADH and 2-Ketoglutarate.

In *E. coli* (*gltA*) as in most Gram-negative bacteria, citrate synthase is allosterically inhibited by NADH and activated by acetyl-CoA (Weitzman 1981). The activity of this enzyme serves to regulate the flow of carbon into the 2-ketoglurate side of the TCA pathway, linking the cellular abundance of NADH and acetyl-CoA to the production of 2-ketoglutarate for biosynthesis (Faloona and Srere 1969; Lee et al. 1994; Walsh and Koshland, Jr. 1985). This enzyme integrates both beneficial effects of added pyruvate, increased electron acceptors (acetaldehyde) and increased carbon skeletons in the 2-ketoglutarate arm of the TCA pathway (2-ketoglutarate). The allosteric control of this enzyme by NADH could restrict the flow of carbon into the biosynthesis of 2-ketoglutarate and other products. This hypothesis can be readily tested by expressing an NADH-insensitive recombinant citrate synthase gene in KO11.

The primary citrate synthase in Gram-positive bacteria is allosterically regulated by ATP and relatively insensitive to NADH (Jin and Sonenshein 1996). Since an over-abundance of ATP is not anticipated during xylose fermentation (Tao et al. 2001), expression of *B. subtilis citZ* in KO11 would be expected to increase carbon flow into the oxidizing arm of the TCA pathway. Primers were used to clone the *citZ* gene (including ribosomal binding site) into pCR2.1-TOPO to produce pLOI2514. Plasmid pLOI2514 was found to complement a *gltA* mutation in *E. coli* W620 on plates containing M9 minimal media supplemented with glucose and thymine. Citrate synthase activity (0.08 U mg protein^{-1}) was also confirmed in strain W620(pLOI2514) and absent in strain W620 lacking *citZ*.

Expression of *citZ* in KO11(pLOI2514) increased growth and ethanol production by approximately 75% (Fig. 2-6) in comparison to the control with vector alone, KO11(pCR2.1-TOPO). The low level of NADH-insensitive citrate synthase produced from pLOI2514 was almost as effective as pyruvate, acetaldehyde and 2-ketoglutarate additions in stimulating growth. Thus the allosteric regulation of the native citrate synthase by high NADH appears to limit the flow of carbon skeletons into biosynthesis in CSL+X medium.

Discussion

The rate of ethanol production and ethanol yield are important factors in determining the cost of large-scale fermentation processes. For KO11, both of these are directly related to the extent of growth of the biocatalyst (Fig. 2-7). In CSL+X medium, more than half of the ethanol was produced after cells entered stationary phase (Fig. 2-1). By increasing cell densities, fermentation times can be reduced without sacrificing ethanol yield. However, previous studies with KO11 have shown that high levels of complex nutrients were needed for cell growth and rapid ethanol production (Martinez et al. 1999; York and Ingram 1996b; York and Ingram 1996b). This apparent requirement for high levels of complex nutrients now appears to reflect a regulatory error in the partitioning of pyruvate skeletons between competing requirements for the oxidation of NADH and biosynthesis. Our study demonstrates that the growth of KO11 was not limited by nutrients, a lack of biosynthetic enzymes, or insufficient ATP from xylose metabolism (0.67 ATP per xylose). During the fermentation of 9% xylose, growth was limited by a lack of carbon skeletons for the biosynthesis of products derived from

2-ketoglutarate. Growth and ethanol production were increased by the addition of pyruvate or 2-ketoglutarate, but not by the addition of oxaloacetate, malate or fumarate.

The apparent starvation for carbon skeletons to produce 2-ketoglutarate was also alleviated by the addition of acetaldehyde, consistent with an involvement of NADH or NADH/NAD^+ ratios. The ratio of NADH/NAD^+ is typically higher during fermentation than during oxidative metabolism (de Graef et al. 1999). High levels of NADH serve as an allosteric inhibitor of citrate synthase, the first committed step for the production of 2-ketoglutarate and a likely bottleneck for the biosynthesis of many amino acids (Walsh and Koshland, Jr. 1985). Addition of acetaldehyde decreased the NADH/NAD^+ ratio by increasing the pool of electron acceptors, potentially increasing the function of the native citrate synthase *in vivo*. This hypothesis was confirmed, in part, using the *B. subtilis citZ* gene encoding an NADH-insensitive citrate synthase (Jin and Sonenshein 1994; Jin and Sonenshein 1996). Expression of *citZ* in KO11 stimulated growth and ethanol production by almost two-fold, substantially reducing the need to supply high levels of complex nutrients.

The pattern of carbon flow in KO11 is summarized in Figure 2-8. Expression of high levels of *Z. mobilis pdc* and *adhB* redirect pyruvate away from native fermentation pathways (pyruvate formate-lyase, lactate dehydrogenase) and into ethanol, even in the presence of competing native enzymes (Ohta et al. 1991). Since the K_m of pyruvate decarboxylase for pyruvate is approximately one-tenth that of the competing enzyme, pyruvate formate-lyase, production of acetyl-CoA would also be limited. Integration of the ethanol-production genes into the chromosomal *pfl* gene may further contribute to this problem by reducing the level of pyruvate formate-lyase activity. Although pyruvate

dehydrogenase has a K_m for pyruvate that is equal to that of pyruvate decarboxylase, pyruvate dehydrogenase is expressed at low levels during fermentation and is allosterically inhibited by the high levels of NADH present during fermentation (de Graef et al. 1999; Graham et al. 1989). In CSL+X medium, a portion of cellular pyruvate was converted to acetyl-CoA by KO11 during the first 24 h as evidenced by the accumulation of acetate as a fermentation product. These acetate levels are presumed to be in excess of biosynthetic needs.

The addition of either pyruvate or acetaldehyde dramatically stimulated the growth of KO11 in CSL+X medium. At least three sites of allosteric regulation may contribute to the increase in growth. Both pyruvate dehydrogenase (de Graef et al. 1999; Graham et al. 1989) and citrate synthase (Faloona and Srere 1969; Weitzman 1981) are allosterically inhibited by NADH. Oxidation of NADH from glycolysis during the reduction of acetaldehyde from added pyruvate or added acetaldehyde would tend to decrease the $NADH/NAD^+$ ratio. This should reduce the allosteric inhibition of pyruvate dehydrogenase and citrate synthase by NADH. Additional acetyl-CoA from pyruvate dehydrogenase would supplement that produced by pyruvate formate-lyase and increase the pool of acetyl-CoA , a substrate for citrate synthase and an allosteric antagonist of NADH inhibition of the native citrate synthase (Weitzman 1981). Together, these regulatory circuits would feed-forward to promote the flow of additional carbon skeletons into 2-ketoglutarate and subsequent products of biosynthesis.

Table 2-1. Effects of additives on the composition of fermentation products (24 h) in 1% CSL+X medium (9% xylose).

Medium or strain	Additive	% of additive remaining	Fermentation Products (mM)					
			Fumarate	Succinate	Lactate[a]	Formate	Acetate	Ethanol
CSL+X medium		–	<0.01	<0.3	9.6	<1.5	<1.0	<1.0
E. coli B, parent	None	–	<0.01	53.4	27.7	95.8	91.4	71
E. coli KO11	None	–	0.03	0.5	12.2	20.1	24.0	150
	Sodium pyruvate (2 mg ml^{-1})	< 5	0.29	0.9	12.1	13.2	27.4	208
	Acetaldehyde (2 x 0.25 mg ml^{-1})	< 5	0.21	0.5	11.1	25.6	26.6	161
	Citric acid (2 mg ml^{-1})[a]	< 5	1.65	0.6	11.8	11.9	27.0	154
	Isocitric acid (2 mg ml^{-1})	78	<0.01	0.5	11.4	23.5	23.0	140
	Sodium 2-ketoglutarate (2 mg ml^{-1})	< 5	0.28	0.9	12.5	37.8	32.1	193
	Oxaloacetic acid (2 mg ml^{-1})	10	0.02	0.8	10.8	17.9	20.4	100
	Sodium malate (2 mg ml^{-1})	6	1.45	0.3	10.9	14.7	17.3	125
	Sodium fumarate (2 mg ml^{-1})	16	1.22	0.4	10.1	19.7	18.8	110
	Sodium succinate (2 mg ml^{-1})	100	0.07	13.0	11.3	20.0	22.7	144

Table 2-2. Effects of additives on growth and ethanol production by KO11 in 1% CSL+X medium (9% xylose).

Additive	No.	Concentration		Cell Yield		Volumetric Production of Ethanol		Maximum Ethanol		Yield[b] (%)
		mg ml^{-1}	mM	Average	% of Control	g liter^{-1} h^{-1}	% of Control	mg ml^{-1}	% of Control	
None (control)	26	–	--	0.94 ± 0.15	100	0.38 ± 0.08	100	31.12 ± 4.64	100	61
Sodium pyruvate	3	0.5	4.6	1.32 ± 0.32	140	0.55 ± 0.17	144	37.60 ± 5.91	121	74
Sodium pyruvate	3	1.0	9.1	1.49 ± 0.30	158	0.66 ± 0.17	173	40.09 ± 5.53	129	79
Sodium pyruvate	15	2.0	18.2	1.99 ± 0.20	212	0.81 ± 0.14	213	44.22 ± 2.69	142	87
Sodium pyruvate	3	4.4	36.4	2.08 ± 0.08	221	0.83 ± 0.01	218	43.23 ± 1.50	139	85
Acetaldehyde (half initially + half after 12 h)	4	0.5 (total)	11.4	1.55 ± 0.07	165	0.60 ± 0.35	158	44.05 ± 1.89	142	86
citric acid	2	2.0	10.4	0.88, 0.75	86	0.35, 0.33	89	30.00, 27.59	93	56
isocitric acid	2	2.0	6.6	1.11, 0.88	105	0.51, 0.36	113	36.45, 29.59	106	65
sodium 2-ketoglutarate	5	2.0	11.9	1.89 ± 0.18	201	0.84 ± 0.08	221	41.54 ± 1.24	133	81
oxaloacetic acid	2	2.0	15.1	0.75, 0.75	80	0.28, 0.28	74	23.58, 23.64	76	46
sodium malate	2	2.0	11.2	0.76, 0.84	85	0.27, 0.27	71	23.89, 24.97	79	48
sodium fumarate	2	2.0	12.5	0.80, 0.96	91	0.37, 0.32	92	27.15, 29.84	92	56
sodium succinate	3	2.0	7.4	1.00 ± 0.12	106	0.41 ± 0.05	108	35.00 ± 1.31	112	69
potassium glutamate	3	2.0	10.8	1.66 ± 0.17	177	0.66 ± 0.13	174	43.14 ± 0.13	139	85
aspartic acid	2	2.0	15.0	0.85, 0.82	88	0.33, 0.32	84	28.40, 27.40	90	55

[a] Volumetric Productivity was calculated as the average hourly rate of ethanol production between 24 h and 48 h after inoculation. When less than 3 replicates are presented, the values of each replicate are shown.

[b] Yield is expressed as a percentage of the theoretical yield (100% = 0.51 g ethanol per g xylose).

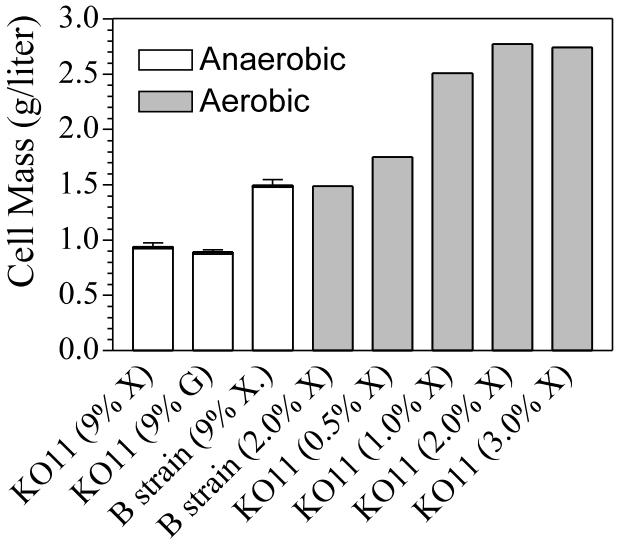

Figure 2-1. Comparison of maximal cell densities achieved during aerobic and anaerobic growth in 1% CSL mineral salts medium containing either xylose or glucose. Thin lines representing the standard error of the mean are shown for averages with three or more replicates.

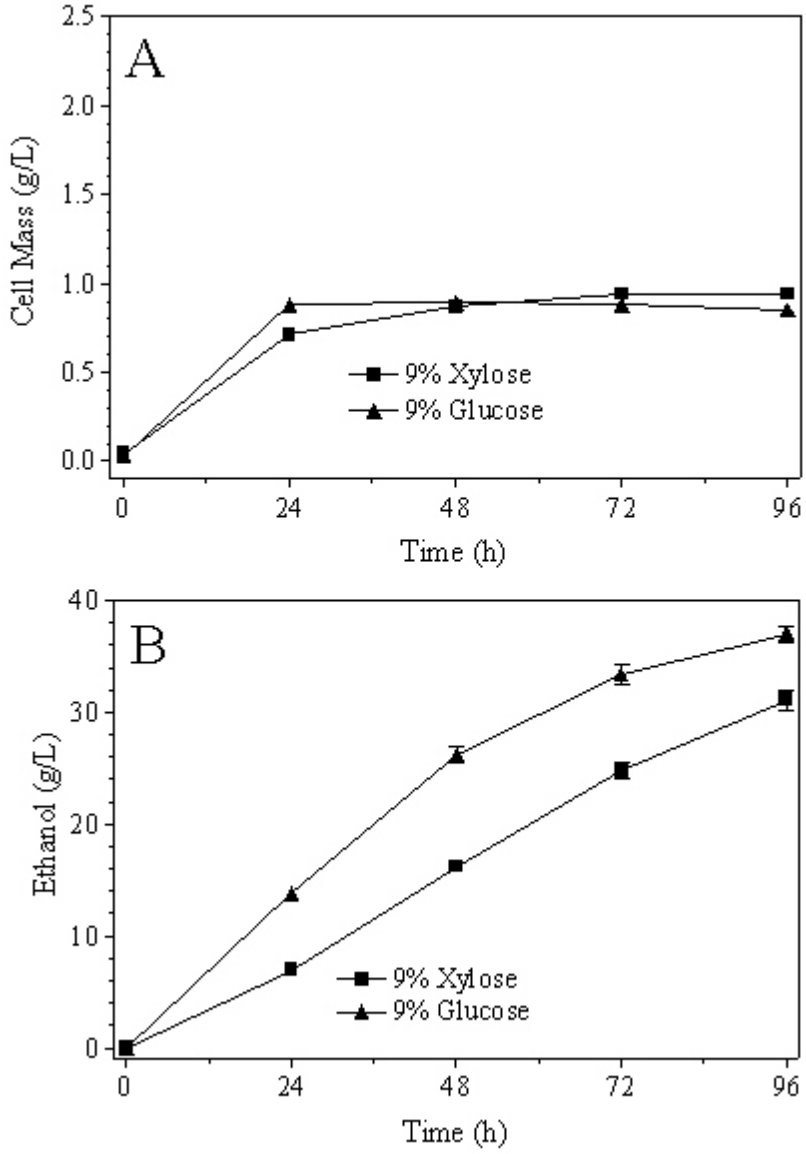

Figure 2-2. Comparison of growth and ethanol production from glucose and xylose by *E. coli* KO11 during the fermentation of 9% sugar in 1% CSL mineral salts medium. A. Growth. B. Ethanol. Thin lines representing the standard error of the mean are shown for averages with three or more replicates.

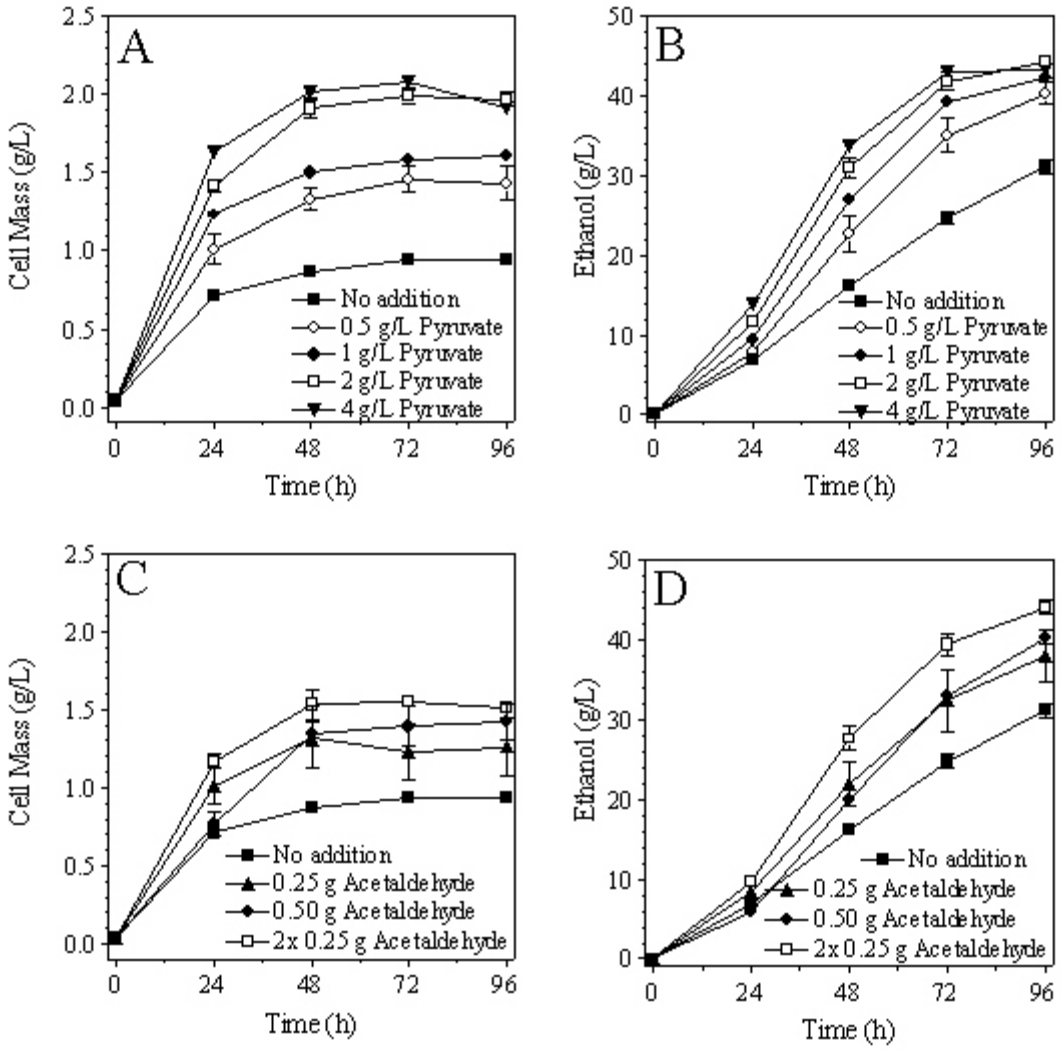

Figure 2-3. Effects of added pyruvate and acetaldehyde on growth and ethanol production by *E. coli* KO11 in CSL+X medium. A. Cell growth with added pyruvate. B. Ethanol production with added pyruvate. C. Cell growth with added acetaldehyde. D. Ethanol production with added acetaldehyde. Thin lines represent the standard error of the mean.

