TRANSCRIPTIONAL ANALYSIS AND ADAPTIVE EVOLUTION OF *ESCHERICHIA COLI* GROWING ON ACETATE

by

EASHWAR RAJARAMAN

(Under the direction of Mark A. Eiteman)

ABSTRACT

Acetate is an unavoidable degradation product in biomass hydrolysis which inhibits microbial production of biochemicals. Acetate can be selectively removed from mixture of sugars and acetate using substrate-selective degradation. This research focuses on improving the growth of *E. coli* on acetate which in turn will help improve the substrate-selective degradation. In a first study, eighteen strains of *E. coli* were compared for maximum specific growth rate (μ_{MAX}) on acetate. We observed that ATCC8739 had the greatest growth rate growth rate (0.405 h^{-1}) while SCS-1 had the slowest growth rate (0.153 h^{-1}). Gene expressions of ATCC8739, SMS-3-5 and BL21 were compared determine why ATCC8739 grows fastest on acetate. From the results, we observed that *folK* of the folate biosynthesis pathway had atleast 2-fold greater expression in ATCC8739 in comparison with the other strains. Also, genes of phenylacetate degradation pathway were more than 2-fold upregulated in ATCC8739 than SMS-3-5 and BL21. These results might explain why ATCC8739 grows faster than other strains on acetate.

An A-stat process was carried out to evolve ATCC8739 for a faster growth on acetate. Three colonies isolated from the A-stat at 0.50 h^{-1} had a greater maximum specific growth rate than the parent strain ATCC8739. Specifically, the MEC136 isolate had a maximum specific growth rate

of 0.51 h^{-1} on acetate. Transcriptome comparison between MEC136 and the parent strain revealed that 10 genes were atleast 2-fold downregulated in MEC136 and 1 gene was 2-fold upregulated in MEC136. Therefore, the MEC136 can be used to improve the substrate selective degradation method.

INDEX WORDS: Acetate, *Escherichia coli*, metabolic engineering, growth rate, ATCC8739, microarray analysis, adaptive evolution, A-stat

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DEDICATION

To my family and friends

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Eashwar Rajaraman

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction and Literature Review

Biofuels are being seriously contemplated as an alternative for petroleum-based liquid fuels. Diminishing reserves of fossil fuels, unsustainable net rates of greenhouse gas emissions and technological advancements in industrial microbiology and bioengineering have revived interest in the development of transportation fuels from renewable resources (Stephanopoulos, 2007; Bartley and Ronald, 2009; Liu and Khosla, 2010). For example, the U.S. DOE Energy Independence and Security Act (EISA) mandated attainment of a national production level of 36 billion gallons of biofuels (to be added to gasoline) by 2022, of which 21 billion gallons must be derived from renewable/sustainable feedstocks (Xu et al., 2009).

Lignocellulosic biomass

Crops such as corn, sugar cane and soybeans are historical sources of renewable feedstocks for biofuels. In order to limit direct food and fuel competition, lignocellulosic biomass is being considered as another source of renewable feedstock for biofuels (Balat and Balat, 2009; Nigam and Singh, 2011). Lignocellulose biomass is comprised of vegetative tissue and plant derived waste products (Waltz, 2008). In addition to being a renewable source of biofuel, lignocellulosic biomass is by far the most abundant raw material obtainable from hardwood, softwood, grasses, and agricultural residues (Lee, 1997). Also, lignocellulosic plant residues containing up to 70% carbohydrates are prominent substrates for cheap ethanol production (Klinke et al., 2004).

Composition of lignocellulosic biomass

Lignocellulose is a complex substrate composed of a mixture of cellulose, hemicellulose and lignin (Lee, 1997). Cellulose and hemicellulose are both carbohydrate polymers. Cellulose comprises 40 – 50 % and hemicellulose comprises 25 – 35 % of the dry wood weight. Cellulose is a glucan polymer consisting of linear chains of β-(1→4)-D-glucopyranose units. Hemicellulose is a mixture of polysaccharides synthesized from glucose, mannose, galactose, xylose, arabinose, 4-O-methylglucuronic acid and galacturonic acid residues (Pettersen, 1984; Mohan et al., 2006). Lignin is a complex, variable, hydrophobic, cross-linked polymer mainly consisting of aromatic alcohols such as coniferyl, sinapyl and p-coumaryl alcohol (Lee, 1997; Klinke et al., 2004; Mohan et al., 2006).

Microorganisms for biofuels production

Selection and development of robust, high yielding microbes is a principal requirement for the microbial production of biofuels. Selection of a host depends on several factors, primarily nature of the raw material and the target fuel chemistry. A considerable amount of research has focused on exploring the feedstock conversion and biofuels production capacity of several species like *Escherichia coli, Saccahromyces cerevisiae, Zymomonas mobilis, Bacillus subtilis, Pichia stipitis, Clostridia* and *Trichoderma reesei* (Dien et al., 2003; Fischer et al., 2008; Alper and Stephanopoulos, 2009; Liu and Khosla, 2010). *Escherichia coli* and *Saccahromyces cerevisiae* are the two preferred microorganisms for biofuels production as they have well-established genetic tools and a long track record of successful industrial applications (Alper and Stephanopoulos, 2009). *S. cerevisiae* lacks the native ability to utilize pentose sugars, the major component of hemicellulose fraction of lignocellulose (Jarboe et al., 2007). In addition to possessing the ability to ferment both hexose and pentose sugars, ethanologenic *E. coli* also has

higher tolerance to lignocellulosic inhibitors than its fermentative counterparts (Zaldivar and Ingram, 1999; Zaldivar et al., 1999, 2000; Jarboe et al., 2007). Jarboe et al. (2007) compared ethanol production between these three microorganisms, determining that *E. coli* is comparable with or surpasses other reported production levels, despite its low membrane tolerance to ethanol. These qualities along with advanced knowledge about the *E. coli* genome and regulation make this bacterium a prime candidate for further development.

Biological conversion of lignocellulose to ethanol

The biological process of converting lignocellulose to fuel ethanol consists of (1) pretreatment with steam, acid, alkali or ammonia to reduce biomass recalcitrance and render cellulose accessible to hydrolytic enzymes, (2) hydrolysis with acids or enzymes to depolymerize cellulose and hemicellulose into monomeric sugars, (3) fermentation of both hexose and pentose sugars to produce ethanol and (4) distillation and product separation to purify ethanol and meet fuel specifications (Himmel et al., 2007; Hendriks and Zeeman, 2009; Margeot et al., 2009; Liu and Khosla, 2010).

Pretreatment methods often referred to as prehydrolysis methods (Olsson and HahnHagerdal, 1996) are required to remove lignin and hemicellulose, reduce cellulose crystallinity and to increase the porosity of the materials (Sun and Cheng, 2002). Several physical, chemical and physico-chemical (combined physical and chemical) methods are used for the pretreatment of lignocellulosic materials (Margeot et al., 2009). Physical methods include milling, steam pretreatment/steam explosion and liquid hot water treatments; chemical methods include acid and alkaline treatments, oxidative pretreatment with the help of an oxidizing agent like hydrogen peroxide; physico-chemical methods consists of combination of thermal pretreatments with acid or alkaline pretreatments (Klinke et al., 2004; Kumar et al., 2008;

Hendriks and Zeeman, 2009). Once cellulose and hemicellulose are released, they are depolymerized into monomeric sugars by hydrolysis. Various methods for the hydrolysis of lignocellulosic materials for ethanol production have been reviewed (Olsson and HahnHagerdal, 1996). Dilute acid hydrolysis and enzymatic hydrolysis are two common examples of hydrolysis processes (Palmqvist and Hahn-Hagerdal, 2000b).