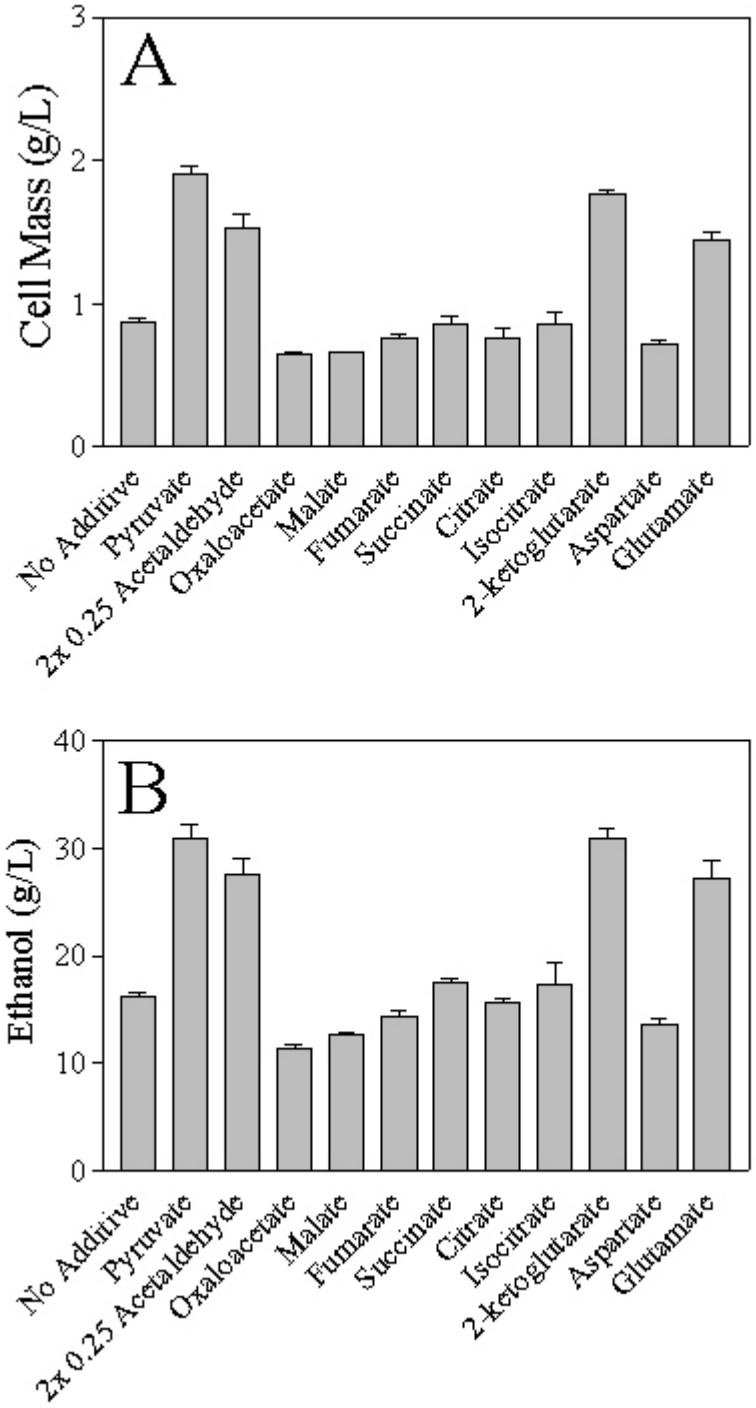

Figure 2-4. Initial effects of added TCA pathway intermediates on growth and ethanol production by *E. coli* KO11 (24 h). A. Growth. B. Ethanol. Thin lines represent the standard error of the mean.

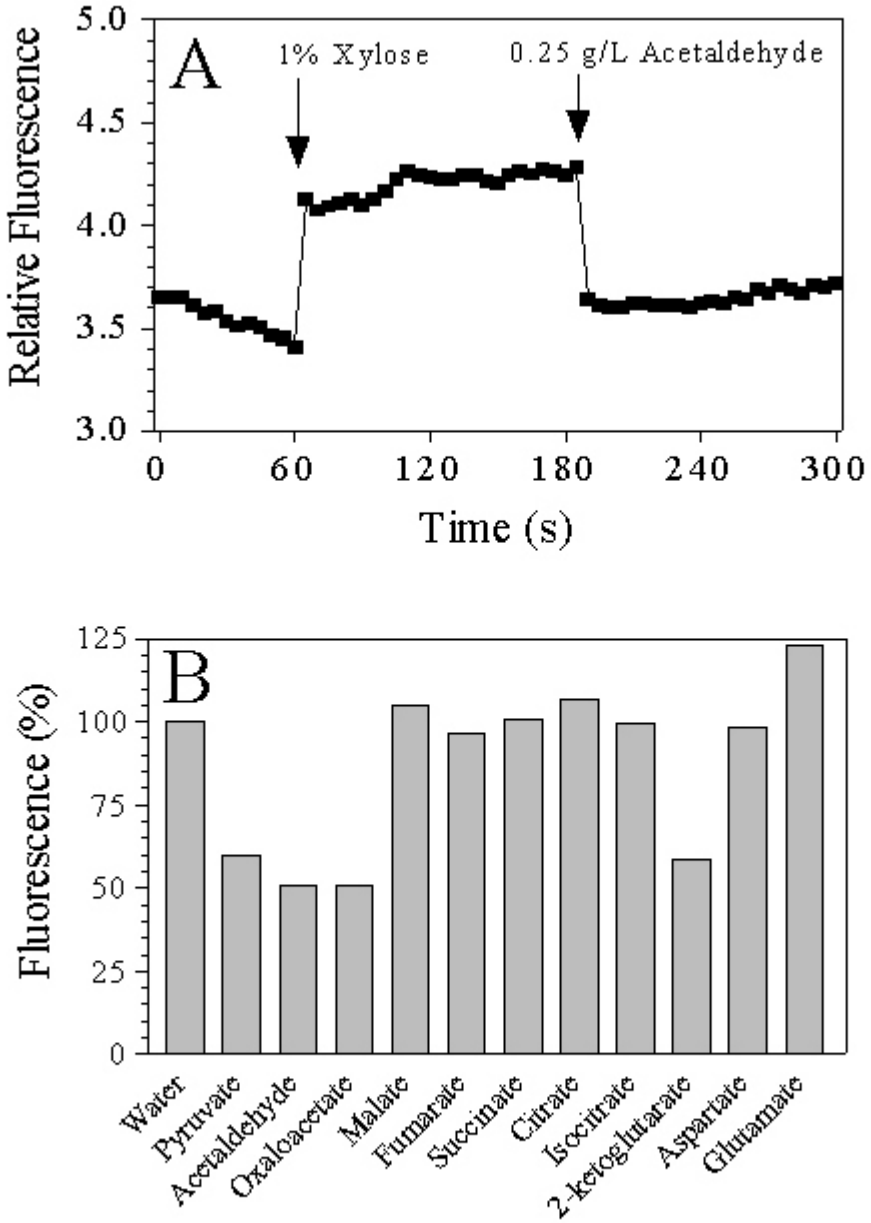

Figure 2-5. Effect of metabolites on whole-cell fluorescence. A. Effects of acetaldehyde on the xylose-dependent increase in fluorescence (time course). B. Effects of metabolites on the xylose-dependent increase in fluorescence. Values in B are expressed as a percentage of the xylose-dependent increase in the fluorescence of whole cells observed in the presence of both xylose and the indicated additive. Note that a decrease in the xylose-dependent fluorescence is interpreted as a decrease in NAD(P)H and the NAD(P)H/NAD(P)+ ratio.

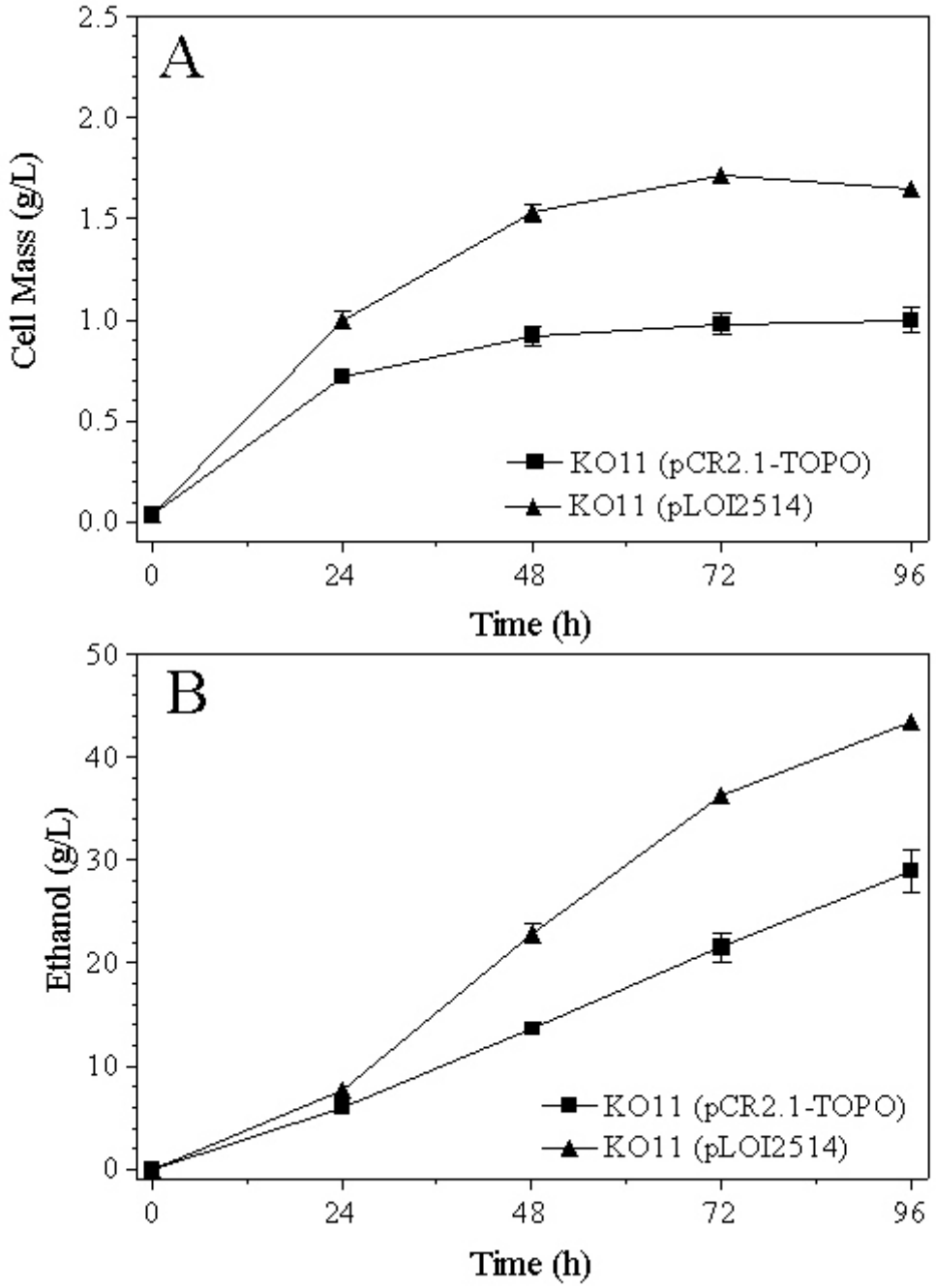

Figure 2-6. *B. subtilis citZ* increases the growth and ethanol production of KO11 in CSL+X medium. A. Growth. B. Ethanol. Thin lines represent the standard error of the mean.

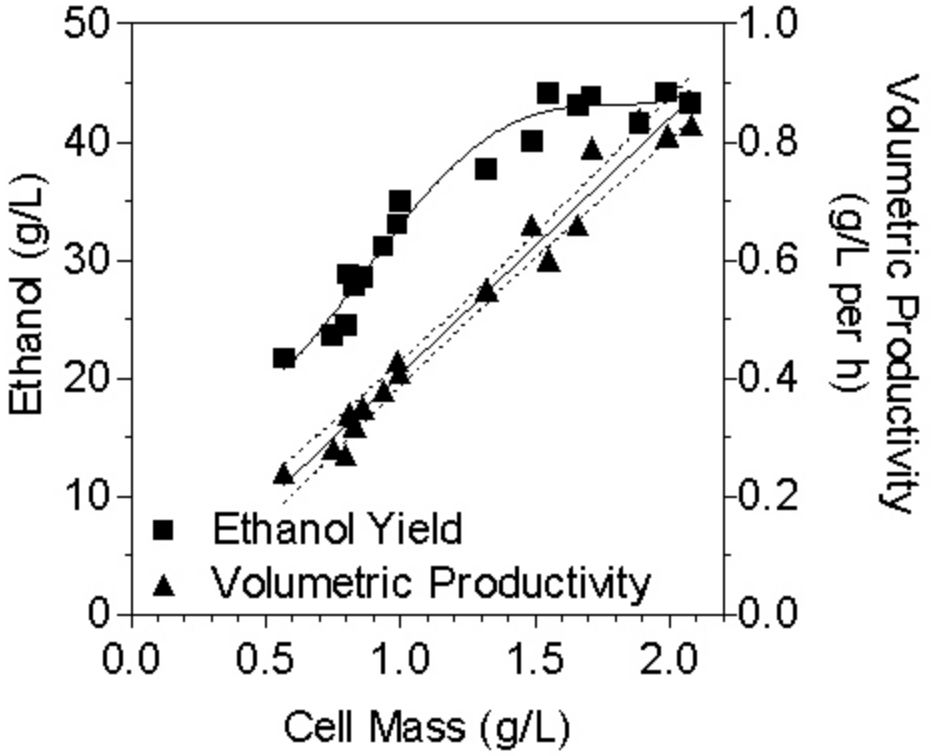

Figure 2-7. Relationship between cell yield and fermentation performance. In this plot, results were combined from fermentations with CSL+X medium alone and with supplements. A computer-generated polynomial was used to approximate cell yields. Results from a linear regression analysis are shown for volumetric productivity. Dotted lines represent the the 95% confidence intervals.