Inhibitors and their effects

During the commonly applied pretreatment and hydrolysis steps, several inhibitory by-products are generated along with the sugar units. These byproducts inhibit cell growth and interfere with the subsequent microbial fermentation (Ma and Liu, 2010). These inhibitory compounds can be classified into three major groups: furaldehydes, weak acids and phenolic compounds (Parawira and Tekere, 2011). Commonly found furaldehydes in the hydrolysate include 2-furaldehyde (furfural) and 5-hydroxymethyl 2-furaldehyde (HMF). Furfural and HMF are formed from dehydration of pentoses and hexoses released from hemicellulose and cellulose respectively (Saeman, 1945; Larsson et al., 1999b; Palmqvist and Hahn-Hagerdal, 2000b). Inhibitory aliphatic acids include formic acid, levulinic acid and acetic acid. Formic acid and levulinic acid are formed by further degradation of HMF (Larsson et al., 1999a). Formic acid is also formed from furfural under acidic conditions at elevated temperatures (Dunlop, 1948). Acetic acid is derived from the hydrolysis of acetylxylan, a component of hemicellulose. Phenolic compounds like 4-hydroxybenzoic acid, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde, and syringic acid are formed from the partial breakdown of lignin (Mills et al., 2009; Parawira and Tekere, 2011).

Furfural and HMF inhibit activities of certain glycolytic and non-glycolytic enzymes, break down DNA and reduce protein and RNA synthesis (Modig et al., 2002; Liu et al., 2004).

Organic acids like formic acid, levulinic acid and acetic acid inhibit yeast fermentation by reducing biomass and ethanol yields. High concentrations can partially deactivate enzymes and may even result in cell death (Taherzadeh et al., 1997; Larsson et al., 1999a; Almeida et al., 2007; Parawira and Tekere, 2011). Also, acetic acid (0 – 10 g/L) and furfural (0 – 3 g/L) are known to synergistically impact the ethanol yield (Palmqvist et al., 1999). Furans and phenolic compounds generally inhibit growth rate, biomass yield and ethanol production rate (Q_{EtOH}) more than ethanol yield (Y_{EtOH}) in *S. cerevisiae* and *Z. mobilis* (Klinke et al., 2004). The aldehydes furfural, syringaldehyde and vanillin are more potent inhibitors than their respective acids. In general, aldehydes and ketones are stronger inhibitors than acids, which, in turn are more toxic than alcohols for both *S. cerevisiae* and *E. coli* (Zaldivar and Ingram, 1999; Zaldivar et al., 1999; Larsson et al., 2000; Zaldivar et al., 2000; Klinke et al., 2003). Furfural, HMF and acetic acid are the degradation products that are often present at detectable concentrations in the hydrolysate and are often used as a measure of the inhibiting effect of the pretreatment liquid (Parawira and Tekere, 2011).

Methods to overcome the problem of inhibition

Different methods have been proposed to deal with the problem of the microbial inhibitors. There are four different approaches to overcome the adverse effects of the inhibitors present in hemicellulosic hydrolyzates: 1) avoiding the formation of the inhibitors during hydrolysis, 2) detoxification of the hydrolyzates before fermentation, 3) development of inhibitor-tolerant strains, and 4) in situ detoxification based on bioconversion of the toxic compounds (Taherzadeh et al., 2000).

Inhibitors like acetic acid, furfural and 5-hydroxymethylfurfural are formed as a result of undesirable side reactions during acid hydrolysis (Saeman, 1945). Since the desired

monosaccharides are formed as intermediate products in a series of sequential reactions it is unlikely that by-products can be completely avoided when using acid hydrolysis. Enzyme hydrolysis also requires a chemical pretreatment to make the wood structure accessible to the enzymes, which generates some inhibitors. Therefore, irrespective of the type of the hydrolysis method used, formation of inhibitors prior to fermentation cannot be avoided (Taherzadeh et al., 2000).

Biological, physical or chemical treatments such as treating with microorganisms or enzymes, vacuum evaporation, lime addition and ion exchange can be employed to detoxify the hydrolysate prior to fermentation (Larsson et al., 1999b; Palmqvist and Hahn-Hagerdal, 2000a; Mussatto and Roberto, 2004). But the detoxification step is seldom complete. Also the extra process step adds to the cost of the process (Taherzadeh et al., 2000).

Engineering microbial tolerance to hydrolysate byproducts is an attractive method to overcome the problem of inhibition which has yielded mixed results. Roe et al. (2002) reported that mutation in *metK* gene lead to intracellular accumulation of methionine which in turn reduced the inhibitory effects of acetate. However, overexpression of *metE* and *glyA* genes involved in methionine biosynthesis did not help the cells overcome acetate inhibition.

In situ detoxification is the conversion of one or several inhibitors by biotransformation reactions. Taherzedah et al. (1999a and 2000) have carried out in situ detoxification to ferment severely inhibiting hydrolyzates using a fed-batch technique. The choice of feed rate was critical to maintain fermentation throughout the entire fed-batch operation, and it was found to be dependent on the composition of the hydrolysate (Taherzadeh et al., 1999, 2000). In another example, Lopez et al. (2004) reported that *Coniochaeta ligniaraia*, which was found by selection

of various microorganism sampled from soil in media containing furfural and HMF, was able to degrade both fufural and HMF (Lopez et al., 2004).

Inhibitory effects of acetic acid

As one of the most abundant organic acids generated through pretreatments, acetate results from the hydrolysis of acetylxylan in hemicellulose (Sakai et al., 2007b). According to Klinke et al. (2002), acetic acid and formic acid are the major degradation products formed from the alkaline wet oxidation pretreatment of wheat straw. Together they accounted for 66-80% (w/w) of the quantified degradation products. Also, concentration of acetic acid is greater than that of formic acid if wet oxidation reaction time is prolonged (Klinke et al., 2002).

Acetic acid is the most studied organic acid inhibitor of *E. coli*. It inhibits cell mass and cell growth (Mills et al., 2009). The presence of acetate in media containing glucose or xylose prolongs the lag phase (Takahashi et al., 1999). Acetate concentrations as low as 8 mM (< 0.5 g/L) inhibit growth rate of *E. coli* by 50 % in minimal media (Roe et al., 1998; Roe et al., 2002). Takahashi et al. (1999) reported that 2.0 – 12.0 g/L acetic acid reduced the growth of *E.coli* KO11 cultured in Luria broth medium containing glucose or xylose. The range of acetate concentrations affected biomass yields, while increasing acetate concentration caused a reduction of growth rate. Glucose consumption was affected at acetate concentrations greater than 10.0 g/L (Takahashi et al., 1999). Acetate affects the fermentation rate of baker's yeast at a pH of 3.5. Increasing acetate concentrations at pH 3.5 decreased the rate of fermentation quantified by the amount of CO_2 formed in 20 minutes (Maesen and Lako, 1952).

Mechanism of acetic acid inhibition

There are a couple of theories for the mechanism of acetic acid inhibition. The first theory is based on chemical interference of acetic acid with the membrane phosphate transport. Acetic acid inhibits the glucose to ethanol fermentation by chemical interference with the cell maintenance functions (Maiorella et al., 1983). Samson et al. (1955) have shown that 0.2 M acetate inhibits phosphate uptake by yeast cells completely. They hypothesized that acetate interferes with the formation of a phosphate-carrier complex responsible for transporting phosphate across the cell membrane. Phosphate transfer through the membrane is an activated transport process and requires the expenditure of ATP. Acetic acid interference results in the increase in the ATP requirement for this maintenance function, which reduces cell yield. Also, the membrane disruption alters the cell morphology with cells becoming irregular and elongated (Samson et al., 1955).

The second theory of acetic acid inhibition is related to the acidification of the cytoplasm. Acetic acid inhibition is pH dependent (Takahashi et al., 1999). At lower pH, acetic acid is present in its undissociated form and freely permeates the membrane. Since the intracellular pH is much higher than the weak acid's pKa (Roe et al., 1998), acetic acid dissociates inside the cell and causes the acidification of the cytoplasm. As a result, the proton gradient across the membrane cannot be maintained and the production of energy and the transport systems dependent on this gradient are uncoupled. In addition to the decrease in the fermentation rate and biomass yield, acidification of cytoplasm also prolongs lag phase and enzyme inhibition (Takahashi et al., 1999; Mills et al., 2009).

Acetate removal by substrate-selective degradation

Substrate selective degradation is a biological method to overcome the problem of acetate inhibition. This method involves acetate removal selectively from a mixture of sugars and acetate (Lakshmanaswamy et al., 2011). It is based on engineering a strain of *E. coli* which will consume *only* acetate in the presence of multiple sugars. To prevent glucose and xylose consumption, genes involved in glucose and xylose uptake were knocked out. The strain MG1655 *ptsG manZ glk xylA* consumed 10 g/L acetate during 30 h of aerobic batch fermentation in a medium containing 20 g/L glucose and 10 g/L xylose. Although acetate was the preferred substrate and less than 1 g/L sugar had been consumed when acetate was exhausted, 7 g/L glucose and 3 g/L xylose were slowly consumed over the subsequent 40 h. Similar results were obtained with a strain containing an additional *crr* knockout and kanamycin resistance. A batch process could be implemented to remove acetate selectively from sugar mixtures without significantly reducing the concentration of glucose or xylose.