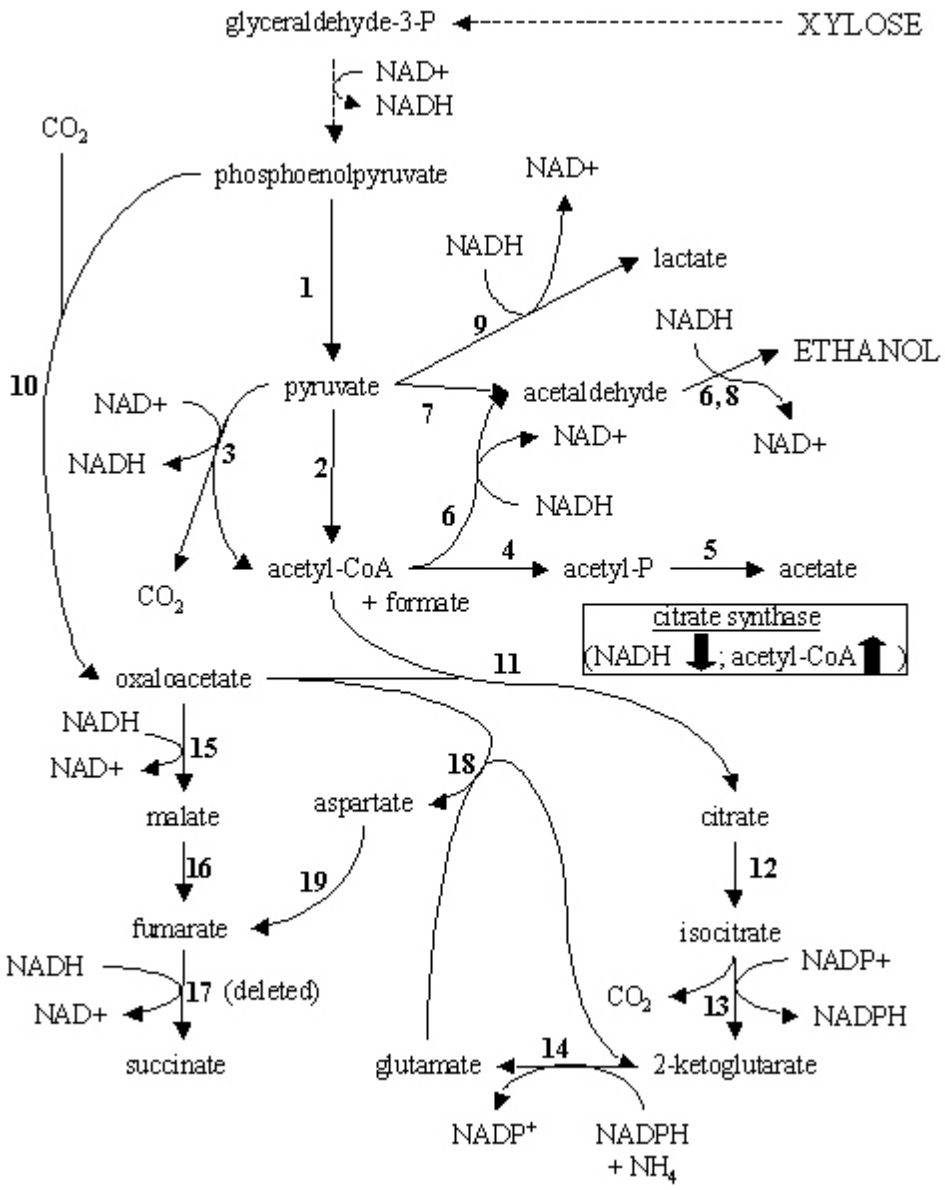

Figure 2-8. Fermentation and TCA pathway. Unless noted otherwise, enzymes listed are native to *E. coli*. Key to enzymes: 1. pyruvate kinase (*pykA, pykF*); 2. pyruvate formate-lyase (*pflB*); 3. pyruvate dehydrogenase (*aceEF,lpd*); 4. phosphotransacetylase (*pta*); 5. acetate kinase (*ackA*); 6. alcohol/aldehyde dehydrogenase (*adhE*); 7. *Z. mobilis* pyruvate decarboxylase (*pdc*); 8. *Z. mobilis* alcohol dehydrogenase II (*adhB*); 9. lactate dehydrogenase (*ldhA*); 10. phosphoenolpyruvate carboxylase (*ppc*); 11. citrate synthase (*gltA*); 12. aconitase (*acn*); 13. isocitrate dehydrogenase (*icd*); 14. glutamate dehydrogenase (*gdhA*); 15. malate dehydrogenase (*mdh*); 16. fumarase (*fumB*); 17. fumarate reductase (*frdABC*); 18. aspartate transaminase (*aspA*); 19. aspartase (*aspC*). Arrows beneath citrate synthase indicate inhibition of activity by NADH and antagonism of NADH inhibition by acetyl-CoA.

CHAPTER 3
GENETIC CHANGES TO OPTIMIZE CARBON PARTITIONING IN ETHANOLOGENIC *Escherichia coli* KO11

Introduction

Citrate synthase, a key enzyme in the partitioning of carbon into biosynthesis (Walsh and Koshland, Jr. 1985), was shown to be growth limiting for KO11 (Chapter 2). Native citrate synthase is allosterically inhibited by high levels of NADH typical of fermentation (Weitzman 1981). Growth and ethanol production were substantially improved in KO11 by expression of an NADH-insensitive citrate synthase (*citZ*) from *Bacillus subtilis*. A similar stimulation of growth and ethanol production was observed during low aeration (oxidation of NADH) and with the addition of pyruvate, 2-ketoglutarate, and acetaldehyde.

An alternative approach to enhance citrate synthase activity in KO11 is to increase available substrate pools (oxaloacetate and acetyl-CoA). *In vitro*, acetyl-CoA has been shown to serve as an allosteric activator of phosphoenolpyruvate carboxylase (Izui et al. 1981) for the production of oxaloacetic acid and to relieve the allosteric inhibition of citrate synthase by NADH (Weitzman 1981). In this chapter, I demonstrate that physiological and genetic approaches which increase the availability of acetyl-CoA for biosynthesis stimulate cell growth and ethanol production. These results were used to engineer a second generation biocatalyst, strain SU102, in which a small additional

portion of substrate carbon was redirected from fermentation products to cellular biosynthesis.

Materials and Methods

Microorganisms and Media

Strains and plasmids used in this study are listed in Table 3-1. KO11 and its derivatives (SU102 and SU104) are prototrophic. Working cultures of ethanologenic strains were transferred daily on solid medium (1.5% agar) containing mineral salts, 2% xylose, and 1% CSL (Chapter 2). Stock cultures were stored frozen at -75°C. Luria-agar plates were used for the maintenance of other strains. Ampicillin (50 μg/ml), kanamycin (50 μg/ml) and tetracycline (5 or 10 μg/ml) were added as appropriate.

Fermentation

Seed cultures and fermentations (35°C and 150 rpm) were grown in mineral salts medium containing 1% CSL and 9% xylose (CSL+X medium; Chapter 2). Fermentations were maintained at pH 6.5 by automatic addition of 2N KOH (Moniruzzaman and Ingram 1998). Supplements were filter sterilized as concentrates and added directly to fermentation broth. Samples were removed during fermentation for the measurement of cell mass, ethanol, organic acids and sugars.

Analytical Methods

Cell mass was estimated from the optical density at 550 nm using a Bausch & Lomb Spectronic 70 spectrophotometer (1 OD_{550} = 0.33 mg ml^{-1} dry cell weight). Ethanol and acetaldehyde were measured by gas chromatography (Varian 3400CX) (Moniruzzaman and Ingram 1998). Organic acids and sugars were analyzed by HPLC (Hewlett Packard 1090 series II chromatograph equipped with refractive index and UV_{210}

detectors) with a BioRad Aminex HPX-87H ion exclusion column. Maximum volumetric productivity in mmol liter^{-1} h^{-1} was estimated as the first derivative of ethanol production using PSI-Plot software (Poly Software International, Salt Lake City, Utah). Specific productivity was estimated by dividing volumetric productivity by cell mass; units are mmol (gram cell dry weight)$^{-1}$ hour^{-1}.

Genetic Methods

Standard methods were used for plasmid construction, DNA amplification (PCR), transformation, electroporation and P1 phage transduction (Miller 1992; Sambrook and Russell 2001). Primers (ORFmers) for the amplification of the *E. coli ackA* and *adhE* coding regions were purchased from the Sigma Genosys (The Woodlands, TX). These primers included *Sap*I sites at both ends of the amplified product. Chromosomal DNA from *E. coli* W3110 (ATCC 27325) served as the template for amplification. This strain was also used as an intermediate during the construction of *adhE* deletion in KO11.

Chromosomal insertion of deleted genes (*adhE* and *ackA*) was facilitated by inserting a *tet* gene flanked by *FRT* sites for removal of the antibiotic marker by the chlorotetracycline-inducible FLP recombinase (pFT-A) in the final construct (Martinez-Morales et al. 1999; Posfai et al. 1997). Integration of linearized DNA was facilitated by using pKD46 (temperature conditional) containing an arabinose-inducible red recombinase (Datsenko and Wanner 2000). Putative deletion mutants were selected for tetracycline resistance (5 mg liter^{-1}) and screened for appropriate antibiotic resistance markers. At each step, mutants were verified by analyses of PCR and fermentation products.

Construction of pLOI2065 Containing a Removable Tetracycline Resistance Cassette

To facilitate antibiotic removal after chromosomal integration, a reusable cassette was constructed from the *tet* gene of pKNOCK-Tc (Alexeyev 1999) and the *FRT* sites in pSG76-A and pSG76-K (Posfai et al. 1997). Both *FRT* sites were oriented in the same direction to allow efficient *in vivo* excision of the *tet* gene by the *flp*-encoded recombinase (Martinez-Morales et al. 1999). This cassette was inserted into a modified pUC18 to produce pLOI2065 (Fig. 3-2). Plasmid pLOI2065 contains two *Eco*RI sites and two *Sma*I sites oriented to allow the isolation of the *FRT-tet-FRT* cassette as a *Sma*I to *Eco*RI fragment for directional insertion, as a blunt fragment (*Sma*I) and as a sticky-ended fragment (*Eco*RI).

Nucleotide Sequence Accession Number

The sequence for plasmid pLOI2065 has been deposited in GenBank under acquisition number AF521666.

Construction of SU102 Containing an Insertion Mutation in *ackA*

Strain SU102 was made by introducing the *ackA* mutation directly into KO11. The PCR-amplified coding region of *ackA* was cloned into pCR2.1-TOPO. After digestion with *Eco*RI, the 1.2 kbp fragment containing the *ackA* coding region was ligated into the unique *Eco*RI site of pLOI2302. A recombinant plasmid was selected in which the direction of transcription of *lac* and *ackA* genes were opposite. The *ackA* gene was disrupted by digestion with *Eco*RV (1 site) and the insertion of a 1.7 kbp *Sma*I fragment from pLOI2065 containing a *tet* gene flanked by two *FRT* sites for FLP recombinase. A 2.8 kbp *Asc*I fragment containing *ackA*'-*FRT-tet-FRT*-'*ackA* was isolated from this plasmid and ligated into the *Asc*I site of pLOI2224 containing a conditional R6K replicon.

The resulting plasmid, pLOI2375 (Fig. 3-2), was used as a template for PCR amplification of the 2.8 kbp *Asc*I fragment with *ackA* primers. After purification by phenol extraction, amplified DNA was used for electroporation of *E. coli* KO11(pKD46) expressing phage lambda red recombinase (Datsenko and Wanner 2000). Recombinants were selected for tetracycline resistance. Plasmid pKD46 was eliminated by growth at 40°C. The integrated *tet* gene was deleted using pFT-A expressing the *flp* recombinase (Martinez-Morales et al. 1999; Posfai et al. 1997). After removal of this plasmid by growth at 40°C, the resulting strain containing a mutation in *ackA* (insertion of 98 bases including stop codons in all three reading frames) was designated SU102.

Construction of SU104 Containing a Deletion in *adhE*

A mutation in *adhE* was initially constructed in W3110 prior to P1 transduction into KO11. The PCR-amplified coding region of *adhE* (2.7 kbp) was cloned into pCR2.1-TOPO. A recombinant plasmid was selected in which the transcription of *lac* and *adhE* were oriented in the same direction. The central region of the *adhE* gene (1.1 kbp) was deleted by digestion with *Hin*CII (2 sites) and replaced with a 1.6 kbp *Sma*I fragment from pLOI2065 containing the *FRT-tet-FRT* cassette (1.7 kbp) to produce pLOI2803 (Fig. 3-2). After digestion of pLOI2803 with both *Pvu*I and *Sca*I, this plasmid served as a template to amplify the 3.2 kbp region containing *adhE'-FRT-tet-FRT-'adhE* using *adhE* primers. This amplified DNA was used for electroporation. Recombinants were selected for tetracycline resistance. Plasmid pKD46 was eliminated by growth at 42°C.

P1 transduction was used to transfer the *adhE* mutation in W3110 to KO11. To circumvent differences in restriction systems, the *adhE::tet* mutation was transduced into a restriction-negative (modification-positive) derivative of *E. coli* B (strain WA837) prior

to transduction into KO11. The tetracycline resistance gene was deleted from the KO11 derivative using pFT-A expressing the *flp* recombinase (Martinez-Morales et al. 1999; Posfai et al. 1997). After removal of this plasmid by growth at 40°C, the resulting strain containing an internal deletion in *adhE* was designated SU104.

Results and Discussion

Acetate Addition Stimulates Growth and Ethanol Production by Reducing Net Acetate Production During Sugar Metabolism.

During the aerobic metabolism of sugars by *E. coli*, acetate production has been associated with a decrease in growth rate. Considerable effort has been made to minimize acetate production as a means of increasing cell density and the production of recombinant proteins (Aristidou et al. 1995; Bauer et al. 1990; Chang et al. 1999; Contiero et al. 2000; Yang et al. 1999a; Yang et al. 1999b). The addition of as little as 2 g liter^{-1} sodium acetate (24 mM) has been shown to decrease growth rate during oxidative sugar metabolism (Luli and Strohl 1990). During xylose fermentation by KO11, however, the addition of acetate stimulated growth and ethanol production (Fig. 3-3A and B; Table 3-2). A portion of the added acetate was initially consumed, in contrast to control fermentations where acetate was continuously produced (Fig. 3-4A). Rates of acetate production declined during subsequent incubation in both control and acetate-supplemented fermentations. Although almost twice as much sugar was metabolized by acetate-supplemented fermentations than by control fermentations (no additions), net acetate production in the acetate-supplemented culture (7.0 mmol liter^{-1}) was less than half that of the control (18.6 mmoles liter^{-1}) after 72 h.

Previous studies have shown that the reversible phosphotransacetylase-acetate kinase pathway can serve as a route for entry of added acetate into the intracellular pool of acetyl-CoA (Brown et al. 1977; Higgins and Johnson 1970). Additional acetate uptake activity may be provided by the inducible acetyl-CoA synthetase, although this gene is typically repressed under fermentative conditions (Kumari et al. 1995). Thus, the stimulation of growth and ethanol production by added acetate is presumed to result from the increased availability of acetyl-CoA. Under anaerobic conditions, the primary role of the TCA pathway is to supply carbon skeletons for biosynthesis. Increasing the availability of acetyl-CoA would promote biosynthesis by relieving the NADH-mediated allosteric inhibition of citrate synthase (Weitzman 1981) and by serving as an allosteric activator of phosphoenolpyruvate carboxylase (Izui et al. 1981).

Stimulation of Growth and Ethanol Production by Added Pyruvate Can Be Primarily Attributed to Increased Acetate Production.

The stimulation of growth and ethanol production by pyruvate reported previously (Chapter 2) appeared quite similar to the effects of added acetate (Fig. 3-3A and B). Analysis of products during fermentation provided further evidence of a related mechanism of action for acetate and pyruvate (Fig. 3-4 A-E). With the exception of formate (Fig. 3-4B), profiles of organic acids were similar for acetate and pyruvate-supplemented cultures. Both were distinctive from the control lacking supplements. Control fermentations produced lower levels of lactate than pyruvate-supplemented and acetate-supplemented fermentations during the initial 72 h (Fig. 3-4C). Addition of pyruvate stimulated the production of acetate to levels equivalent to that of acetate-supplemented fermentations (Fig. 3-4A). In both pyruvate and

acetate-supplemented fermentations, acetate concentrations were approximately 2-fold higher than in the control after 36 h. Acetate concentrations in all fermentations remained relatively constant during further incubation.

Most of the supplemental pyruvate (22 mM) was metabolized during the initial 3 h of incubation (Fig. 3-5) although the benefits for growth and ethanol production persisted throughout fermentation. During the initial 3 h, the largest change was an increase in acetate (Fig. 3-6A). Smaller pyruvate-dependent increases were observed for ethanol, formate, lactate and acetaldehyde. Biosynthetic needs were estimated to be small (increase of approximately 0.06 mg dry cell weight liter^{-1}) and did not represent a significant sink for the added pyruvate (2 g liter^{-1}). The partitioning of pyruvate between these different fermentation products (and biosynthesis) in KO11 is generally regarded as the result of 5 competing reactions: pyruvate decarboxylase (PDC), pyruvate formate-lyase (PFL), pyruvate dehydrogenase (PDH), lactate dehydrogenase (LDH) and phosphoenolpyruvate carboxylase (PPC). On a triose basis, relative activities can be estimated from the distribution of fermentation products (Fig. 3-1; de Graef et al. 1999). The large pyruvate-dependent increase in acetate after 3 h (Fig. 3-6A) reflects an increase in acetyl-CoA production (PFL and PDH activities). In the absence of formate hydrogen lyase induction (Bock and Sawers 1996), formate production provides an independent measure of PFL activity and exhibited a modest increase compared to acetate. These results indicate that PDH activity (estimated as acetate minus formate) serves as the primary source of additional acetyl-CoA during the metabolism of added pyruvate (Fig. 3-6B). Production of ethanol also increased immediately after the addition of pyruvate due to an increase in the production of acetaldehyde by PDC. Increased pyruvate

oxidation by PDH is presumed to provide the additional NADH required to reduce acetaldehyde produced from added pyruvate. The increase in LDH activity (estimated as lactate production) can be attributed to substrate activation (Tarmy and Kaplan 1968). Elevated extracellular levels of acetate in pyruvate-supplemented fermentations may serve to increase intracellular acetyl-CoA pools, extending the period of growth and thereby increasing the volumetric rate of ethanol production.

The channeling of pyruvate to acetyl-CoA and acetate by the addition of pyruvate can be readily explained based on known allosteric controls (Fig. 3-1). Pyruvate is both a substrate for acetyl-CoA production and a strong allosteric activator of phosphotransacetylase (Suzuki 1969). Addition of pyruvate has also been shown to increase acetaldehyde and decrease the level of NADH (Chapter 2), an allosteric inhibitor of phosphotransacetylase (Suzuki 1969) and PDH (de Graef et al. 1999; Hansen and Henning 1966). These actions would also tend to increase the partitioning of carbon into acetate.

Higher levels of succinate and fumarate (3-fold to over 10-fold, respectively) were produced by acetate- and pyruvate-supplemented fermentations (Fig. 3-4C and D). PPC (Izui et al. 1981) and citrate synthase (Weitzman 1981) are both activated by acetyl-CoA and link the supply of this important intermediate to fermentation and biosynthesis. Under anaerobic conditions, the reductive portion of the TCA pathway is used to produce succinate. Due to the deletion of fumarate reductase (*frd*) in KO11, little succinate was produced and a small amount of fumarate accumulated. The increases in succinate and fumarate levels in acetate and pyruvate-supplemented fermentations may result from an excess of citrate. Excess citrate can be cleaved into acetate and oxaloacetate by an

inducible citrate lyase (Lutgens and Gottschalk 1980). Additional succinate can be produced from isocitrate by isocitrate lyase (Weitzman 1981).

Pyruvate and free CoA are co-substrates for formate production by PFL (Fig. 3-1). Formate levels increased during the initial 12 h of incubation in all fermentations and declined thereafter (Fig. 3-4B). The decline in formate can be attributed to the formate-inducible formate hydrogen lyase (Bock and Sawers 1996). Supplementing with acetate and pyruvate had opposite effects on formate production (Fig. 3-4B), higher concentrations in pyruvate-supplemented fermentations and lower levels in acetate-supplemented fermentations in comparison to those of the control. Both differences are in general agreement with the central role of acetyl-CoA in metabolism (Chang et al. 1999b; Contiero et al. 2000; Kirkpatrick et al. 2001). In acetate-supplemented fermentations, formate production by PFL may be limited by a lack of free CoA. Conversely, higher formate levels produced by pyruvate-supplemented fermentations may result from an increase in free CoA due to the allosteric activation of phosphotransacetylase by pyruvate (Suzuki 1969).

Stimulation of Growth and Ethanol Production by Acetaldehyde Can Be Attributed to Increased Acetyl-CoA.

Growth and ethanol production were also stimulated by acetaldehyde (Chapter 2; Fig 3-3A and B). At concentrations above 5.6 mM, acetaldehyde strongly inhibited growth. It was empirically determined that stimulation equivalent to that of pyruvate could be achieved by the addition of 11.2 mM acetaldehyde, 5.6 mM initially and 5.6 mM after 12 h of fermentation (Chapter 2). Previous studies also demonstrated that the

addition of acetaldehyde caused a rapid decrease in the intracellular concentration of NADH (Chapter 2).

The initial portion of added of acetaldehyde was metabolized within 3 h (Fig. 3-5A). During this time, ethanol increased by an amount equal to 70% of the added acetaldehyde (Fig. 3-6A). Increased pyruvate flux through PDH appears to provide the additional NADH required for acetaldehyde reduction (Fig. 3-6B). The second acetaldehyde addition was metabolized within 1 h (Fig. 3-5A) although benefits persisted throughout fermentation (Fig. 3-3A and B). Following the second addition, production of acetate and ethanol was increased while formate production was reduced. The persisting benefit of acetaldehyde additions for growth and ethanol production are presumed to result from an increase in the intracellular acetyl-CoA pool as a consequence of higher extracellular levels of acetate. High levels of NADH and global regulation by ArcA and FNR (de Graef et al. 1999) may also limit PDH function in the absence of supplements. Increased production of acetyl-CoA by PDH (and perhaps increased synthesis of PDH) would be expected in response to NADH oxidation.

The production of formate by PFL may be limited by competition with PDH for free CoA. Patterns of organic acid production in acetaldehyde-supplemented cultures provide further support for a mechanism of action similar to that for pyruvate and acetate (Fig. 3A-E). Acetate levels were higher in all three supplemented cultures than in the unsupplemented control. Each supplemented fermentation also produced higher levels of succinate, lactate and fumarate than the control.

Stimulation of Growth and Ethanol Production by Inactivation of Non-biosynthetic Pathways Which Consume Acetyl-CoA.

Acetyl-CoA serves as the single most important intermediate for cellular biosynthesis, providing over half of the cellular carbon during sugar metabolism (Neidhardt et al. 1990). Previous studies have shown that cell growth is limited by the availability of carbon skeletons during the fermentation of xylose (Chapter 2), a limitation which was relieved (Fig. 3-1A and B) by supplements which increase the extracellular levels of acetate (acetate, pyruvte, acetaldehyde). During fermentation (Fig. 3-1), two pathways drain acetyl-CoA from the intracellular pool but provide limited benefit to biosynthesis. Acetyl-CoA can be reduced to acetaldehyde and ethanol by alcohol dehydrogenase E (*adhE*) as an alternative route for NADH oxidation in KO11 (Fig. 3-1). Acetyl-CoA can also be converted to acetate by phosphotransacetylase (*pta*) and acetate kinase (*ackA*), increasing the production of ATP. Mutations in these pathways were investigated as a means of sparing acetyl-CoA for biosynthetic needs.

Inactivation of *ackA* rather than *pta* was chosen to minimize potential problems associated with global regulation. Acetyl-P is proposed to serve as an important global regulator in *E. coli* (Bouche et al. 1998; Kirkpatrick et al. 2001; McCleary et al. 1993), affecting gene expression and fundamental processes such as the turnover of RpoS. During oxidative metabolism, inactivation of the acetate pathway (*pta, ackA*) is detrimental to growth (Chang et al. 1999b; Contiero et al. 2000; Kirkpatrick et al. 2001). Although not fully understood, this detrimental effect has been attributed to depletion of free CoA due to low rates of acetyl-CoA turnover (Chang et al. 1999b). In contrast to that found in previous studies concerning oxidative metabolism, inactivation of *ackA* (SU102)

stimulated growth and ethanol production during the fermentation of xylose (Fig. 3-3C and D). An *adhE* mutation in strain KO11 (SU104) was of no benefit during xylose fermentation. Together, these results suggest that ADH contributes little to metabolism in KO11. The beneficial effect of inactivating *ackA* is presumed to result from an increase in the availability of acetyl-CoA for biosynthesis, the genetic equivalent of adding acetate, pyruvate, or acetaldehyde.

Strains SU104 (*adhE* mutant) and SU102 (*ackA* mutant) were also tested in fermentations with supplements that had been shown to increase the growth and ethanol production in KO11 (Table 3-2). Addition of acetate, pyruvate and acetaldehyde to SU104 increased growth and ethanol production indicating that the native alcohol dehydrogenase (*adhE*) was not essential for this response. Growth and ethanol production by SU102 (*ackA*) without supplements were equivalent to that of KO11 with supplements. The addition of pyruvate, acetate, 2-ketoglutarate, or acetaldehyde to SU102 provided little further improvement in growth or ethanol production.

HPLC analysis of organic acids revealed similarities in the patterns of fumarate (Fig. 3-3J) and succinate (Fig. 3-3I) production between SU102 (*ackA* mutant) and KO11 supplemented with acetate, pyruvate or acetaldehyde (Fig. 3-4E). The *ackA* mutation in SU102 also increased lactate production (Fig. 3-4I) and delayed the production of formate (Fig. 3-4G) and acetate (Fig. 3-4F). The delay in formate production in SU102 could result from increased acetyl-CoA, reducing the pool of free CoA (co-substrate for PFL) analogous to acetate-supplemented KO11 (Fig. 3-4B). Both acetate addition and mutations in the acetate pathway have been shown to cause a similar repression of 37 genes (Kirkpatrick et al. 2001), attributed to an increase in the acetyl-CoA pool.

Inactivation of acetate kinase (SU102) caused an initial delay in acetate production but did not block later synthesis. The pathway responsible for acetate production during the latter stages of fermentation remains unknown but may be the result of spontaneous dephosphorylation of acetyl-P as previously proposed (Brown et al. 1977) or from induction of cryptic enzyme(s). Despite the potential benefit of increased ATP production by acetate kinase, the increased drain of acetyl-CoA to acetate through this pathway appears to be more detrimental for growth and ethanol production by KO11 than the reduction in ATP. With the exception of acetate (Fig. 3-4F), the production of fermentation products by the *adhE* mutant (strain SU104) was essentially the same as for the parent strain, KO11 (Fig. 3-4A). Acetate production by SU104 continued throughout fermentation and reached higher final concentrations than KO11.

Conclusions

Increasing the availability of acetyl-CoA stimulated growth and ethanol production from xylose by prolonging the growth phase of ethanologenic *E. coli*. The resulting increase in biocatalyst rather than an increase in cellular activity was responsible for the increased rate of ethanol production (Table 3-2). Similar benefits were obtained by minimizing the loss of acetyl-CoA as acetate (*ackA* mutation) and by increasing intracellular levels of acetate (supplementing with acetate, pyruvate, or acetaldehyde). Inactivation of the native *E. coli* alcohol/aldehyde dehydrogenase (*adhE*) had little effect indicating that this pathway has limited function in ethanologenic KO11.

ATP production during xylose fermentation does not appear to limit growth or cell yield in KO11. Including the energy required for xylose uptake and activation, less than 1 ATP (net) is produced from the metabolism of each xylose converted to ethanol

(Tao et al. 2001; Chapter 2). During the initial 12 hours of growth, up to 31% of the ATP (net) produced by KO11 is provided by the acetate pathway (calculated by assuming 1 ATP per acetate from acetate kinase and 0.4 ATP per pyruvate from glycolysis). Disruption of this pathway (*ackA*) in SU102 increased cell yield by 2-fold (Table 3-2). Thus, the partitioning of carbon skeletons rather than the production of ATP appears to limit the growth of ethanologenic *E. coli* during xylose fermentation.

The mechanism for the stimulation of growth in ethanologenic *E. coli* KO11 is consistent with established patterns of allosteric regulation although further controls of gene expression (FNR, ArcA) may also contribute to the observed effects. More than half the amino acids produced in the cell are derived from the TCA pathway. Flux through this pathway is controlled by PPC and citrate synthase (Lee et al. 1994; Walsh and Koshland, Jr. 1985), activities which can be stimulated by acetyl-CoA (Weitzman 1981). The individual addition of pyruvate, acetate, and acetaldehyde increased the extracellular levels of acetate which can in turn serve to elevate intracellular pools of acetyl-CoA by reversible reactions. Additional benefits of supplements include a reduction in the level of NADH (added pyruvate and acetaldehyde), an allosteric inhibitor of citrate synthase which is antagonized by high levels of acetyl-CoA. Based on these results with mutants and with supplements, we conclude that the regulation of acetyl-CoA production and consumption can be used to make small changes in the partitioning of carbon between biosynthesis and fermentation during the ethanol production by *E. coli* KO11.

Table 3-1. Strains and plasmids used in Chapter 3.

Strain or Plasmid	Relevant Characteristics	Reference or source
Strains		
KO11	*frd cat pfl*$^{+}$ *pfl::*(*Z. mobilis pdc*$^{+}$ *adhB*$^{+}$)	Ohta et al. 1991
SU102	KO11 *ackA*	This work
SU104	KO11 *adhE*	This work
W3110	wild type	ATCC 27325
WA837	r_{B-} m_{B+} *gal met*	Wood 1966
Plasmids		
pKD46	γ β *exo repA*101 pSC101 replicon^{ts} (red recombinase)	Datsenko and Wanner 2000
pFT-A	*bla flp* pSC101 replicon^{ts} (FLP recombinase)	Posfai et al. 1997
pCR2.1-TOPO	*bla kan* ColE1	Invitrogen
pKNOCK-Tc	*tet* R6K (*pir* dependent replicon)	Alexeyev 1999
pSG76-K	*kan FRT* R6K (*pir* dependent replicon)	Posfai et al. 1997
pSG76-A	*bla FRT* R6K (*pir* dependent replicon)	Posfai et al. 1997
pLOI2065	*bla FRT-tet-FRT* ColE1	This work
pLOI2224	*kan* R6K (*pir* dependent replicon)	Martinez-Morales 1999
pLOI2302	*bla* ColE1 (*Eco*RI flanked by *Asc*I sites)	Zhou and Ingram 1999
pLOI2375	*ackA::FRT-tet-FRT kan* R6K (*pir* dependent replicon)	This work
pLOI2803	*adhE::FRT-tet-FRT kan* ColE1	This work

Table 3-2. Effects of mutations and additives on cell yield and ethanol productivity.

Strain and additive	Concentration (mM)	N	Cell Mass			Ethanol			
			Maximum (g liter^{-1})	Time (h)	μ [a] (h^{-1})	Maximum (mM)	Max VP [b] (mmol liter^{-1} h^{-1})	Sp. Prod.[c] (mmol g^{-1} h^{-1})	Theoretical Yield [d] (%)
KO11	none	44	0.95 ± 0.13	72	0.63	641 ± 93	8.3	10.1	63
+ pyruvate	21	18	2.00 ± 0.18	72	0.76	955 ± 55	18.3	11.5	94
+ acetate	24	2	1.92 ± 0.01	48	0.75	930 ± 11	18.7	12.0	92
+ acetate	19	6	1.61 ± 0.30	72	ND [f]	901 ± 118	ND	ND	89
+ acetaldehyde [e]	11	6	1.51 ± 0.15	96	0.47	909 ± 98	18.2	11.3	90
+ 2-ketoglutarate	12	6	1.84 ± 0.20	72	ND	907	ND	ND	90
SU102	none	8	1.94 ± 0.14	48	0.66	946 ± 20	17	13.7	93
+ pyruvate	21	4	1.93 ± 0.13	48	0.44	926 ± 19	17.4	10.0	92
+ acetate	19	2	2.24 ± 0.17	48	ND	933 ± 26	ND	ND	92
+ acetaldehyde	11	2	2.02 ± 0.17	48	ND	952 ± 6	ND	ND	94
+ 2-ketoglutarate	12	2	1.83 ± 0.01	96	ND	901 ± 1	ND	ND	89
SU104	none	8	1.02 ± 0.08	96	0.71	550 ± 89	8.9	13.7	55
+ pyruvate	21	2	1.96 ± 0.06	48	ND	885 ± 24	ND	ND	87
+ acetate	19	2	1.68 ±0.02	48	ND	889 ± 10	ND	ND	88
+ acetaldehyde	11	2	1.57 ±0.31	48	ND	856 ± 100	ND	ND	84
+ 2-ketoglutarate	12	2	1.97 ± 0.15	48	ND	971 ± 20	ND	ND	96

[a] Specific growth rate at 2 h.

[b] VP, maximum volumetric productivity, mM ethanol produced per liter per hour.

[c] Specific productivity at 12 h, mmol ethanol produced per gram cell dry weight per hour.

[d] Theoretical yield from 91g liter^{-1} xylose (1.667 mmol ethanol/mmol xylose).

[e] Half added initially, half added after 12 h.

[f] ND, not determined. Estimates of specific and volumetric productivity were not calculated due to the limited number of sampling times.

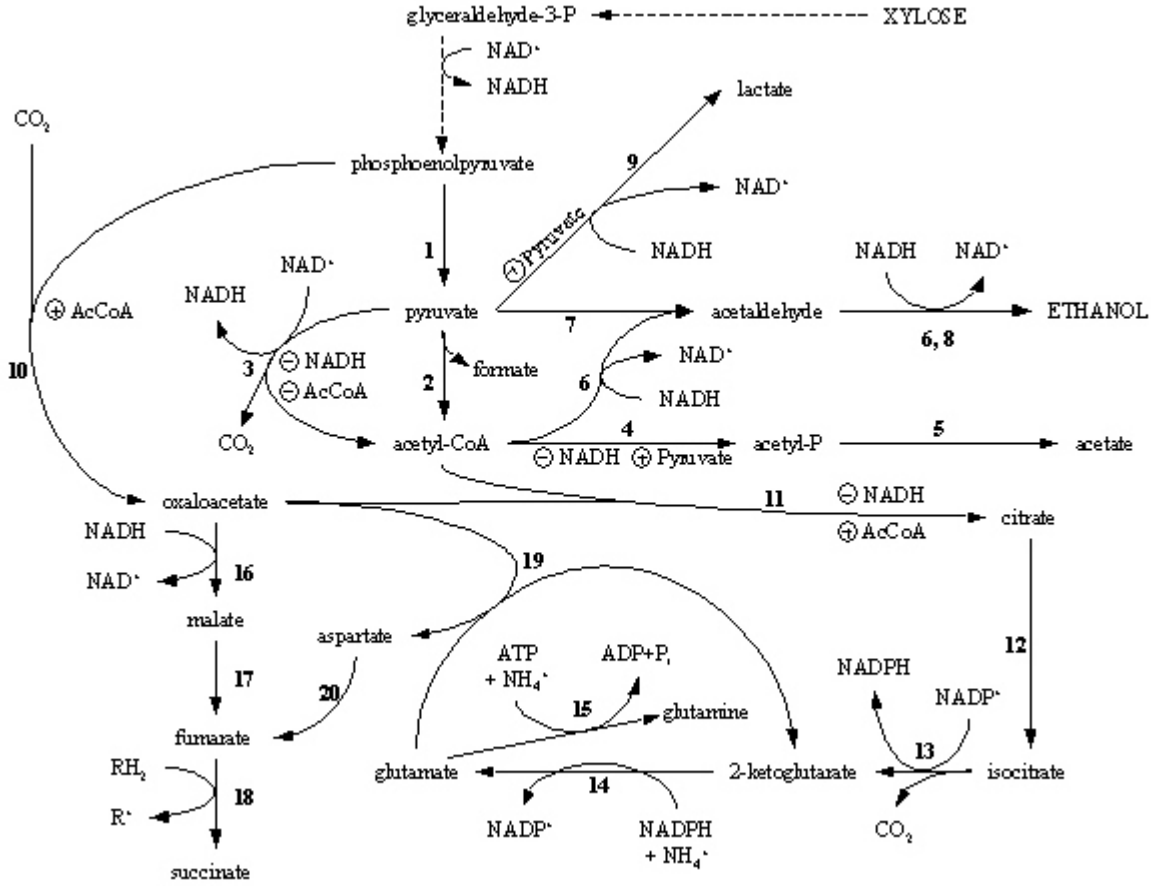

Figure 3-1. Allosteric control of central metabolism. Unless noted otherwise, enzymes listed are native to *E. coli*. Enzymes: 1. pyruvate kinase (*pykA, pykF*); 2. pyruvate formate-lyase (*pflB*); 3. pyruvate dehydrogenase (*aceEF,lpd*); 4. phosphotransacetylase (*pta*); 5. acetate kinase (*ackA*); 6. alcohol/aldehyde dehydrogenase (*adhE*); 7. *Z. mobilis* pyruvate decarboxylase (*pdc*); 8. *Z. mobilis* alcohol dehydrogenase II (*adhB*); 9. lactate dehydrogenase (*ldhA*); 10. phosphoenolpyruvate carboxylase (*ppc*); 11. citrate synthase (*gltA*); 12. aconitase (*acn*); 13. isocitrate dehydrogenase (*icd*); 14. glutamate dehydrogenase (*gdhA*); 15. glutamine synthetase (*glnA*); 16. malate dehydrogenase (*mdh*); 17. fumarase (*fumB*); 18. fumarate reductase (*frdABCD*); 19. aspartate transaminase (*aspA*); 20. aspartase (*aspC*). ⊕ indicates allosteric activation, ⊖ indicates allosteric inhibition.