Acetate assimilation and metabolism

Acetate freely permeates the membrane in its undissociated form (Kihara and Macnab, 1981; Repaske and Adler, 1981; Salmond et al., 1984; Booth, 1985), and its assimilation does not require a dedicated transport system. However, under certain circumstances acetate uptake is saturable, suggesting that such a system exists. Gimenez et al. (2003) reported an acetate permease (*actP* formerly *yjcG*) and provided evidence for the existence of a second acetate transporter. The authors proposed that these systems play critical roles when cells scavenge for micromolar concentrations of acetate (Gimenez et al., 2003).

The cellular assimilation of acetate in *E. coli* first involves the conversion of acetate to acetyl-CoA. The organism possesses two distinct enzymatic mechanisms by which acetate is

converted to acetyl-CoA. The first pathway (called the AMP-ACS pathway) is catalyzed by the enzyme acetyl-CoA synthetase (ACS) (acetate: CoA ligase [AMP forming]; EC 6.2.1.1), and converts acetate and ATP to the enzyme-bound intermediate acetyladenylate (acetyl-AMP) and pyrophosphate. ACS then reacts with acetyl-AMP and CoASH to form acetyl-CoA, releasing AMP (Chou and Lipmann, 1952; Berg, 1956). Although reversible *in vitro*, this reaction is irreversible *in vivo* because of the presence of intracellular pyrophosphatases (PPase). Brown et al. (1977) have shown that ACS binds acetate with a high affinity (K_M of 0.2 mM for acetate) and low V_{MAX}. The second pathway (called the PTA-ACK pathway) is catalyzed by two enzymes: acetate kinase (ACK) [acetyl-CoA(CoA):Pi acetyltransferase; EC 2.7.2.1] and phosphotransacetylase (PTA) [ATP:acetate phosphotransferase; EC 2.3.1.8] (Kumari et al., 1995). The enzymes ACK and PTA normally play a catabolic role in excreting acetate (when grown on glucose), but they can also act in the opposite direction to mediate the first step in the net conversion of acetate to cell components during growth. ACK catalyzes the conversion of acetate to acetyl-phosphate with the cleavage of ATP to ADP. In the second step, PTA transfers the acetyl moiety from acetyl phosphate to coenzyme A, resulting in the formation of acetyl-CoA and inorganic phosphate (Brown et al., 1977). In contrast to ACS, ACK binds acetate poorly with a K_M of 7 to 10 mM for acetate and high V_{MAX} (Brown et al., 1977; Fox and Roseman, 1986; Kumari et al., 1995). Therefore, this reversible low affinity PTA-ACK pathway may assimilate acetate only when acetate is present at a relatively large concentration. Evidence for this conclusion is the observation that cells which express acetate kinase (ACK) and phosphotransferase (PTA) but not ACS grow poorly on low concentrations of acetate (Kumari et al., 1995). In contrast cells which express ACS, but not ACK and PTA, grow poorly on high concentrations of acetate (Kumari et al., 1995). Those that do not express any of the three

proteins ACS, PTA and PTA do not grow on acetate. They proposed that possession of both activation pathways permits *E. coli* to survive in environments that contain widely varying concentrations of acetate with the high-affinity ACS pathway functioning primarily anabolically, scavenging for small amounts of environmental acetate (Brown et al., 1977; Kumari et al., 1995). While an *acs* knockout in *E. coli* showed decreased growth rate on acetate, overexpression of the *acs* gene resulted in efficient acetate assimilation. MG1655 strain with overexpressed *acs* consumed 64 mM acetate and reached 2.5 OD_{600} in less than 36 hours in a defined medium while MG1655 did not consume any acetate in 36 h (Lin et al., 2006). Interestingly, Castano-Cerezo et al. (2009) reported that the growth rate and acetate consumption rate of a *pta* mutant (*E. coli* BW25113 *Δpta*) was almost half compared to the control strain (*E. coli* BW25113) while the acetate consumption rate of an *acs* mutant was found to be higher than the control strain. The biomass yield was reduced in both the mutant strains (Castano-Cerezo et al., 2009). These seemingly contradictory results nevertheless demonstrate the significant impact of ACS on acetate-grown cultures.

Once acetate forms acetyl-CoA, it is mainly catabolized by the tricarboxylic acid cycle. Since with each turn of the cycle two carbon atoms are lost as CO_2, no net material would be available for cellular synthesis from acetyl-CoA if this were the only means of assimilation (Nimmo, 1984). Thus, growth on acetate or fatty acids as the sole carbon source requires operation of an anaplerotic pathway, the glyoxylate shunt, to replenish dicarboxylic acids drained from the tricarboxylic acid cycle for cellular biosynthesis (Kornberg, 1966).

The glyoxylate shunt consists of six of the eight reactions of the tricarboxylic acid cycle but bypasses the two oxidative steps in which carbon dioxide is evolved (Kornberg and Madsen, 1957, 1958). This cycle prevents the loss of acetate carbon by redirecting the substrate isocitrate

from the TCA cycle via the two key enzymes isocitrate lyase and malate synthase. Isocitrate lyase (ICL, EC 4.1.3.1) catalyzes the conversion of isocitrate by an aldol cleavage to form glyoxylate and succinate (Campbell et al., 1953). Malate synthase (EC 4.1.3.2) catalyses the condensation of acetyl-CoA with glyoxylate to yield malate, a second intermediate of the tricarboxylic acid cycle (Wong and Ajl, 1956). Acetate-grown *E. coli* contained these enzymes of the glyoxylate cycle in activities greater than those found in cells grown on substances other than acetate (such as glucose or C_4 acids) (Kornberg et al., 1960). Also, mutants devoid of isocitrate lyase did not grow on acetate but grew on glucose or other utilizable intermediates of the tricarboxylic acid.

Isocitrate dehydrogenase (ICDH, EC 1.1.1.41) is another enzyme important in acetate metabolism. ICDH catalyzes the conversion of isocitrate to α-ketoglutarate. About 75% of ICDH is converted to an inactive phosphorylated form by the binding of NADP during growth of *E. coli* on acetate in order to direct carbon flux through the glyoxylate bypass (Bennett and Holms, 1975; Borthwick et al., 1984). The inactivation of isocitrate dehydrogenase is reversible (Bennett and Holms, 1975; Borthwick et al., 1984) and is mediated by phosphorylation/dephosphorylation (Garnak and Reeves, 1979; Borthwick et al., 1984) catalyzed by a bifunctional kinase/phosphatase (Nimmo et al., 1984). When acetate (or fatty acids) serves as the energy/carbon source, the channeling of metabolites from the Krebs cycle into the glyoxylate bypass occurs at isocitrate. Isocitrate lyase and isocitrate dehydrogenase compete for the common substrate. This competition favors isocitrate lyase by the inactivation of isocitrate dehydrogenase (Holms and Bennett, 1971; Negre et al., 1992). Also, K_m of isocitrate lyase for isocitrate is much higher than that of isocitrate dehydrogenase (Elmansi et al., 1985). Based on this observation, Elmansi et al., (1985) proposed that phosphorylation of the dehydrogenase is

believed to facilitate flux through the glyoxylate bypass by rendering the dehydrogenase rate-limiting in the tricarboxylic acid cycle and thus increasing the intracellular concentration of isocitrate. Therefore, during growth on acetate flux through glyoxylate shunt is facilitated by maintaining high concentrations of isocitrate and by controlling the phosphorylation state of isocitrate dehydrogenase (Nimmo and Nimmo, 1984; Walsh and Koshland, 1984; Elmansi et al., 1985).

Objectives

This research is an important component of a project aimed at an efficient conversion of lignocellulosic sugars to several biochemicals by metabolic engineering of a suite of bacteria. The ultimate objective of this research is to achieve a complete consumption of acetate present in the hydrolysate using a metabolically engineered strain of *E. coli*.

The three main objectives are

1. Compare the maximum specific growth rates of different *E. coli* strains for growth on acetate and identify the best strain with respect to growth on acetate.
2. Find a correlation between differences in growth rate on acetate and genotypic differences.
3. Increase the growth rate of the best growing strain on acetate.

Hypotheses

1. Different strains of *E. coli* have different specific growth rates, different acetate consumption rates, and different biomass yields.
2. The differences observed are correlated with genotypic differences.
3. Growth rate of the best strain on acetate can be increased further by the process of adaptive evolution.
4. Positive characteristics on a strain can be conferred by providing for those genotypic differences.

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CHAPTER 2

COMPARISON OF ESCHERICHIA COLI STRAINS FOR GROWTH ON ACETATE[1]

[1] Rajaraman, E., Eiteman, M.A. and Altman, E. To be submitted to *Appl. Microbiol. Biotechnol.*

Abstract

Acetate is an unavoidable degradation product in biomass hydrolysis and it inhibits microbial production of biochemicals. Acetate can be selectively removed from mixture of sugars and acetate using substrate-selective degradation. This research focuses on understanding the growth of *E. coli* on acetate. First, eighteen strains of *E. coli* were compared for maximum specific growth rate (μ_{MAX}) on acetate. We observed that ATCC8739 had the greatest growth rate growth rate (0.405 h^{-1}) while SCS-1 had the slowest growth rate (0.153 h^{-1}). Second, microarray analysis of 7 genome sequenced strains was conducted to determine why ATCC8739 had the greatest maximum growth rate on acetate. We observed that the *folK* gene in the folate biosynthesis pathway was more than 2-fold upregulated only in ATCC8739 in comparison to SMS-3-5 and BL21. Since products of folate biosynthetic pathway are vital for growth of *E. coli*, the overexpression of *folK* gene might explain why ATCC8739 grow fast on acetate when compared to other strains. Genes of phenylacetate degradation pathway were also upregulated in ATCC8739 compared with SMS-3-5 and BL21. Also, there was a positive correlation between differences in expression levels of phenylacetate degradation genes and differences in growth rates of ATCC8739, SMS-3-5 and BL21. These observations indicate towards the possibility of an alternate acetate degradation pathway in *E. coli*.

Introduction and Literature Review

Diminishing reserves of fossil fuels, unsustainable net rates of greenhouse gas emissions and technological advancements have revived interest in the microbial formation of transportation fuels from renewable resources (Stephanopoulos, 2007; Bartley and Ronald, 2009; Liu and Khosla, 2010). Hardwood, softwood, grasses, and agricultural residues (Lee, 1997) contain as great as 70 % carbohydrates as cellulose and hemicellulose, closely associated with lignin (Klinke et al., 2004). Pretreatment methods are required to remove lignin and make the hemicellulose and cellulose fractions available for fermentation (Sun and Cheng, 2002; Klinke et al., 2004). Degradation products formed in the pretreatment step, such as furaldehydes, weak acids and phenolic compounds (Parawira and Tekere, 2011) are fermentation inhibitors (Klinke et al., 2002; Klinke et al., 2004) which must be removed prior to the biological conversion of lignocellulosic feedstock into a transportation fuel.

Acetic acid (acetate) is one of the most abundant inhibitor generated through the pretreatment process (Pettersen, 1984; Sakai et al., 2007a). Any acetate remaining in a fermentation medium containing glucose or xylose affects the specific growth rate and biomass yield (Luli and Strohl, 1990; Roe et al., 1998; Takahashi et al., 1999; Roe et al., 2002). For example, Takahashi et al. (1999) reported that acetate negatively affected the growth of *E. coli* KO11 on Luria broth containing glucose or xylose as a sugar source and different concentrations of acetate (2.0 – 12.0 g/L). For example, only 67% of glucose and 45% of xylose were consumed in a media containing 15.0 g/L acetate. Luli and Strohl (1990) similarly reported that 10 g/L acetate added during exponential growth of *E. coli* JM105 on glucose reduced the growth rate and biomass yield by more than 50%. Roe et al. (1998) observed that acetate concentrations as low as 8 mM (< 0.5 g/L) decreased the growth rate of *E. coli* by 50%. Also, acetate affects the

fermentation rate of Baker's yeast: increasing acetate concentration from 34 mM to 68 mM at pH 3.5 decreased the rate of CO_2 formation by more than 60% (Maesen and Lako, 1952).

Several approaches have been proposed to remove acetate prior to microbial fermentation. Um et al. (2011) reported 76% acetate removal in a single stage liquid-liquid extraction with trioctylphosphine oxide (TOPO) as an extractant (pH < 3) with hardwood-derived hemicellulose extracts. The low pH requirement to insure protonation of the acid is the principal shortcoming of this method for acetate removal (Um et al., 2011). Electrodialysis (ED) (Wong et al., 2009) and bipolar electrodialysis (BPED) (Wong et al., 2010) removed acetate during *E. coli* fermentations, with the latter having lower energy consumption with simultaneous pH control. Neither of these methods completely removed acetate (76% using BPED and 62 % using ED). In their detailed comparison of different detoxification methods, Larsson et al. (1999b) conclude that the choice of method depends on the composition of hydrolysate and the type of feedstock (Larsson et al., 1999b). These approaches for acetate removal involve an additional processing step.

Recently, a novel microbial method to remove acetate selectively from a mixture of sugars and acetate was reported (Lakshmanaswamy et al., 2011). This method is based on the genetic engineering of a strain of *E. coli* which will consume only acetate in the presence of multiple sugars. To prevent glucose and xylose consumption, genes involved in glucose and xylose uptake were knocked out. The strain MG1655 *ptsG manZ glk xylA* consumed 10 g/L acetate and less than 1 g/L sugar during 30 h of aerobic batch fermentation from a medium also containing 20 g/L glucose and 10 g/L xylose, demonstrating that a batch process could be implemented to remove acetate selectively from sugar mixtures without significantly reducing the concentration of glucose or xylose.

Microbial "detoxification" of acetate-containing lignocellulosic hydrolysates has a few important advantages. First, the acetate carbon is retained at least partially in the form of biomass to serve as nutrients in a subsequent microbial process to convert the sugars to fuels or chemicals. The inhibitor is essentially upgraded to a nutrient. Second, as a microbial process itself, selective removal of acetate is very compatible with existing conversion steps (e.g., pH, temperature).

One potential disadvantage with the microbial detoxification is that the strains unable to consume sugars grow slower than the wild-type on acetate in the presence of the unmetabolizable sugars. For example, MG1655 *ptsG manZ glk crr xylA* consumed 10 g/L acetate at a maximum specific growth rate of 0.23 h^{-1} in the absence of glucose and xylose and at 0.12 h^{-1} in the presence of sugars in batch fermentations. Understanding and improving the growth of *E. coli* on acetate generally would therefore improve the microbial acetate-removal process

Completion of the genome sequence of *E. coli* K12 strain MG1655 (Blattner et al., 1997) followed by the genome sequence of several other strains has helped in determining the precise functions of all genes by global transcriptional analysis (Richmond et al., 1999). DNA microarray is one example of transcriptional analysis and is a powerful tool for strain improvement and bioprocess development (Oh and Liao, 2000). For examples, Oh and Liao (2000) have demonstrated the use of DNA microarrays to measure differential transcript levels of genes related to central metabolic pathways, regulatory and protein processing genes of *E. coli* MC4100 grown in acetate, glucose and glycerol media.

The first goal of this study is to compare several strains of *E. coli* using acetate as a sole carbon source. The fastest growing strain will ultimately be preferred for the selective

consumption of acetate from lignocellulosic hydrolysates. The second goal of this study is to correlate the differences in growth rate with differences in gene expression as measured with microarrays. The underlying hypothesize is that growth rate differences are correlated with differences in gene expression based on the general observation that changes in growth rate changes gene expression.

Materials and Methods

Bacterial Strains

Eighteen *Escherichia coli* were used in this study and they are listed in Table 2.1.