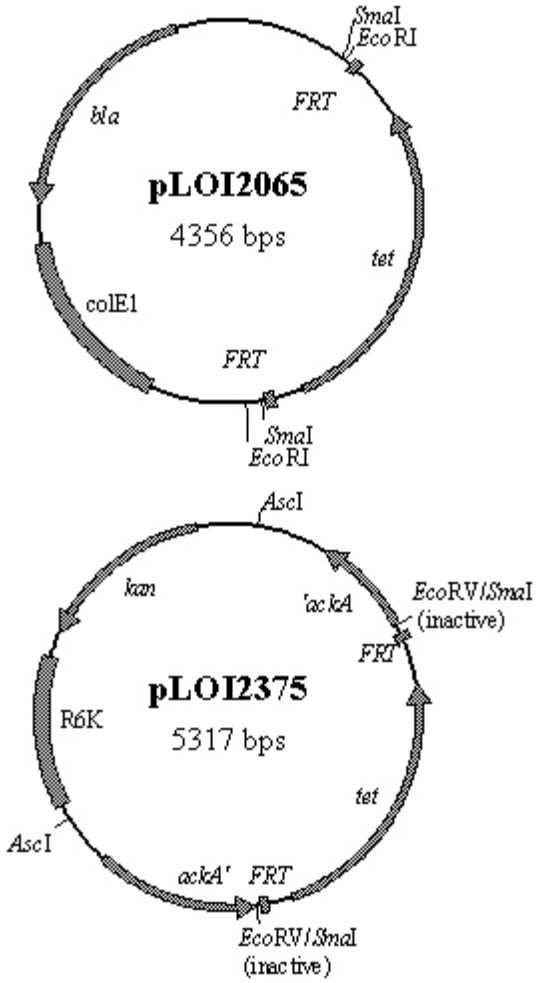

Figure 3-2. Plasmids used to construct mutations in KO11. *FRT* sites allow *in vivo* excision of the *tet* gene after integration using FLP recombinase (*flp*). A. Plasmid pLOI2065 containing a *tet* gene flanked by *FRT* sites. B. Plasmid pLOI2375 containing an interrupted *ackA* gene.

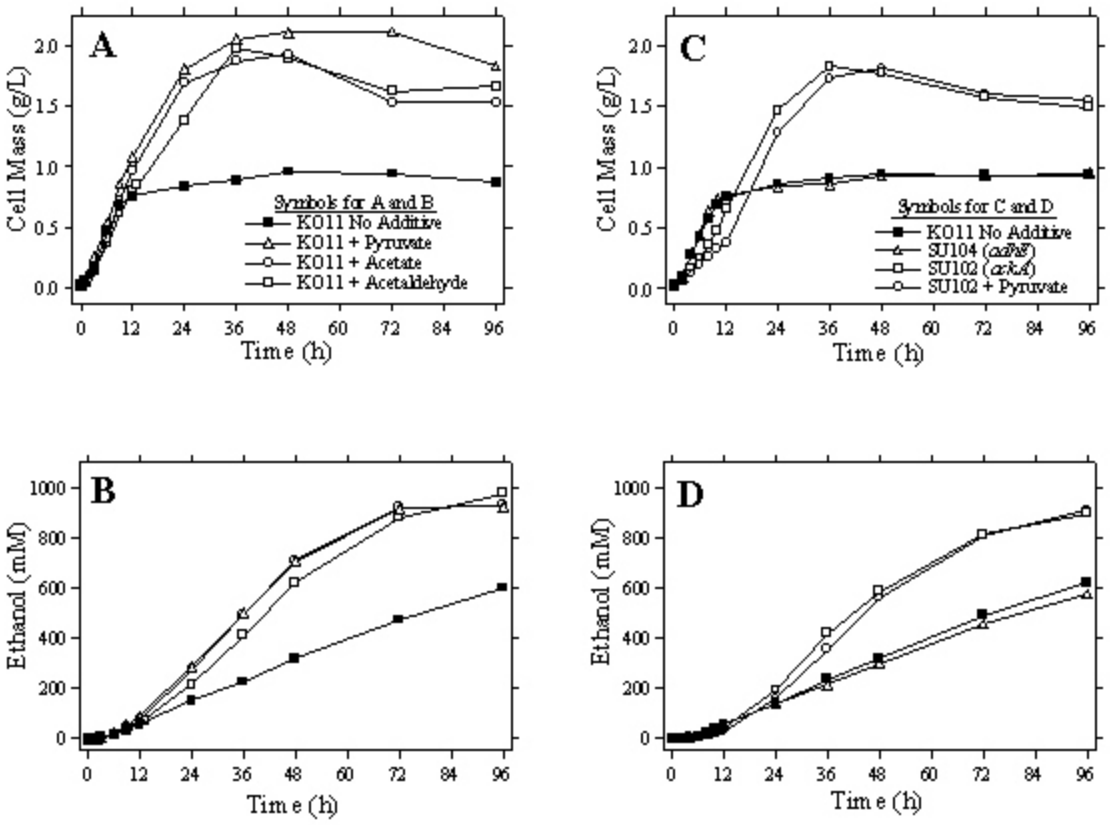

Figure 3-3. Effect of media additions and mutations on growth (A, C) and ethanol production (B, D). Symbols for A and B: ■, KO11 no additive; Δ, KO11+ pyruvate; ❍, KO11 + acetate; and ❒ , KO11+ acetaldehyde. Symbols for C and D: ■, KO11 no additive; ❒, SU102 (*ackA*) no additive; Δ, SU104 (*adhE*) no additive; and ❍, SU102 + pyruvate.

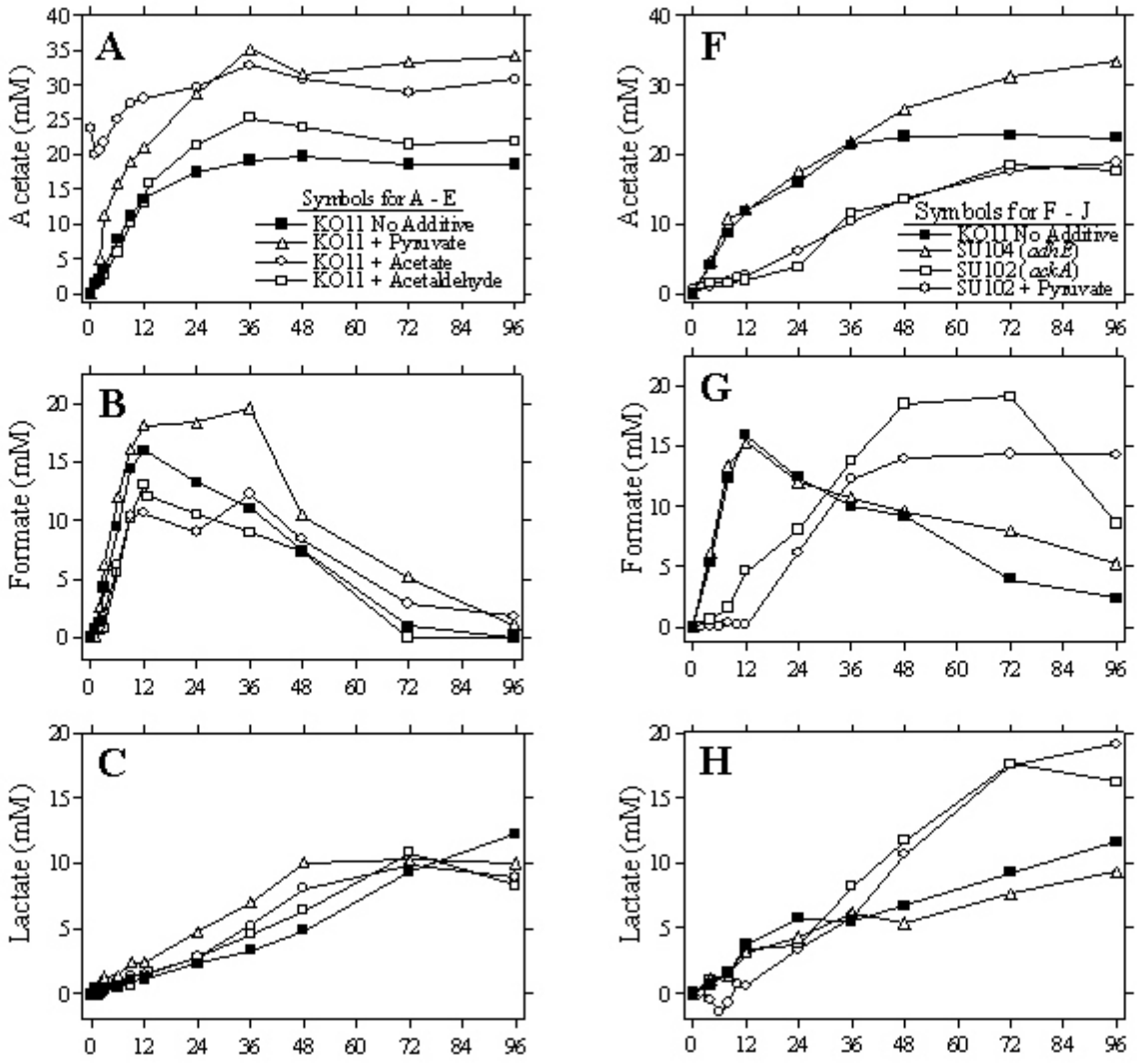

Figure 3-4. Effect of media additions and mutations on organic acid production: acetate (A/F), formate (B/G), lactate (C/H), fumarate (D/I) and succinate (E/J). Symbols for A-E: ■, KO11 no additive; Δ, KO11+ pyruvate; ❍, KO11 + acetate; and ❒, KO11+ acetaldehyde. Symbols for F-J: ■, KO11 no additive; ❒, SU102 no additive; Δ, SU104 no additive; and ❍, SU102 + pyruvate.

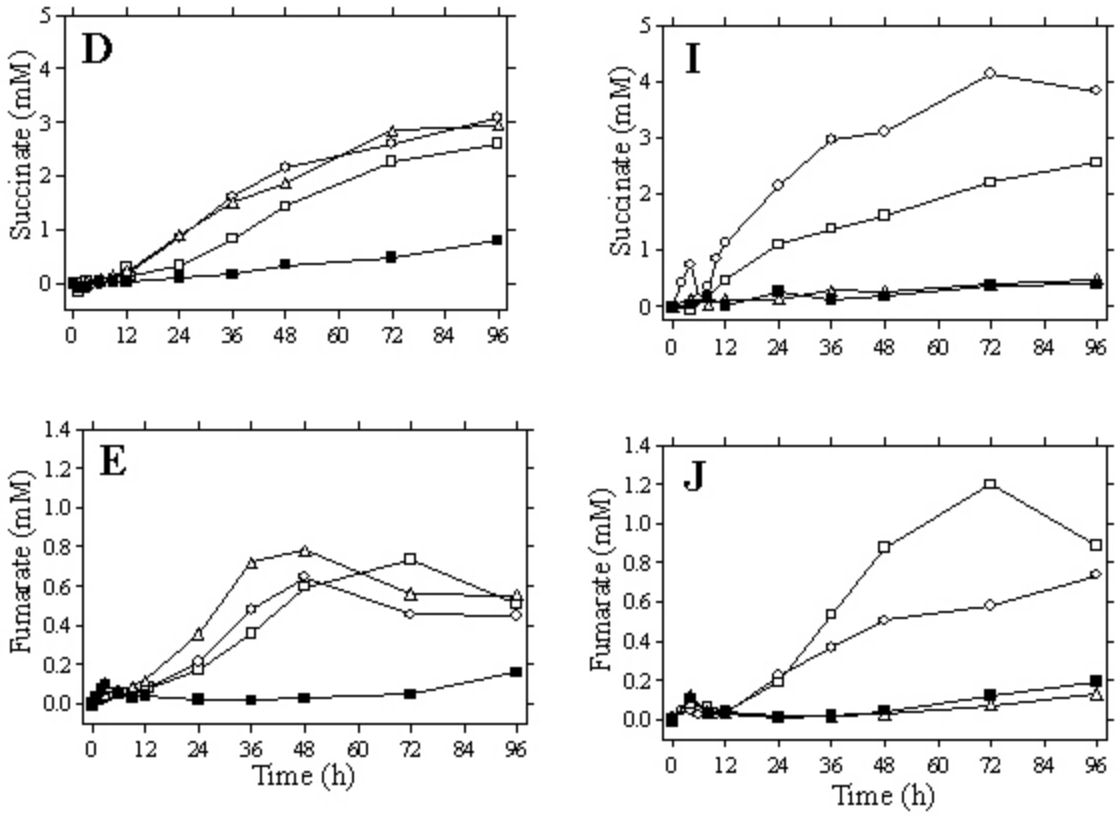

Figure 3-4. continued.

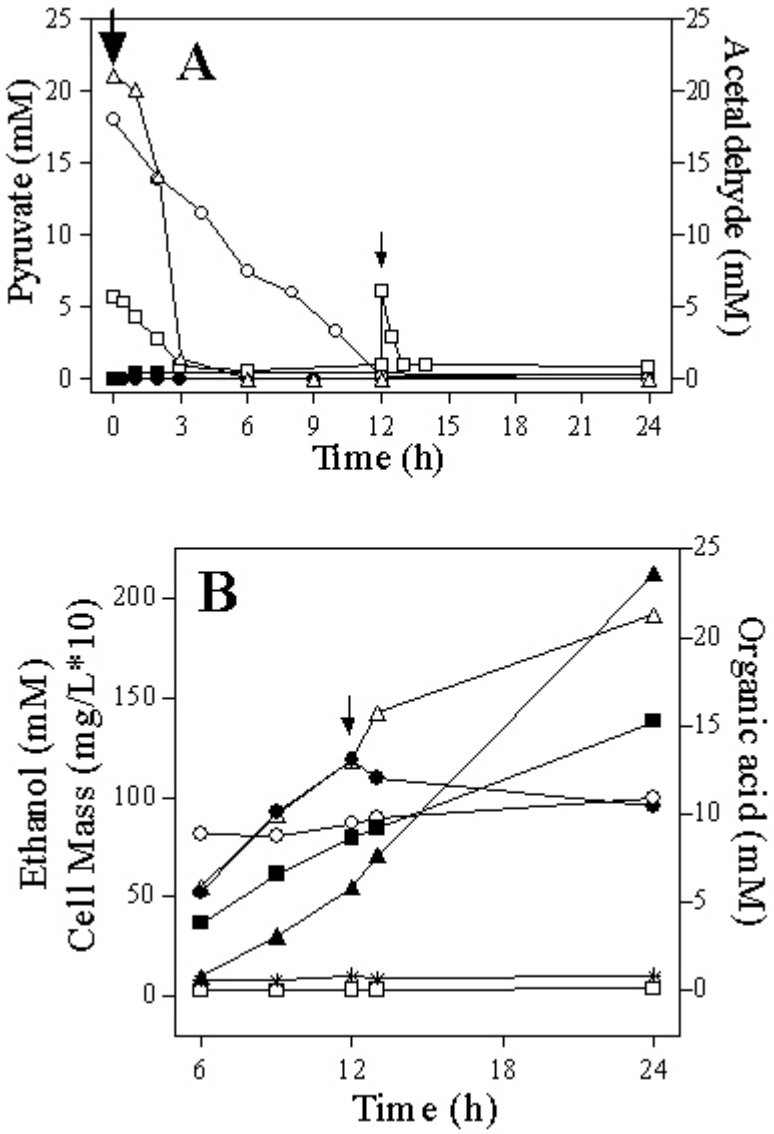

Figure 3-5. Metabolism of added acetaldehyde and pyruvate during fermentation. Pyruvate addition is indicated by the large arrow. Acetaldehyde additions (5.6 mM each) are indicated by the large arrow (initial addition) and the small arrow (second addition at 12 h). A. Utilization of added pyruvate and acetaldehyde. Symbols: Δ, pyruvate utilization by KO11; ❍, pyruvate utilization by SU102; ❒, acetaldehyde utilization by KO11; ■, acetaldehyde in KO11 broth with no additions; and ●, pyruvate in KO11 broth with no additions. B. Effect of second acetaldehyde addition on production of fermentation products by KO11. Symbols: ■, cell mass; ▲, ethanol; ●, formate; ❍, lactate; Δ, acetate; ✳, succinate; and ❒, fumarate.

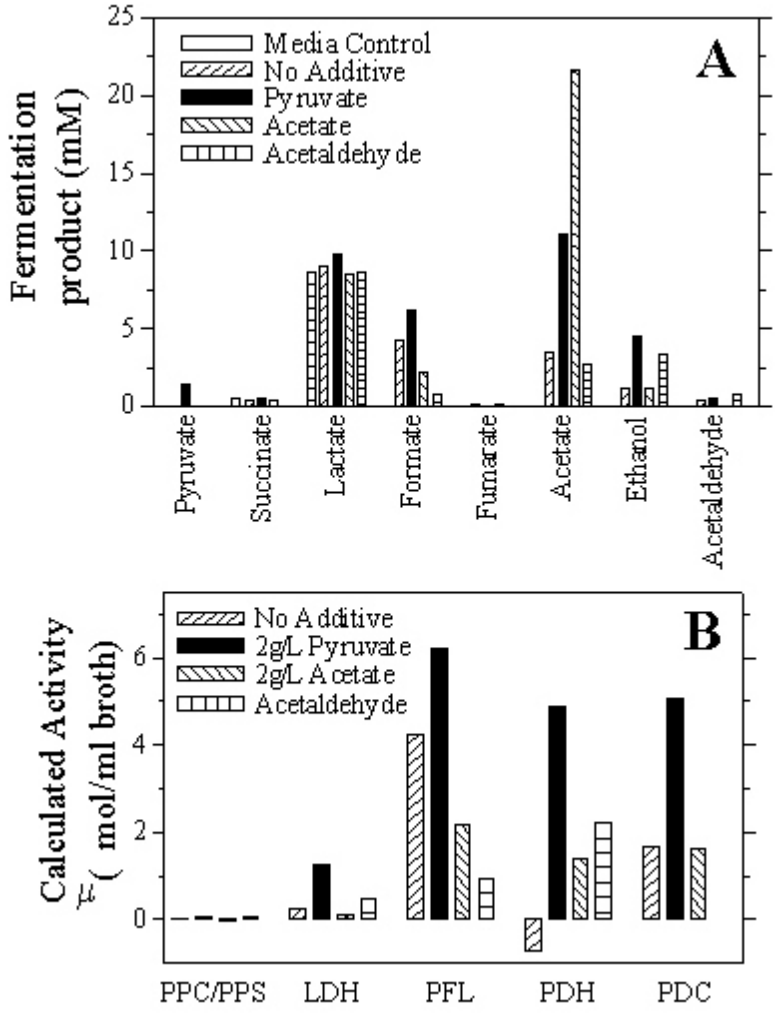

Figure 3-6. Partitioning of carbon among competing pathways during the initial 3 h of fermentation. A. Fermentation products after 3 h. B. Relative activity of primary enzymes that partition 3-carbon intermediates carbon (pyruvate and phosphoenolpyruvate) through competing pathways. Relative activities were estimated using fermentation products, expressed as μmol of product per ml during the initial 3 h of incubation. Endogenous production of acetate in acetate-supplemented fermentations was assumed to be equal to that for the control fermentation without additives. Pyruvate decarboxylase activity was assumed to be equal to ethanol production, except for the acetaldehyde-supplemented fermentations where it could not be calculated. Pyruvate dehydrogenase was calculated as the difference between acetate and formate production. Lactate dehydrogenase, pyruvate formatelyase, and phosphoenolpyruvate carboxylase activities were assumed to equal to the production of lactate, formate, and succinate, respectively. Abbreviations: PPC, phosphoenolpyruvate carboxylase; PPS, phosphoenolpyruvate synthetase; LDH, lactate dehydrogenase; PFL, pyruvate formatelyase; PDH, pyruvate dehydrogenase; PDC, pyruvate decarboxylase.

CHAPTER 4
A DEFICIT IN PROTECTIVE OSMOLYTES IS RESPONSIBLE FOR THE DECREASED GROWTH AND ETHANOL PRODUCTION DURING XYLOSE FERMENTATION

Introduction

Maintaining inexpensive sources of fuels and commodity chemicals for the U. S. is a matter of national security. Increasing the production of fuel ethanol offers a potential solution to this problem. The conversion of lignocellulose to fuel ethanol and other chemicals typically derived from petroleum would decrease the U. S. dependance on imported oil (Artzen and Dale 1999). Enteric bacteria are noted for their broad range of growth substrates, including all the sugars present in the polymers of lignocellulose. *Escherichia coli*, a microbial platform for the commercial production of amino acids and recombinant proteins (Chotani et al. 2000; Akesson et al. 2001), was previously engineered for the production ethanol by integrating a synthetic operon containing the ethanol pathway from *Z. mobilis* (*pdc* and *adhB*) into the chromosome (Ohta et al. 1991). The resultant strain, designated KO11, fermented all the sugar constituents of lignocellulose to ethanol with yields approaching 100% (Ohta et al. 1991; Martinez et al. 1999; Ingram et al. 1999).

During batch fermentations with strain KO11, the volumetric rate of ethanol production was directly related to the growth of the biocatalyst (Martinez et al. 1999; Chapter 2; Chapter 3). Cell yield for both the ethanologenic strain and its parent (*E. coli* B) was dependent upon the availability of nutrients in a variety of media, despite the

absence of any specific auxotrophic requirements. During the batch fermentation of xylose (90 g liter^{-1}) to ethanol by strain KO11, cell growth appeared to be limited by the availability of carbon skeletons derived from the citrate arm of the anaerobic TCA pathway (Chapter 2; Chapter 3) (Fig. 4-1).

During fermentative metabolism, the TCA pathway is interrupted by the repression of 2-ketoglutarate dehydrogenase (Iuchi and Lin 1988). The ultimate product of this pathway, 2-ketoglutarate, is a substrate for glutamate biosynthesis. During growth in minimal media, glutamate is the most abundant free amino acid in the cytoplasm of *E. coli* (Cayley et al. 1991). In addition to its roles in metabolism and protein synthesis, glutamate biosynthesis is part of the primary response to osmotic stress (Csonka 1989; Csonka and Hanson 1991). The high osmolarity of the medium used for ethanol production (0.6 M xylose) would be expected to increase the requirement for glutamate as a protective osmolyte.

Potassium ions are rapidly accumulated by *E. coli* in response to osmotic stress. This is rapidly followed by the accumulation of glutamate (McLaggan et al. 1994), a negatively charged amino acid and protective osmolyte, to balance the positive charge of the accumulated potassium. In the closely related organism *Salmonella typhimurium*, cells that are restricted in their ability to synthesize glutamate have been demonstrated to grow poorly during osmotic stress (Csonka 1988; Yan et al. 1996). Mutations preventing glutamate production were associated with the inability to balance the charge of intracellular potassium. The resulting decrease in steady-state potassium levels has been proposed to limit cell growth (Yan et al. 1996).

Alternate protective osmolytes such as glycine betaine (hereafter referred to as betaine), proline, taurine, dimethylsulfoniopropionate and many others can be accumulated from the environment. A hierarchy for these osmoprotectants was empirically determined for salt stress (Randall et al. 1995). Although there have been conflicting reports concerning this hierarchy for sugar-mediated osmotic stress (Glaasker et al. 1998), betaine is generally regarded as the most effective protective osmolyte for *E. coli.* The effectiveness of betaine for restoration of growth has been shown to cary with the sugar used for osmotic stress (Dulaney et al. 1968)..

In this study, NMR was used to examine changes in the intracellular pools of osmolytes in response to genetic changes and nutrient supplements that stimulated cell growth and ethanol production. Low cell yield and low ethanol production in the absence of supplements appears to result from a deficit in intracellular glutamate or alternative protective osmolytes.

Materials and Methods

Microorganisms and Media.

The ethanologenic *E. coli* strains KO11 and SU102 (KO11 Δ*ackA*; Chapter 3) are prototrophic. Working cultures were transferred daily on solid medium (1.5% agar) containing mineral salts, 2% xylose, and 1% corn steep liquor (CSL+X medium; Chapter 2) alternating between 40 mg liter^{-1} and 600 mg liter^{-1} chloramphenicol. Stock cultures were stored frozen at -70°C in 40% glycerol.

Fermentation.

Seed cultures (35°C and 120 rpm) and fermentations (35°C and 100 rpm) were grown in either mineral salts medium containing 1% corn steep liquor and 9% xylose

(Chapter 3) or Luria broth (10 g liter $^{-1}$ tryptone, 5 g liter^{-1} yeast extract, 5 g liter^{-1} NaCl) with 9% xylose. For fermentations, sufficient cell mass to achieve and initial concentration of 33 mg liter^{-1} were harvested by centrifugation (5000 x g; 5 min) and suspended in appropriate fresh medium. Fermentations were maintained at pH 6.5 by automatic addition of 2N KOH (Moniruzzaman and Ingram 1998). Stock solutions (100 mM) of betaine (Sigma, St. Louis, MO) and dimethylsulfoniopropionate (TCI America, Portland, OR) were dissolved in deionized water and filter sterilized directly into the fermentation vessel. Glutamate and acetate were added as described previously (Chapter 2; Chapter 3).

~~^{13}C NMR.~~

Intracellular osmolytes were analyzed by NMR (Park et al. 1997). After a 24 h incubation in the fermentation chamber, 700 mL culture was harvested by centrifugation, washed twice in mineral salt solution containing NaCl (0.6 M) , and resuspended in 3 volumes of ethanol (95%). Suspensions were rocked gently 16-24 hr at 4ºC. Cellular debris was removed by centrifugation (4˚C, 10,000 x g, 30 min). Extracts were dried under vacuum, disloved in deionized water, dried under vacuum, resuspended in 33% D_2O and filtered (0.2µm) . Acetone (10µL) was used as an internal reference. Data was obtained using a modified Nicolet NT300 spectrometer operating in the Fourier transform mode as follows: 75.46 MHz; excitation pulse width, 25 us; pulse repetition delay, 40s; spectral width 18 kHz and broadband (bi-level) decoupling of protons. For cell extracts, at least 1000 scans were obtained.

Analytical Methods.

Cell mass was determined from the optical density at 550 nm using a Bausch & Lomb Spectronic 70 spectrophotometer (1 OD_{550} = 0.33 g $liter^{-1}$ dry cell weight), and ethanol was measured by gas chromatography (Varian 3400CX) (Moniruzzaman. and Ingram 1998). For the quantitation of the compounds detected by NMR, a standard curve was generated. The average of all the chemical shift values are reported normalized to cell mass.

Results and Discussion

Citrate Synthase Flux Limits the Biosynthesis of Glutamate, a Primary Intracellular Osmolyte.

E. coli typically maintains large cytoplasmic pools of potassium, glutamate and trehalose during growth in media containing high concentrations of sugars or salt (Cayley et al. 1991; Lewis et al. 1990). Previous studies with the ethanologenic *E. coli* strain KO11 demonstrated that the flux through citrate synthase, the first step in glutamate biosynthesis, limited growth and ethanol production during the fermentation of 90 g $liter^{-1}$ xylose (0.6 M) to ethanol (Chapter 2; Chapter 3). This limitation was proposed to be due to the drain of pyruvate to ethanol by the recombinant pathway (pyruvate decarboxylase-alcohol dehydrogenase) which has a higher affinity for pyruvate than pyruvate formate-lyase or pyruvate dehydrogenase, competing pathways for aceyl-CoA biosynthesis (Chapter 2; Chapter 3). Supplementing the CSL+X medium with acetate increased the availability of acetyl-CoA (Chapter 3), an activator of citrate synthase (Weitzman 1966). During fermentations with strain KO11, supplementing the CSL+X

medium with glutamate increased the final cell yield and ethanol productivity (Chapter 2) by bypassing the growth-limiting citrate synthase.

The intracellular osmolyte pools were compared between conditions of lower growth (CSL+X medium without additives) and higher growth (supplemented with glutamate or acetate) to investigate whether the inability of strain KO11 to accumulate glutamate resulted from restricted citrate synthase flux (Fig. 4-2; Table 4-1). Fermentations without additives accumulated only proline, while those supplemented with acetate (activating citrate synthase) or glutamate (bypassing citrate synthase) resulted in approximately 2-fold higher growth and ethanol productivity. Cells from these fermentations accumulated approximately the same level of intracellular glutamate, supporting the hypothesis that a deficit in the accumulation of this protective osmolyte limited growth.

The intracellular proline, a known osmoprotectant (Csonka 1989;Csonka and Hanson 1991), was likely accumulated from the CSL provided as a source of complex nutrients. Though some organisms synthesize proline in response to osmotic challenge (Kawahara et al. 1989), *E. coli* can only accumulate this protective osmolyte by active transport (Smith et al. 1984). However, mutants have been isolated that are less sensitive to the feedback-inhibition of the proline biosynthetic pathway (Smith 1985). Strains expressing these genes accumulated high levels of intracellular proline (derived from glutamate) and were more resistant to high osmotic environments (Csonka 1981; Csonka et al. 1988). Presumably, the intracellular proline in strain KO11 may have resulted from a similar spontaneous mutation. Accordingly, such a mutation would also reduce the glutamate pool.

Glutamate was accumulated in fermentations with increased growth yield, and the intracellular concentration of proline decreased (Table 4-1). This further supports the hypothesis that the intracellular proline was taken up from the medium and was not a result of biosynthesis. If the drain of intracellular glutamate for proline production had starved the cells for glutamate, supplementing the medium with proline should have a sparing effect on the consumption of glutamate. However, proline addition only increased the intracellular proline pool without affecting either growth or ethanol production (Table 4-1).

Glutamate is a product of proline degradation (McFall and Newman 1996), but the degradation of proline has been shown to be inhibited in media of high osmotic strength (Csonka 1988). Supplementing the medium with an excess of proline (17 mM) should provide excess proline for glutamate production. However, the absence of increased growth and intracellular glutamate in these fermentations confirms the previously observed inhibition of proline degradation during osmotic stress (Csonka 1988).

The accumulation of proline was previously shown not to affect glutamate pools (Cayley et al. 1992). During experiments in a glucose-mineral salts medium buffered with MOPS and high NaCl, the primary osmolytes accumulated by *E. coli* were K^+, glutamate, MOPS and trehalose (Cayley et al. 1991; Cayley et al. 1992; Lewis et al. 1990). Cultures in this medium supplemented with proline accumulated this protective osmolyte and reduced the biosynthesis of trehalose (Cayley et al. 1992). However, the intracellular glutamate concentration was not significantly altered by the accumulation of proline.

Thus, the intracellular accumulation of proline (from the CSL) by strain KO11 should not have affected the glutamate requirement.

Strain KO11 failed to synthesize detectable levels of the osmoprotectant trehalose (<10 mM) under these conditions. While the presence of proline would have decreased the synthesis of trehalose (Cayley et al. 1992), significant trehalose should have been detected. This may be a result of growth on xylose, a pentose. Perhaps the relatively low ATP yield from xylose catabolism (0.4 ATP/pyruvate) restricts the gluconeogenic production of glucose and uridine diphosphate-glucose, substrates for trehalose biosynthesis.

Genetic Changes to Optimize Carbon Partitioning Increased the Glutamate Pool.

The functional expression of *citZ* by strain KO11 (pLOI2514) was previously shown to increase growth and ethanol production. To confirm that the expression of this enzyme aides in glutamate accumulation, the intracellular osmolyte pool during fermentations in the CSL+X medium were analyzed (Fig 4-2; Table 4-1). Similar to the fermentations supplemented with glutamate or acetate, cells from these fermentations had an increased glutamate pool. The intracellular accumulation of proline was similar to that of other experiments with increased growth yields. Thus, the expression of *citZ* provided more citrate, ultimately increasing glutamate biosynthesis and the glutamate pool.

A mutation in the primary acetate production pathway (Δ*pta*) was previously shown to increase glutamate biosynthesis (Chang et al. 1999b). This likely resulted from the accumulation of acetyl-CoA, an activator of citrate synthase (Weitzman 1966). Acetyl-CoA is also an activator of phosphoenolpyruvate carboxylase (*ppc*) (Izui et al. 1981), the controlling step in the biosynthesis of oxaloacetate and co-substrate for citrate

synthase. Thus, the biosynthesis of citrate is regulated, in part, by the availability of acetyl-CoA. In an analogous study presented here, blocking acetate production (Δ*ack*) increased the intracellular glutamate pool level (Fig. 4-2, Table 4-1).

Glutamate Accumulation Functions in Osmoprotection.

Three different osmoprotectants were tested for their ability to restore growth and ethanol production, replacing the additional glutamate requirement.(Fig. 4-3). The addition of 1.0 mM betaine or dimethylsulfoniopropionate (DMSP) increased the cell yield and ethanol production similar to experiments where glutamate production had been increased. Taurine, a weak osmoprotectant for *E. coli* (McLaggan and Epstein 1991), failed to increase growth or ethanol production. Neither betaine nor presumably DMSP should provide a source of glutamate. Thus, supplying osmoprotectants to the medium replaced the need for the accumulation of intracellular glutamate.

To determine the optimal concentration required to restore growth, betaine and DMSP were added from 0.1-2.0 mM and 0.1-1.0 mM, respectively (Fig. 4-4). Growth and ethanol were increased in a dose-dependant manner in each instance. The maximum stimulation of growth and ethanol production by betaine was at the highest level of betaine tested, 2.0 mM. However, only 0.25 mM DMSP was required for maximum benefit. Surprisingly, during the fermentation of xylose to ethanol, DMSP is 10-fold more effective in restoring growth and ethanol production than betaine. Though this is contrary to previous reports that betaine is the most effective protective osmolyte (Randall et al. 1995), these studies were done using high levels NaCl for osmotic challenge. The ability of betaine to restore growth has been reported to vary during osmotic challenge with

different sugars (Dulaney et al. 1968). Thus, a specific protective osmolyte may be more effective during challenge by different osmolytes (sugars and salts).

Replacement of Glutamate by Other Osmoprotectants.

The intracellular osmolyte pools of cells in fermentations supplemented with betaine and DMSP were examined by NMR. Cells from fermentations supplemented with betaine were found to contain only detectable levels of betaine (Fig. 4-2), consistent with previous studies (Cayley et al. 1992). This was likely a result of the properties of the osmotically activated transport pathways. While the K_m of ProP and ProU for proline are 0.3 mM and 2 μM, respectively, ProU has a 1.3 μM K_m for betaine (Lucht and Bremer 1994). The high level of betaine in the medium (2.0mM) coupled with the low Km of the primary betaine transport pathway (ProU) explains the exclusive accumulation of this protective osmolyte. Additionally, the ProP system has a periplasmic, high-affinity betaine binding protein (K_D 1 μM) which aids in the accumulation of this preferred substrate.