Growth Medium and shake flask conditions

Defined medium was used for all the shake flask experiments and this medium contained (per liter): 1.70 g citric acid, 13.30 g KH_2PO_4, 4.50 g $(NH_4)_2HPO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$, 13 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 1.5 mg $CuCl_2 \cdot 2H_2O$, 15 mg $MnCl_2 \cdot 4H_2O$, 2.5 mg $CoCl_2 \cdot 6H_2O$, 3.0 mg H_3BO_3, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 100 mg Fe(III) citrate, 4.5 mg thiamine·HCl, 8.4 mg $Na_2(EDTA) \cdot 2H_2O$, and 5 g (84.7 mM) acetate using $Na(CH_3COO) \cdot 3H_2O$ (concentrations of acetate ion are reported). Acetate solutions were sterilized separately and later combined with other components. The pH was adjusted to 7.0 with a 20% (w/v) NaOH solution. Cells were routinely stored on Luria-Bertani agar plates and then transferred to 50 mL medium in 250 mL shake flasks, incubated at 37 °C and agitated at 350 RPM. When the OD reached 2.0, 5 mL were transferred to 2-3 identical flasks for growth rate and acetate measurement and microarray analysis.

Table 2.1: List of *E. coli* strains used in this study

Strain	Genotype	Reference
C (ATCC8739)	Wild type	ATCC8739
W	Wild type	ATCC9736
SE11	Wild type	JCM16754
SE15	Wild type	JCM16755
SMS-3-5	Wild type	ATCCBAA-1743
B	Wild type	CGSC5365
MC4100	F- *araD*139 Δ(*argF-lac*)U169 *rpsL150 relA1 deoC1 rbsR fthD*5301 *fruA*25 λ -	Laboratory collection
MACH1	F- Φ80(*lacZ*) ΔM15 Δ*lac*X74 *hsd*R(r_K^- m_K^+) Δ*recA*1398 *endA1 tonA*	Invitrogen
C (ATCC13706)	Wild type	ATCC13706
JM101	F´*traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)M15/* Δ*(lac-proAB)supE thi-1* λ-	ATCC33876
JM105	F- Δ(*lac proAB*) *lacI*^{q} *thi repsL endAl slcB15 hadR4 traD36 proAB* Δ(ZM15)	Pharmacia
BL21	B F- *dcm ompT hsdS* (r_B^-, m_B^-) *gal*	Stratagene
DH1	F- λ- *supE44 hsdR17 recA1 gyrA96 relA1 endA1 thi*-1	ATCC33849
MG1655	F- λ- *ilvG rfb*-50 *rph*-1	ATCC700926
C (CGSC3121)	Wild type	CGSC3121
W3110	*F- λ- rph-1 INV(rrnD, rrnE)*	CGSC4474
IAI1	Wild type	Touchon et al., 2009
SCS-1	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1*	Stratagene

Analytical Methods

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth.

All shake flask experiments and fermentations were stored at -20°C for analyses. These samples were centrifuged (8400×g for 10 minutes), and the supernatant solution used for HPLC measurement of acetate concentrations. A Shimadzu HPLC (LC-6A pump, SCL-6B system controller and auto-injector) with a refractive index detector (Waters Model 2410), a Coregel 64-H ion-exclusion column (transgenic) and a mobile phase of 4 mN H_2SO_4 was used for analyte analyses (Eiteman and Chastain, 1997).

Dry cell weight measurements

The dry cell weights of strains were measured to determine the biomass yield. Samples at different ODs were centrifuged (8400×g for 10 minutes) using a Sorvall T21 centrifuge. The pellets were washed twice with distilled water and were dried on an aluminum weighing boat at 60°C for 24 h, and the boat reweighed.

Transcriptome analysis

Escherichia coli K12 (4 × 72K format) microarrays were used in this study (Roche NimbleGen catalog #A6697-00-01.The cDNA samples were shipped to a core facility at Florida State University for microarray analysis.

Cell lysis and RNA Extraction

Samples having OD_{600} of 0.5 to 0.6 were mixed with the QIAGEN RNA protect reagent in the volumetric ratio of 1:2, incubated for 5 min at room temperature (15 – 25°C) and centrifuged (5000 ×g for 10 min). Supernatant was discarded, and the pellet was stored at -20°C. 200 µL of TE buffer containing 10 mg/mL lysozyme and 15 µL proteinase K was used as the

lysis reagent. Addition of lysis reagent was followed by incubation in a water bath at 37 °C for 10 min and three freeze-thaw cycles (Lahtvee et al., 2009). Total RNA was extracted using QIAGEN RNeasy Mini kit and genomic DNA was removed using QIAGEN RNase-free DNase set.

cDNA synthesis

Single stranded cDNA was synthesized from total RNA using superscript III first strand synthesis system (Invitrogen, Carlsbad, CA). 5 µg of total RNA was combined with the following components on ice (per sample): 2 µL of 50 ng/µL random hexamers, 2 µL of 10 mM dNTP Mix, and diethylpyrocarbonate (DEPC)-treated water to make up the total volume to 26 µL. The samples were incubated at 65 °C for 5 min in a thermocycler (Finnzymes, Vantaa, Finland). The first strand cDNA synthesis mix consisted of the following reagents (per reaction): 8 µL 5× 1^{st} strand buffer, 2 µL 0.1 M DTT, 2 µL RNase OUT, 2 µL Superscript reverse transcriptase III, and 14 µL of first strand cDNA synthesis master mix. After mixing thoroughly, samples were incubated at 50°C for 4 hours and at 70°C for 15 minutes in the thermocycler. 2 µL of RNase H was added to the PCR product to remove RNA complementary to the cDNA and was followed by incubation at 37°C for 20 min in the thermocycler. cDNA cleanup was carried out using QIAGEN Qiaquick PCR purification kit.

Microarray analysis

The labeling, hybridization, washing and image scanning steps were performed at a core facility in Florida State University. The samples were labeled with cy3 random nonamers (dye) on an Applied Biosystems Veriti, hybed on a 4 bay NimbleGen hybridization (Applied Biosystems, Foster City, CA). Scanning of the array was performed using a NimbleGen MS200 2 µm scanner (Roche NimbleGen, Madison, Wisconsin). Raw data extraction, image analysis

and normalization steps were performed using NimbleGen's DEVA software. ArrayStar software was used for data analysis (DNASTAR, Madison, WI).

Results

Growth rate on acetate

Eighteen strains were compared for growth in defined medium containing 5 g/L acetate as the sole carbon source. ATCC8739 had the greatest specific growth rate of 0.405 h^{-1}, and SCS-1 had the lowest specific growth rate of 0.153 h^{-1} (Table 2.2). A wide distribution of growth rates were observed, even from presumably closely related strains. For example, Figure 2.1 illustrates the differences in growth rates between the 3 C strains: ATCC8739, ATCC13706 and CGSC3121. In Table 2.2, the nine strains marked with an asterisk have their genome sequenced. Seven of these strains were selected for the microarray analysis.

Table 2.2: Maximum specific growth rate (μ_{max}) of eighteen *E. coli* strains. The strains marked with an asterisk (*) are genome sequenced strains.

***E. coli* Strains**	**Maximum specific growth rate (h^{-1})**
ATCC8739*	0.405 ± 0.007
W	0.372 ± 0.009
SE11*	0.369 ± 0.016
SE15*	0.356 ± 0.007
SMS-3-5*	0.356 ± 0.001
B	0.343 ± 0.055
MC4100	0.340 ± 0.047
MACH1	0.340 ± 0.020
ATCC13706	0.337 ± 0.012
JM101	0.328 ± 0.011
JM105	0.304 ± 0.020
BL21*	0.301 ± 0.010
DH1*	0.295 ± 0.038
MG1655*	0.292 ± 0.007
CGSC3121	0.265 ± 0.009
W3110*	0.255 ± 0.005
IAI1*	0.180 ± 0.016
SCS-1	0.153 ± 0.008