Cells from the fermentations supplemented with 0.25 mM DMSP accumulated both proline and DMSP. There are three possible explanations for the contemporaneous accumulation of proline and DMSP under these conditions. It is possible that there is an independent transporter for DMSP. However, DMSP is structurally similar to betaine (Fig. 4-5), and it is likely transported by the same mechanism. Possibly, the accumulation of both proline and DMSP results from the K_m for proline and DMSP being more similar to each other. Alternatively, the low level of DMSP in the medium (0.25 mM) compared to the concentration of betaine used (2.0 mM) may have allowed for the proline (equal

concentrations in both experiements) to more effectively compete for transport by ProP and/or ProU.

Neither the betaine nor DMSP supplemented fermentations contained detectable levels of glutamate (>10 mM; Fig 4-2), confirming that the high glutamate pool was not needed for biosynthesis, per se. Thus, the requirement for additional glutamate is presumed to be associated with adaptation to the higher sugar environment. This is consistent with previous reports using strains of *E. coli* or *S. typhimurium* which were deficient in glutamate biosynthesis (McLaggan et al. 1994; Csonka et al. 1994; Yan et al. 1996). While the growth of these strains was poor in high osmotic environments, normal growth was observed in more optimal osmotic environments. Thus, the observed deficiency in glutamate biosynthesis in strain KO11 was attributed to the inability to accumulate large quantities of intracellular glutamate for osmoadaptation but not necessarily for macromolecular biosynthesis.

Betaine from Difco Yeast Extract Restores Growth in Luria Broth Fermentations.

Dulaney and coworkers (1968) demonstrated that Difco yeast extract , a component of Luria broth (10 g liter^{-1} tryptone, 5 g liter^{-1} yeast extract and 5 g liter^{-1}), contains betaine by extracting and fractionating Difco yeast extract. Xylose fermentations with these nutrients yielded high biocatalyst concentrations and high ethanol productivity (Table 4-1). Cells harvested from these fermentations at 12 h (when the sugar concentration would be similar to that of the CSL fermentations at 24h) accumulated proline and betaine were accumulated by strain KO11 (Fig 4-2). The ratio of proline to betaine was much higher in Luria broth than in CSL+X medium supplemented with 2.0 mM betaine, thus allowing proline to more effectively compete with betaine for transport

by the osmotically active tranporters, ProP and ProU. The lower growth yield in betaine-supplemented fermentations indicated that although betaine aided in restoring growth, it was not as effective as the rich Difco nutrients. Some other nutrient in the Luria broth may be necessary for even higher growth yields (>2 g liter^{-1}). The high availability of carbon skeletons, essential vitamins and minerals in the Luria broth would also have a sparing effect on all biosynthetic pathways. The higher growth yield and ethanol productivity observed in fermentations with these nutrients may have resulted from this general sparing effect.

Conclusions

Growth and ethanol production in the CSL+X medium was restricted by a deficit in the accumulation of protective osmolytes. Glutamate, typically accumulated in response to osmotic stress, failed to accumulate due to restricted citrate biosynthesis. Supplementing the medium with potassium glutamate (2 g liter^{-1}) bypassed this limitation and restored the intracellular glutamate pool. Alternately, the addition of sodium acetate (2 g liter^{-1}), an activator of citrate synthase and precursor of glutamate biosynthesis, restored the intracellular glutamate pool and increased growth and ethanol production. Together, these results suggested that a deficit in the production of glutamate restricted growth.

Betaine and DMSP increased the growth and ethanol production in a dose-dependent manner when added to the medium. NMR analysis of the intracellular osmolytes during these fermentations demonstrated the accumulation of these protective osmolytes. While the addition of DMSP to the CSL+X medium resulted in the accumulation of both proline and DMSP, cells from betaine supplemented cultures

accumulated betaine exclusively. Although growth and ethanol production were stimulated by betaine and DMSP, the glutamate pool was not restored. Thus, the accumulation of protective osmolytes (glutamate, betaine or DMSP) was required for adaptation to the high sugar environment rather than macromolecular synthesis.

Fermentations in rich media (Luria broth) achieve comparatively higher cell densities (3.5 g liter^{-1} cell dry weight) than those in the CSL+X medium supplemented with betaine (1.6 g liter^{-1} cell dry weight) or DMSP (1.8 g liter^{-1} cell dry weight). Betaine (from Difco yeast extract) and proline were accumulated in fermentations with Luria broth (90 g liter^{-1} xylose), consistent with a need for osmoprotectants. While supplementing the CSL+X medium with protective osmolytes (glutamate, betaine or DMSP) restored growth, the maximum biocatalyst concentration was still less than that of fermentations with Luria broth. Based on these results, a deficit in the accumulation of protective osmolytes appears to be the primary factor that restricts cell growth and ethanol production in the CSL+X medium.

Table 4-1. Intracellular accumulation of protective osmolytes by KO11.

Fermentaion conditions[a]	Fermentation Parameters		Intracellular Osmolytes[b]				
	Max. Cell conc.[c]	Avg Vol Productivity[d]	Proline	Glutamate	Betaine	DMSP	Total
No Additive	0.95 ± 0.13	0.33 ± 0.05	135 ± 6	<10[e]	<10	<10	128
+ Proline (17 mM)	0.98 ± 0.10	0.35 ± 0.05	158	<10	<10	<10	158
+ Glutamate (11 mM)	1.78 ± 0.20	0.56 ± 0.03	88	31	<10	<10	119
+ Acetate (24 mM)	1.77 ± 0.12	0.57 ± 0.05	82	28	<10	<10	111
KO11 (pLOI 2514)	1.72 ± 0.11	0.50 ± 0.04	91	34	<10	<10	125
SU102 (Δ*ackA*)	1.94 ± 0.12	0.54 ± 0.02	84	38	<10	<10	122
+ Betaine (2.0 mM)	1.58 ± 0.25	0.49 ± 0.06	<10	<10	107	<10	107
+ DMSP (0.25 mM)	1.84 ± 0.08	0.58 ± 0.03	41	<10	<10	54	95
+ Taurine (1.0 mM)	0.96; 0.94	0.33; 0.31	133	<10	<10	<10	133
LB Xylose strain KO11	3.53 ± 0.34	0.88 ± 0.02	67 ± 6	<10	81 ± 0.3	<10	147

[a] All fermentations were in the CSL+X medium unless otherwise indicated

[a]Concentrations (mM) were determined by ^{13}C NMR and assumed 1 mg DCW = 1 mL intracellular volume.

[b]Maximum cell mass (dry weight), g liter^{-1}. Standard deviations are given in experiments with more than 3 replicates. Otherwise, individual replicates are given.

[c]Average volumetric productivity (g ethanol liter^{-1} h^{-1}) for the first 72 hours for CSL+X fermentation, 48 h for LB fermentation.

[d] Limit of detection for intracellular osmolytes was > 10 mM.

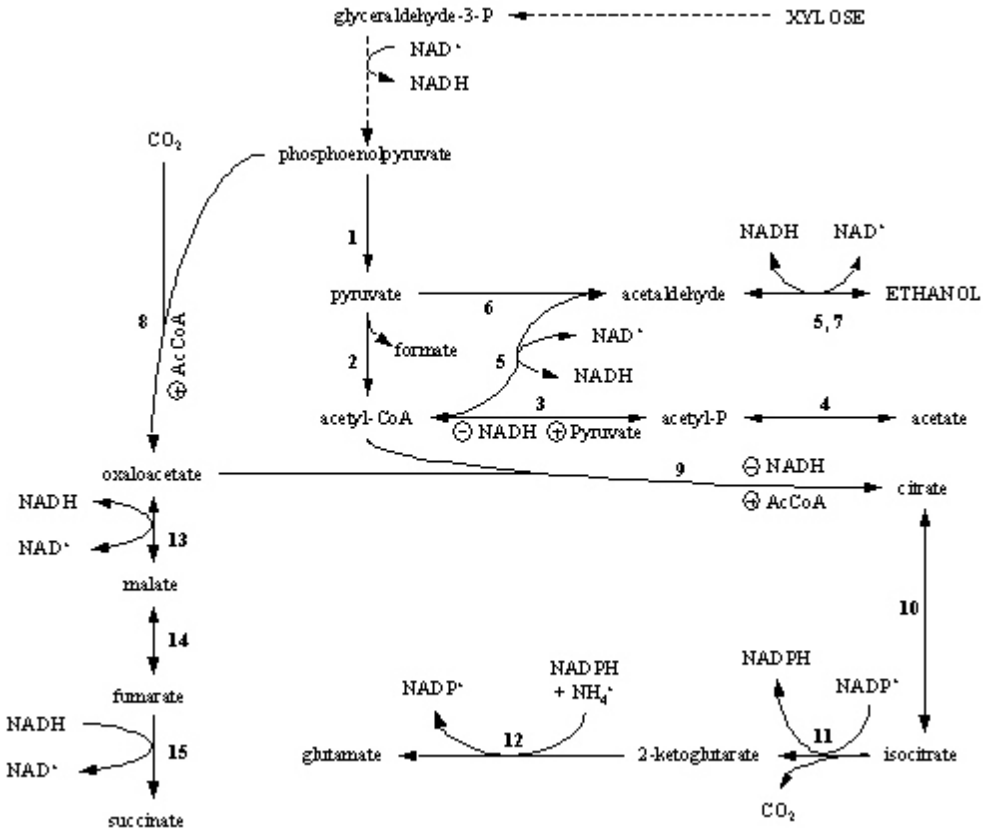

Figure 4-1. Carbon flow during fermentation of xylose by *E. coli* KO11. Unless noted otherwise, enzymes listed are native to *E. coli*. Enzymes: 1. pyruvate kinase (*pykA, pykF*); 2. pyruvate formate-lyase (*pflB*); 3. phosphotransacetylase (*pta*); 4. acetate kinase (*ackA*); 5. alcohol/aldehyde dehydrogenase (*adhE*); 6. *Z. mobilis* pyruvate decarboxylase (*pdc*); 7. *Z. mobilis* alcohol dehydrogenase II (*adhB*); 8. phosphoenolpyruvate carboxylase (*ppc*); 9. citrate synthase (*gltA*); 10. aconitase (*acn*); 11. isocitrate dehydrogenase (*icd*); 12. glutamate dehydrogenase (*gdhA*); 13. malate dehydrogenase (*mdh*); 14. fumarase (*fumB*); 15. fumarate reductase (*frdABC*); ⊕ indicates allosteric activation, ⊖ indicates allosteric inhibition.

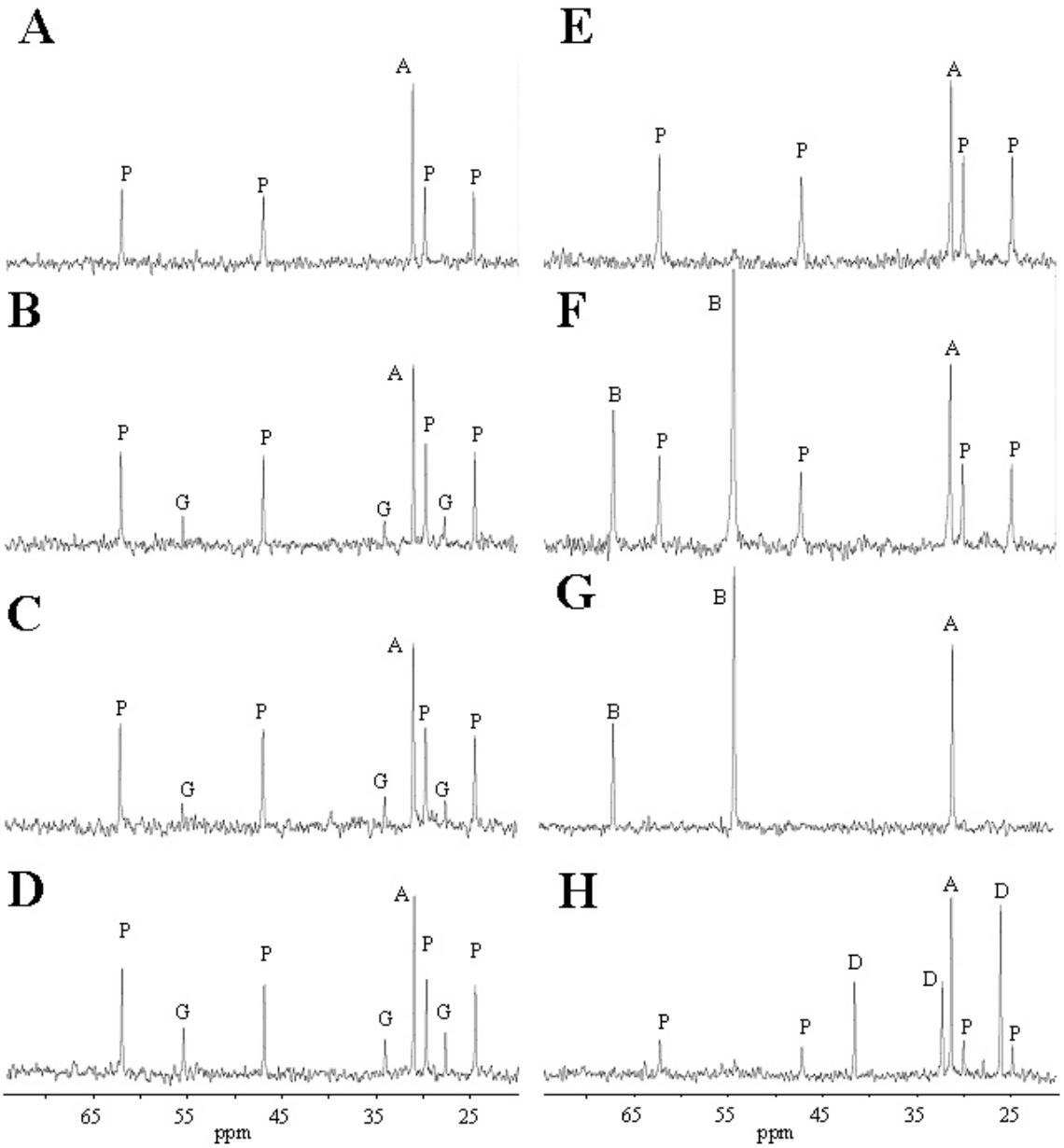

Figure 4-2. Major intracellular osmolytes accumulated by ethanologenic *E. coli* during fermentation of 90 g liter^{-1} xylose to ethanol. ^{13}C NMR spectra (1000 scans) were obtained as described in the materials and methods. (A) CSL+X medium with out additives, strain KO11. (B) CSL+X medium supplemented with 11 mM (2 g liter^{-1}) potassium glutamate, strain KO11. (C) CSL+X medium supplemented with 24 mM (2 g liter^{-1}) sodium acetate, strain KO11. (D) CSL+X medium without additives, strain SU102 (Δ*ackA*). (E) CSL+X medium with 17 mM (2 g liter^{-1}) proline (F) Luria broth, strain KO11. (G) CSL+X medium supplemented with 2 mM betaine, strain KO11. (H) CSL+X medium supplemented with 0.25 mM DMSP, strain KO11. Signals are labeled as follows: acetone, A; proline, P; glutamate, G; betaine, B; DMSP, D.

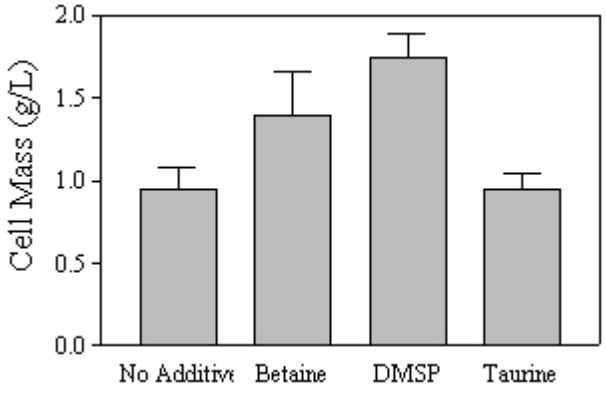

Figure 4-3. Effect of osmoprotectants (1.0 mM) on maximum cell concentration. Fermentations were supplemented with the indicated supplements. Small bars indicate the standard deviation.

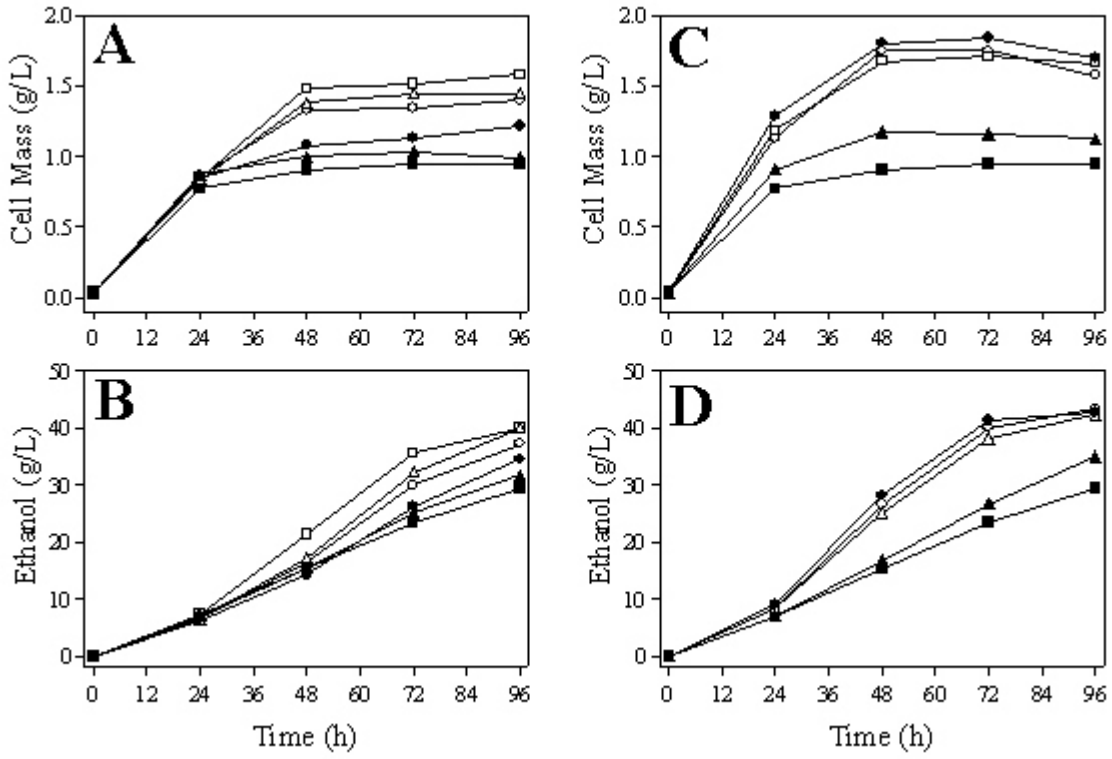

Figure 4-4. Effects of Betaine and DMSP on growth and ethanol production. Dose-dependent increase in biocatalyst and ethanol with the addition of betaine (A and B) or DMSP (C and D): ■, no addition; ▲, 0.1 mM; ●, 0.25 mM; Δ, 0.5 mM; ❍, 1.0 mM; and ❒, 2.0 mM.

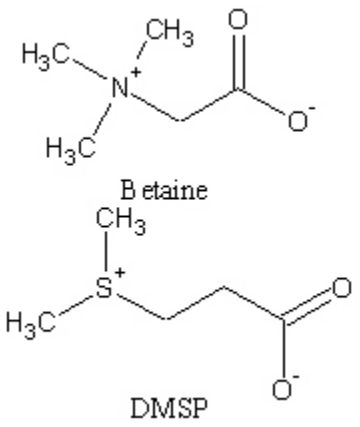

Figure 4-5. The chemical structure of betaine and DMSP. Both osmoprotectants are zwitterionic and proposed to be transported by either the ProP or ProU transport systems due to structural similarity of the compounds.

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

This study investigated the basis for the high nutrient requirement of ethanologenic *E. coli* strain KO11 during fermentation of xylose to ethanol, with the ultimate goal of minimizing the requirement for nutritional supplements. As a starting point, the fermentation of 9% xylose to ethanol in a 1% CSL medium was evaluated. Despite the absence of any specific auxotrophic requirement, the growth of strain KO11 was limited by the availability of complex nutrients. Cell growth was limited due to an imbalance in carbon partitioning between biosynthesis (glutamate) and product formation (ethanol), likely resulting from the metabolic engineering of the strain. The high expression of the low K_m *pdc* from *Z. mobilis* out-competed PFL for pyruvate, restricting citrate synthase activity resulting in a glutamate deficiency. Several approaches were evaluated that significantly increased the growth and ethanol production by strain KO11 in this medium. Ultimately, genetic and physiological approaches decreased the nutrient requirement 5-fold.

Increased Acetyl-CoA Availability Stimulated Growth.

Aerobic growth experiments were the first indication that an imbalance in carbon partitioning restricted growth (Chapter 2). Under these conditions, the oxidative conversion of pyruvate to acetyl-CoA (PDH) is active due to decreased feedback inhibition by NADH (de Graef et al. 1999; Hansen and Henning 1966) which are known to be higher during fermentative or anaerobic growth (de Graef et al. 1999; Snoep et al. 1990). The PDH K_m for pyruvate (0.52 mM; Nemeria et al. 2001) is more similar to that

of the to the recombinant PDC (Km 0.4 mM; Raj et al. 2002) than the fermentative PFL (2.0 mM; Knappe and Sawers 1990). Thus, PDH is more competitive for essential carbon skeletons than the fermentative pathway (PFL). Supplementing the CSL+X medium with acetate reduced its production to less than half that of fermentations without additives (Chapter 3), increasing the availability of acetyl-CoA for biosynthetic pathways. Added pyruvate was consumed within the first three hours, resulting in increased acetate production. Increased PDH activity accounted for the additional acetyl-CoA biosynthesis required for acetate production. This also resulted in a transient increase in the availability of acetyl-CoA during the first 3 h of incubation. However, the beneficial effects on growth were not realized until after more than 9 h of incubation. The final acetate concentrations in these fermentations were similar to the acetate supplemented cultures. Thus, the long-term benefit resulting from pyruvate supplementation was due to the accumulation of acetate in the medium, increasing the availability of acetyl-CoA.

Acetaldehyde supplemented experiments were shown to have decreased relative intracellular NADH concentration (decreased $NADH/NAD^+$ ratio), increasing growth yield and ethanol productivity (Chapter 2). Though the initial 0.25 g $liter^{-1}$ were consumed in the first three hours of incubation (Chapter 3), the effects of this addition were not realized, in terms of increased growth, until after 12 hours. The second addition of acetaldehyde at this time increased PDH flux and produced moderately more acetate. This is indicative of increased acetyl-CoA production during a critical time of growth where fermentations without additives ceased to grow.

Some TCA Intermediates Increase Growth.

Addition of intermediates from the succinate production pathway (oxaloacetate, malate or fumarate) did not increase growth yields or ethanol productivity. Fermentations supplemented with succinate did not noticeably affect the performance of strain KO11 in this medium. Supplementing fermentations with malate or fumarate resulted in elevated fumartate levels compared to those without additives (Chapter 2). As the production of succinate by this pathway is blocked in strain KO11 (Δ*frdABCD*), the elevated levels of fumarate were expected. However, fermentations with added oxaloacetate had lower biocatalyst yields and decreased ethanol productivity. While these cultures consumed all of the added oxaloacetate within the first 24 h, the carbon could not be accounted for in the fermentation broth as succinate or fumarate (Table 2-1). This might be explained as the induction of a futile cycle for ATP generated by the contemporaneous expression of phosphoenolpyruvate carboxylase (*ppc*) and the gluconeogenic phosphoenolpyruvate carboxykinase (*pck*). The creation of this futile cycle has been previously shown to restrict growth (Chao et al. 1993; Chao and Liao 1994; Gokarn et al. 2000).

While citrate and isocitrate did not increase growth of strain KO11, their derivatives 2-ketoglutarate and glutamate were beneficial (Chapter 2). The addition of citrate resulted in higher levels of fumarate and acetate, while formate production was lower than fermentations without supplements. This is consistent with citrate lyase induction (Lutgens and Gottschalk 1980), which cleaves citrate into acetate (without formate production) and oxaloacetate (leading to fumarate). Most of the isocitrate added to the medium remained at 24 hours. However, 2-ketoglutarate and its amino acid derivative, glutamate, stimulated growth and ethanol production.

Citrate Synthase–A Unifying Hypothesis.

Based on these results, citrate synthase activity was proposed to limit the growth of strain KO11 in the CSL+X medium. Supplementing the medium with glutamate or 2-ketoglutarate bypassed the need for additional citrate biosynthesis. Acetyl-CoA, increased with the addition of acetate, pyruvate or acetaldehyde, is both an activator and co-substrate for *E. coli* citrate synthase (Weitzman 1969). The biosynthetic pathway for the other co-substrate, oxaloacetate, is also activated by acetyl-CoA (Izui et al. 1981), linking the availability of the two substrates. The decreased $^{NADH/NAD^+}$ ratio in the cell resulting from supplementing the medium with pyruvate, acetaldehyde or 2-ketoglutarate, decreased the NADH-mediated inhibition of citrate synthase. Thus, all of the beneficial supplements can be linked by a common activity, citrate biosynthesis.

The biosynthesis of citrate is regulated by the allosteric controls of this enzyme. By varying the levels of citrate synthase expression in *E. coli* from a plasmid, Walsh and Koshland, Jr. (1985) demonstrated that the over-expression of citrate synthase did not increase flux through this pathway. The carbon flow through this pathway is linked to the regeneration of NAD^+. As the electron transport chain becomes more reduced, NADH accumulates in the cell (de Graef et al. 1999). Under these conditions, the further production of NADH is not favored. This is indicated by the inhibition of the oxidative cleavage of pyruvate to acetyl-CoA (PDH) which produces NADH (de Graef et al. 1999; Snoep et al. 1990). Furthermore, the oxidation of acetyl-CoA by the TCA cycle (producing NADH) is decreased by the NADH-mediated inhibition of citrate synthase (Weitzman 1966), the first committed step of the TCA cycle.

In strain KO11, the normal patterns of carbon flow were altered by the expression of the *Z. mobilis pdc* which competes for pyruvate. During fermentation, acetyl-CoA is produced by PFL which has a higher K_m for pyruvate (2.0 mM; Knappe and Sawers 1990) than the recombinant PDC (0.4 mM; Raj et al. 2002). Thus, most of the pyruvate produced from glycolysis is directed toward ethanol, resulting in high ethanol yields. However, this decreased the production of acetyl-CoA, an essential substrate and allosteric regulator of many biosynthetic pathways including citrate synthase (Weitzman 1966). Thus, carbon partitioning between biosynthesis and cofactor regeneration was disrupted, resulting in decreased biocatalyst yield in the nutrient-poor medium.

Two genetic solutions to increase citrate biosynthesis were tested. The functional expression of *citZ* from *B. subtilis* increased growth and ethanol production in strain KO11 (Chapter 2). This could be due to two factors, neither of which are mutually exclusive. Typical of the citrate synthases from Gram-positive or eukaryotic organisms, this enzyme is insensitive to NADH (Jin and Sonenshein 1996). Additionally, this enzyme has a significantly lower K_m for acetyl-CoA than the native citrate synthase. In light of the evidence presented in these studies, either of these factors may have contributed to the increased growth of strain KO11. Expressing citrate synthases with a range K_m values between that of the *E. coli* and *B. subtilis* may provide further evidence as to the exact cause of the increased growth during the expression of the heterologous citrate synthase.

Eliminating the non-biosynthetic drain of the acetyl-CoA through the primary acetate production pathway (Δ*ackA*) also increased growth and ethanol production (Chapter 3). Though the ATP resulting from the acetate pathway may have caused the

slight growth lag initially observed with this strain, the maximum growth rate was not affected. The specific productivity was also unchanged by this mutation. Thus, the elimination of competing pathways for acetyl-CoA increased the availability of acetyl-CoA, partitioning more carbon into biosynthesis. Similar effects have been achieved in other *E. coli* strains engineered for the production of optically-pure lactic acid (Zhou et al. 2002; Zhou et al. 2003).

A Deficit in Protective Osmolytes Limited Growth

Intracellular osmolyte pools are regulated by cells for adaptation to the osmotic strength of the medium (Csonka 1989; Csonka and Hanson 1991) . During growth in minimal media of high osmotic strength, *E. coli* and other related organisms will accumulate large quantities of K^+, glutamate and trehalose (Cayley et al. 1991; Lewis et al. 1990). The biosynthesis of glutamate requires 2-ketoglutarate, a product of citrate. However, citrate biosynthesis has been demonstrated to limit growth under the conditions of this study (Chapter 2; Chapter 3). In the CSL+X medium (0.6M xylose), strain KO11 accumulated only the protective osmolyte proline. When this medium was supplemented with additives that increased growth and ethanol production, glutamate accumulated. Thus, the accumulation of the protective osmolyte glutamate was necessary for extended growth.

Trehalose likely failed to accumulate to detectable levels (<10 mM) because of the carbon source used for growth. Glucose-6-phosphate and uridine diphosphate glucose, both derived from glucose, are substrates for trehalose biosynthesis. During growth on xylose, fructose-6-phosphate and glyceraldehyde-3-phosphate enter glycolysis at their respective steps of glycolysis. Trehalose biosynthesis would require 2 molecules of

fructose-6-phosphate be converted to the substrates for trehalose biosynthesis. This would result in an expenditure of ATP. However, the ATP yield during growth on xylose (0.4 ATP per pyruvate produced) is low compared to that of glucose (1 ATP per pyruvate). Additionally, ATP can only be made by substrate-level phosphorylation during fermentation. Thus, the energetic demands for the biosynthesis would have further restricted growth.

The accumulation of proline from the medium should not have affected the steady-state glutamate pool (Cayley et al. 1992). The accumulation of intracellular glutamate in fermentations with higher growth yields (supplements or genetic manipulation) indicated that additional protective osmolytes were necessary for continued growth under these conditions. Though proline can be degraded to glutamate, this pathway was inhibited by high osmotic environment. Thus, even an excess of proline (added as a supplement) did not result in increased growth or accumulation of intracellular glutamate.

Supplementing the medium with betaine or DMSP increased growth and ethanol production. Analysis of the intracellular osmolytes accumulated by cells from these fermentations did not detect increased glutamate pools. Thus, the rate of glutamate biosynthesis is sufficient for growth of strain KO11 in the CSL+X medium, but not for adapting to the high osmolarity of the growth medium. This is consistent with previous reports with strains restricted in their ability to make glutamate. By deleting the glutamate pathway with a high affinity for ammonium (glutamine synthase-glutamate synthase) and growing these strains in media with low ammonium and high osmolarity, cells continued to grow at a lower rate (Csonka et al. 1994; Yan et al. 1996), indicating that the

accumulation of glutamate for adaptation to the osmotic environment (and not for biosynthesis) limited growth.

While DMSP supplemented fermentations (0.25 mM) accumulated both proline and DMSP, cells from the betaine supplemented fermentations (2.0 mM) contained only betaine (Chapter 4). This supports previous observations that betaine is the preferred substrate for the osmotically activated transport mechanisms ProP and ProU (Randall et al 1995). However, significantly less DMSP (0.25 mM) than betaine (2.0 mM) was required to restore the growth of strain KO11, in contrast to previous reports establishing betaine as the most effective osmoprotectant (Randall et al. 1995). It is important to note that studies of osmoprotectants commonly use NaCl for increasing the osmolarity of the medium, while the experiments presented here used xylose as both a growth substrate and most predominant osmolyte in the medium. There has been at least one report of betaine not being as effective in restoring growth when different sugars were the challenging osmolyte and growth substrate (Dulaney et al. 1968). Thus, during fermentation in the CSL+X medium, DMSP seems to be the more effective protective osmolyte.

Difco yeast extract has been reported to have betaine (Dulaney et al. 1968), despite the absence of a biosynthetic pathway for betaine in yeast. Fermentations with Difco nutrients had higher growth yields (>3 g liter^{-1}) and ethanol productivity than that of fermentations in CSL+X medium. In fermentations with Difco nutrients, both proline and betaine were accumulated by the cells. This suggests that the ratio of betaine to proline in the medium was much lower, thus allowing proline to more effectively compete with betaine for the transport mechanisms. While supplementing the CSL+X medium with betaine or DMSP restored growth (~2 g liter^{-1}), cell yields were still

somewhat lower than those during fermentations with Difco nutrients. Further studies are necessary to determine the basis for this apparent requirement for additional nutrients.

Beet molasses and cane molasses are natural sources of betaine and DMSP, respectively. While these byproducts of the sugar processing industry are more expensive than CSL, they are inexpensive sources of protective osmolytes. Their potential to increase ethanol productivity and replace the high levels of complex nutrients required by strain KO11 should be investigated.

Future Prospects for Metabolic Engineering.

With recent advances in DNA technology, it is now possible to engineer microorganisms to produce a wide variety of products by fermentation. Researchers have examined the possibility of using a variety of host strains for the production of ethanol (Ingram et al. 1999; Gong et al,1999), succinate (Donnelly et al. 1998a; Donnelly et al. 1998b; Vemuri et al. 2002), optically-pure lactic acid (Zhou et al. 2002; Zhou et al. 2003; Dien et al. 2001; Kyla-Nikkila et al. 2000; Bianchi et al. 2001; Chang et al. 1999), adipic acid (Niu et al. 2002), 1,3-propandiol (Nakamura et al. 2000; Tong et al. 1991; Diaz-Torres et al. 2000) and many other compounds (Chotani et al. 2000). The success of these efforts is reflected by the recent commercialization of lactic acid production for poly-lactide synthesis, a biodegradable thermoplastic, by Cargill Dow LLC. DuPont is examining the feasibility of using genetically engineered *E. coli* for the production of 1,3-propandiol, a copolymer in their latest polyester material, Sorona. Archer Daniels Midland Company is currently using genetically engineered yeast for the production of ethanol from corn fiber hydrolysate. Several other companies are trying to commercialize the production of ethanol from biomass using a variety of biocatalysts, including *E. coli*

KO11. The economic viability of these and other fermentation processes for value-added compounds is reliant upon the development of the most efficient biocatalyst for each specific process. The studies presented here have provided insight into the physiological effects of metabolic engineering and offered physical and genetic solutions to alleviate the deleterious effects of altering carbon flow on growth and productivity.

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BIOGRAPHICAL SKETCH

Stuart was born in Clanton, Alabama, in 1975. After a short stay in Texas, in 1979 his family moved to the Pensacola, Florida, area where he attended Gulf Breeze Elementary and Middle School. After moving to Destin, Florida, he attended Fort Walton Beach High School for two years. Seeking a better education, Stuart enrolled in Deerfield Academy in Deerfield, Massachusetts. In addition to several Advanced Placement courses, he was on the varsity water polo, swimming and golf teams. Following graduation, he began his pursuit of a Bachelor of Science degree in microbiology and cell science from the University of Florida in the fall of 1994. Following graduation in the fall of 1997, he worked for a small start-up biotechnology company. After the company collapsed, he moved to Research Triangle Park, North Carolina, where he worked with Novartis Biotechnology and then Duke University. Seeking further education and a return to microbiology research, he came back to the University of Florida for his doctoral degree in the spring of 1999. Under the guidance of Dr. Lonnie O. Ingram, Stuart studied the metabolism and physiology of the ethanol producing *E. coli* strain KO11. While attending graduate school, he met and married his wife Beverly, a Ph. D. candidate in plant molecular biology. Together, they are looking forward to building a family and long careers in scientific pursuits.