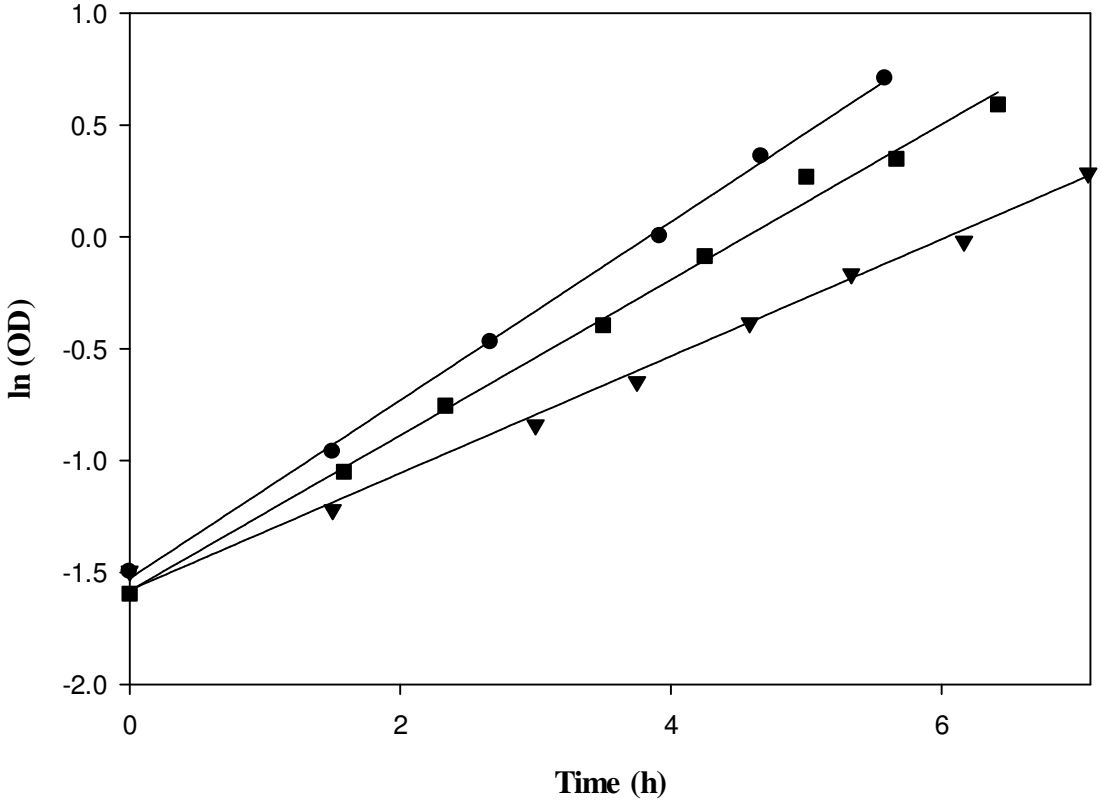

Figure 2.1: Growth rate comparison of the 3 C strains, ATCC8739 (●), ATCC13706 (■), CGSC3121 (▼) and the parent strain on a defined medium containing 5 g/L acetate.

Biomass yield

Biomass yield for 14 strains was calculated as the ratio of observed biomass generated to the acetate consumed. At least three samples of six *Escherichia coli* strains (ATCC8739, ATCC13706, CGSC3121, W, MG1655 and SCS-1) at different ODs were used to generate the dry cell weigh (DCW) calibration. The biomass produced at each OD was calculated using a quadratic model between OD and DCW.

(Equation 2.1):

$$X = 0.0741*(OD)^2 + 0.2403*(OD) \qquad \text{Equation 2.1}$$

For each strain, a plot was constructed between the biomass concentration (X) at each OD and the corresponding acetate concentration (A). A line of best fit was constructed and slope of the best fit line ($\Delta X/\Delta A$) was calculated as biomass yield ($Y_{X/A}$).

The biomass yields (Table 2.3) of fourteen strains were calculated using the method described above. There was no correlation between biomass yields and specific growth rate.

Table 2.3: Biomass yields of fourteen *E. coli* strains (listed in order of greatest to lowest specific growth rate).

***E. coli* Strains**	**Biomass Yield ($Y_{X/A}$)**
C (ATCC8739)	0.371 ± 0.029
W	0.389 ± 0.036
B	0.303 ± 0.052
MC4100	0.314 ± 0.028
MACH1	0.407 ± 0.017
C (ATCC13706)	0.383 ± 0.006
JM101	0.379 ± 0.003
JM105	0.359 ± 0.024
BL21	0.264± 0.021
DH1	0.441 ± 0.055
MG1655	0.334 ± 0.064
C (CGSC3121)	0.412 ± 0.124
W3110	0.314 ± 0.003
SCS-1	0.255 ± 0.009

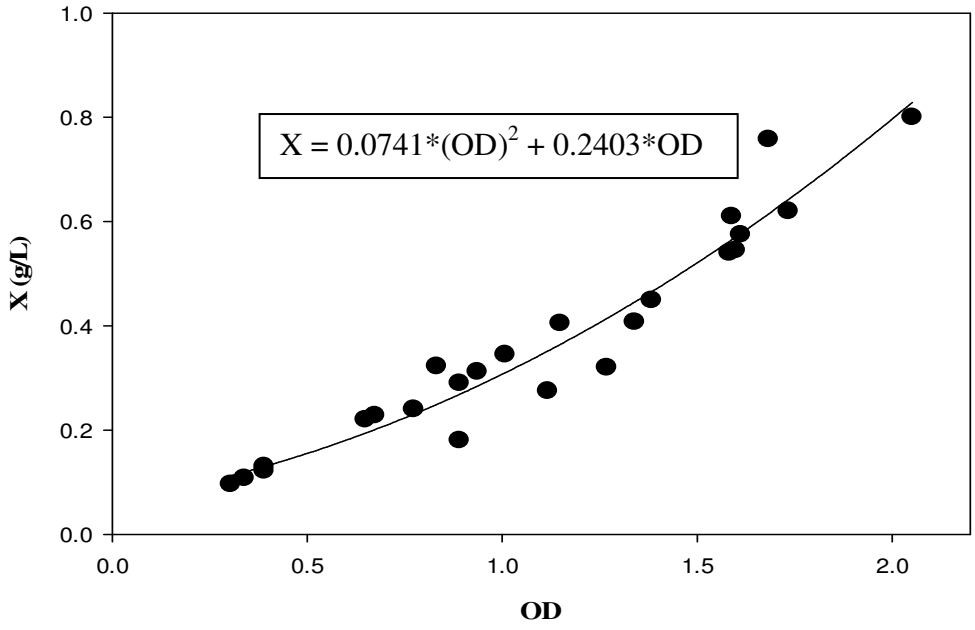

Figure 2.2: A quadratic fit between biomass concentrations (X) vs OD values of 6 strains

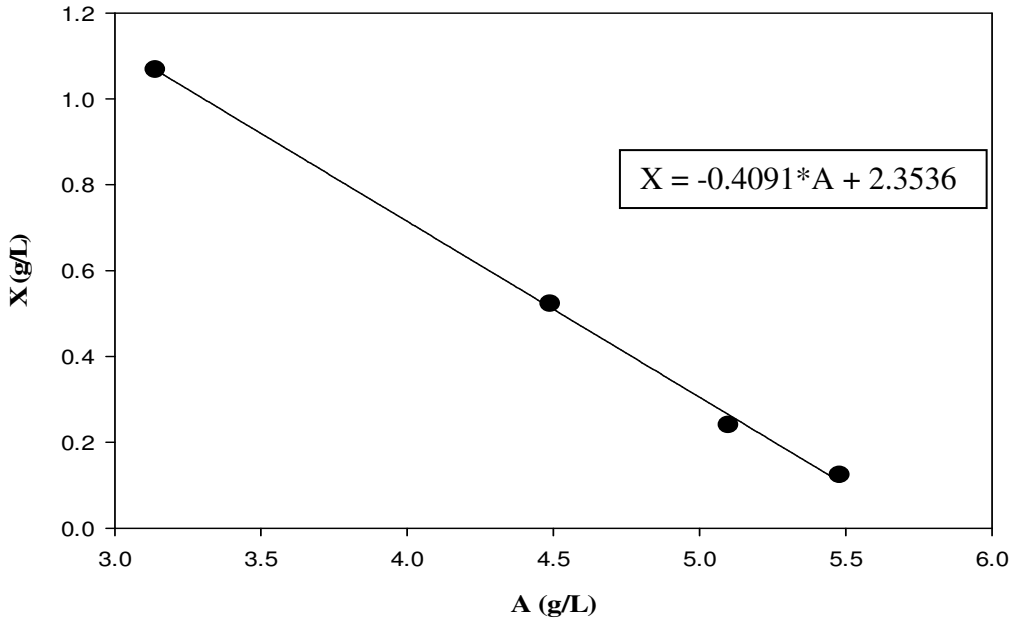

Figure 2.3: A plot between biomass concentration (X) and acetate consumption (A) for MG1665. The positive slope represents the biomass yield.

Transcriptome analysis

Seven strains whose genome has been sequenced (Table 2.1) were selected for transcriptome analysis using microarrays. Transcriptome analysis was carried out to understand why the ATCC8739 grows faster than other strains on acetate when compared to SMS-3-5 and BL21. 71 genes were atleast 2-fold upregulated in ATCC8739 in comparison to SMS-3-5 and BL21. Interestingly, we observed that, for more than 90 % of the genes (65 genes), the expression level of SMS-3-5 was higher than the expression level of BL21. This indicate that

For 65 genes: $(\text{expression level})_{ATCC8739} > (\text{expression level})_{SMS\text{-}3\text{-}5} > (\text{expression level})_{BL21}$

Also, the growth rates of ATCC8739, SMS-3-5 and BL21 are 0.405 h^{-1}, 0.356 h^{-1} and 0.301 h^{-1} respectively (Table 2.2). Therefore, the expression level differences of these 65 genes are correlated with growth rate differences of ATCC8739, SMS-3-5 and BL21. The list of 71 genes includes prophage related genes, phenylacetate metabolic genes, pseudo genes and

transcriptional regulators. 10 genes yqeI, yqeJ, yqeK, ygeF, ygeG, ygeH, ygeI, ygeK, ygeN, ygeO belong to the type three secretion system pathogenicity island ETT2. The ETT2 gene cluster is present in majority of *E. coli* strains (pathogenic or commensal) and has been subjected to varying degree of mutational attrition that render it unable to encode a functioning secretion system (Ren et al., 2004). The phenylacetate metabolic genes, the transcriptional regulator *ybdO*, peptidoglycan binding enzyme gene *pbI* might be relevant to growth on acetate and are Also, 129 genes were atleast 2-fold down regulated in ATCC8739 in comparison with SMS-3-5 and BL21 (Table 2.5).

Table 2.4: List of 71 genes which are atleast two-fold up-regulated in ATCC8739 in comparison with SMS-3-5 and BL21

Gene(GeneID)	Function	Fold change		p-value
		SMS-3-5	BL21	
Phenylacetate Degradation				
paaA (945833)*	subunit of phenylacetate-coA oxygenase, hydroxylates phenylacetyl-CoA	7.77	15.55	1.66E-08
paaB (947595)*	subunit of phenylacetate-coA oxygenase, hydroxylates phenylacetyl-CoA	8.03	15.19	2.24E-08
paaC(945956)*	subunit of phenylacetate-coA oxygenase, hydroxylates phenylacetyl-CoA	7.57	16.66	2.15E-07
paaD(945959)*	subunit of phenylacetate-coA oxygenase, hydroxylates phenylacetyl-CoA	5.37	11.61	4.35E-06
paaE(945962)*	subunit of phenylacetate-coA oxygenase, hydroxylates phenylacetyl-CoA	6.55	11.28	2.88E-10
paaF(946011)*	putative 2,3-dehydroadipyl-CoA hydratase	7.94	17.10	5.15E-07
paaG(946263)*	putative 1,2-epoxyphenylacetyl-CoA isomerase	8.90	17.85	1.02E-07
paaH(945940)*	putative 3-hydroxyl-acyl-CoA dehdydrogenase	8.44	16.38	1.45E-07
paaI(945265)*	Thioesterase,	7.33	13.44	2.21E-08
paaJ(946121)*	ketoadipyl-CoA thiolase	6.07	9.82	4.13E-12
paaK(945963)*	phenylacetyl-CoA ligase	10.35	33.17	3.28E-07
paaX(945966)*	DNA-binding transcriptional repressor of paa operon	9.70	12.39	1.04E-09
paaY(945965)*	predicted hexapeptide repeat acetyltransferase	38.96	217.13	4.50E-04
paaZ(945954)*	Fused oxepin-coA hydrolase involved in phenylacetate catabolism	6.51	14.54	7.73E-08
Transcriptional Regulators				
yqeI(947327)*	predicted transcriptional regulator	7.65	25.16	5.56E-09
ygeH(946265)*	predicted transcriptional regulator	64.90	140.30	1.22E-08
ybdO(945216)*	predicted DNA-binding transcriptional regulator	58.04	160.90	1.18E-06
CRISPR-based defense system				
ygbF(947213)*	probable ssRNA endonuclease, CRISP-associated protein	6.21	13.69	1.71E-09
ygbT(947228)*	DNA endonuclease, CRISP-associated protein	6.95	12.82	3.71E-14
casE(947226)*	CRISPR RNA precursor cleavage enzyme	6.03	12.33	4.7E-06
casD(947225)*	subunit of CRISP RNA containing cascade antiviral complex	10.92	24.74	4.72E-07
casC(947224)*	subunit of CRISP RNA containing cascade antiviral complex	13.93	69.76	1.63E-06
casB(947223)*	subunit of CRISP RNA containing cascade antiviral complex	10.51	59.19	2.1E-05
casA(947222)*	subunit of CRISP RNA containing cascade antiviral complex	37.43	59.65	9.8E-05
ygcB(947229)*	predicted helicase needed for cascade antiviral activity	11.65	29.49	8.64E-07

Pseudo genes				
*pbI(947338)**	pseudo; predicted peptidoglycan-binding enzyme	72.22	145.61	5.13E-10
insB(4056034)	pseudo; transposon-related function	2.06	2.02	4.14E-12
ymdE(945589)*	Pseudo	43.55	76.68	1.86E-05
icdC(4056026)*	Pseudo	4.18	4.61	5.22E-13
insZ(4056037)*	Pseudo	3.41	15.42	3.38E-12
exoD(947902)*	Pseudo	5.79	12.82	1.45E-07
ysaD(5625575)*	Pseudo	3.47	8.07	1.68E-12
Type three secretion system pathogenicity island ETT2				
yqeJ(947328)*	predicted protein	58.53	187.00	2.91E-07
yqeK(947329)*	predicted protein	77.05	185.06	2.64E-08
ygeF(946823)*	Pseudo	34.21	100.10	3.96E-10
ygeG(946986)*	predicted protein	51.46	135.04	3.61E-07
ygeI(947336)*	predicted protein	50.21	157.03	5.53E-08
ygeJ(947338)*	Pseudo	72.22	145.61	5.13E-10
ygeK(947332)*	Pseudo	84.32	237.78	6.29E-08
ygeN(2847705)*	Pseudo	80.84	112.80	1.13E-12
ygeO(947325)*	Pseudo	39.57	83.60	1.89E-06
Prophage-related (name of the prophage)				
aaaD(5625560)*	pseudo; predicted tail fiber assembly protein (DLP12)	8.37	14.32	5.26E-13
tfaR(946062)*	predicted tail assembly protein (Rac)	8.11	15.30	9.99E-05
ydfO(945992)*	predicted protein (Qin)	74.37	136.98	1.59E-10
gnsB(946054)*	multicopy suppressor of secG(Cs) and fabA6(Ts) (Qin)	36.34	51.42	2E-10
ynfN(946097)*	cold shock induced protein (Qin)	21.45	62.89	1.66E-08
cspI(946099)*	cold shock protein (Qin)	46.40	132.33	2.23E-07
rzpQ(946101)*	RZ-like equivalent (Qin)	4.49	7.13	3.65E-10
ydfR(946095)*	predicted protein (Qin)	27.98	55.04	7.58E-05
essQ(946093)*	predicted lysis protein (Qin)	5.67	9.10	2.86E-14
cspB(946091)*	cold shock protein (Qin)	84.37	123.51	4.93E-13
cspF(946090)*	cold shock protein (Qin)	5.36	6.85	1.91E-10
quuQ(946103)*	predicted anti-termination protein Q (Qin)	8.48	16.08	4.3E-08
ydfU(946108)	predicted protein (Qin)	5.83	3.41	5.94E-11
relE(947549)*	toxin of the RelE-RelB toxin-antitoxin system (Qin)	13.67	81.05	8.6E-06

relB(948308)*	bifunctional antitoxin/transcriptional repressor (Qin)	15.40	62.12	6.02E-06
ydfV(948801)*	predicted protein (Qin)	11.23	59.45	7.83E-06
flxA(947392)*	predicted protein (Qin)	16.67	54.68	9.41E-06
intK(946111)*	pseudo (Qin)	10.26	120.24	2.67E-05
dicA(946241)*	transcriptional repressor (Qin)	8.03	19.05	1.78E-08
ydfA(946082)*	predicted protein (Qin)	3.97	8.51	1.66E-14
ydfB(946176)*	predicted protein (Qin)	2.27	94.42	1.68E-06
ynfO(1450267)*	hypothetical protein (Qin)	65.18	160.07	7.15E-06
Others				
ycdU(945592)*	predicted inner membrane protein	13.52	32.37	1.31E-06
rfbA(945154)*	Glucose-1-phosphate thimydylyltransferase	2.10	3.005	3.99E-05
rfbB(945276)	dTDP-glucose 4,6 dehydratase, NAD(P)-binding	10.87	2.88	5.48E-06
gspD(947822)	general secretory pathway component, cryptic	10.87	5.33	2.15E-06
yjeN(948678)*	predicted protein	3.82	21.76	4.02E-10
folK(948792)*	leads to synthesis of folate cofactors	2.16	2.49	3.28E-07
yfgJ(946984)	conserved protein related to swarming	21.42	2.67	0
? (2487750)*	Gene doesn't exist in NCBI	3.35	11.58	8.94E-12
? (2487704)*	Gene doesn't exist in NCBI	13.21	190.59	6.79E-11

***-** Genes positively correlated with growth rate

Table 2.5: List of 129 genes which are atleast two-fold down-regulated in ATCC8739 in comparison with SMS-3-5 and BL21

Gene(GeneID)	**Function**	**Fold change**		**p-value**
		SMS-3-5	**BL21**	
Prophage or phage related (name of the prophage)				
intD (947162)	predicted integrase of the cryptic lamboid prophage (DLP12)	2.42	22.11	3.15E-11
renD (945024)	pseudo (DLP12)	3.76	2.01	9.67E-03
emrE (948442)	multidrug resistance protein (DLP12)	2.85	128.83	5.34E-07
ybcK (945166)	predicted recombinase (DLP12)	2.25	205.15	4.49E-10
ybcL (945165)	probable kinase regulator; secreted protein (DLP12)	4.10	70.12	3.58E-08
ybcM (945163)	predicted DNA-binding transcriptional regulator (DLP12)	5.46	207.83	4.32E-08

ybcN (945162)	predicted protein (DLP12)	6.42	59.44	3.19E-09
nine (945151)	conserved protein (DLP12)	13.24	293.18	1.64E-07
ybcO (945147)	predicted protein (DLP12)	2.39	9.84	9.89E-09
quuD (945177)	transcriptional antiterminator (DLP12)	4.89	50.29	2.76E-07
nmpC (946786)	pseudo; truncated outer membrane porin protein (DLP12)	3.65	16.97	2.64E-06
xisD (5625559)	pseudo; exisionase (DLP12)	2.35	18.74	7.23E-10
ylcG (1450240)	expressed protein (DLP12)	3.20	31.42	1.04E-09
ylcH (5061504)	hypothetical protein (DLP12)	2.95	35.60	2.64E-08
essD (947545)	predicted phage lysis protein (DLP12)	2.92	13.47	8.18E-11
rrrD (947539)	predicted lysozyme (DLP12)	2.17	52.63	6.93E-08
rzoD (1450242)	predicted lipoprotein (DLP12 prophage)	2.35	19.24	1.33E-09
rzpD (945929)	predicted murein endopeptidase (DLP12)	5.39	97.04	5.75E-10
intR (946976)	integrase (Rac)	2.45	19.65	2.50E-10
ydaC (947504)	predicted protein (Rac)	3.58	207.74	3.35E-07
ralR (945914)	restriction alleviation protein (Rac)	4.03	192.77	3.77E-07
recT (945917)	recombination and repair protein (Rac)	4.12	52.34	1.33E-07
recE (945918)	$5' \rightarrow 3'$ specific dsDNA exonuclease (Rac)	4.73	26.68	2.22E-07
racC (945920)	predicted protein (Rac)	4.02	189.79	4.74E-07
kilR (945921)	responsible for the killing activity of the Rac prophage (Rac)	5.87	107.24	2.02E-07
sieB (945913)	prevents phage superinfection (Rac)	4.24	177.30	1.7E-07
ydaG (945907)	predicted protein (Rac)	3.92	41.59	8.21E-07
trkG (945932)	potassium ion transporter subunit (Rac)	3.57	51.89	6.00E-08
ynaK (947417)	conserved protein (Rac)	2.13	12.45	7.53E-10
rzpR (945928)	predicted defective peptidase (Rac)	2.07	19.12	1.73E-10
ydaE (948959)	conserved protein (Rac)	4.32	250.62	7.86E-07
yeeW (946535)	pseudo (CP4-44)	16.43	2.16	0
yagJ (949094)	pseudo (CP4-6)	3.19	2.07	6.32E-02
yffR (946931)	predicted protein (CPZ-55)	5.92	2.91	5.26E-02
rnlB (947113)	predicted protein (CP4-57)	5.68	2.03	7.31E-03
rzoR (1450262)	predicted lipoprotein (Rac)	2.36	19.52	1.47E-09
Pseudo				
intB (948782)	Pseudo	37.32	32.87	1.35E-11
yjhR (944740)	Pseudo	7.61	94.83	5.5E-08

ypdJ (1450283)	pseudo (exisonase remnant)	2.11	5.44	1.24E-06
ykfN (5625552)	Pseudo	3.48	2.09	3.28E-01
yeeH (5625565)	Pseudo	25.34	18.98	5.64E-07
yoeG (5625566)	pseudo (CP4-44 putative prophage remnant)	235.80	193.50	0
yoeH (5625567)	pseudo (CP4-44 putative prophage remnant)	126.35	122.75	1.11E-12
yoeD (5625568)	pseudo (CP4-44 putative prophage remnant)	21.19	11.80	3.46E-13
yjiV (2847669)	pseudo conserved hypothetical protein	4.04	46.52	1.14E-10
yjhD (948809)	pseudo (KpLE2 phage-like element)	2.68	28.93	3.93E-09
yjdQ (5625580)	Pseudo	95.77	129.45	1.86E-09
ykfJ (944924)	Pseudo	3.86	3.42	2.48E-11
yafU (946644)	Pseudo	5.10	2.39	1.28E-01
ylbH (945114)	Pseudo	3.08	168.76	6.51E-06
rhsE (946026)	Pseudo	6.07	194.53	4.94E-04
yncL (945121)	Pseudo	4.68	195.20	4.19E-07
yghE (945897)	pseudo; secretion pathway protein	15.24	18.94	0
yghF (947469)	pseudo; secretion pathway protein	17.11	9.07	0
yrhA (947951)	Pseudo	2.86	114.08	4.39E-07
Transposon-related				
insG (948805)	IS4 transposase; involved in integration	25.52	14.66	0
insM (1450310)	pseudo; transposon related functions	2.95	43.02	1.05E-10
insD (945203)	IS2 transposase	27.86	10.96	0
insD (946118)	pseudo; transposon related functions	24.46	13.00	0
insJ (948082)	IS150 transposase A	18.48	30.72	6.00E-13
insK (948081)	IS150 transposase B	166.71	283.80	0
Transporters				
gltJ (945443)	glutamate, aspartate ABC transporter permease subunit	3.82	2.54	0
yjhB (948807)	member of the major facilitator superfamily (MFS) of transporters	3.39	150.34	1.55E-07
sgcB (1450295)	predicted enzyme IIb component of PTS	3.08	33.65	6.32E-08
sgcC (946849)	predicted phosphotransferase enzyme IIC component	2.56	25.42	1.85E-09
idnT (948798)	L-idonate and D-gluconate transporter	25.07	19.90	0
yidK (948185)	transporter of the SSS superfamily of sodium dependent transporters	19.10	22.82	0
yihO (948377)	predicted transporter of the major facilitator superfamily of transporters	165.14	112.76	2.68E-12
gntP (948848)	fructuronate transporter	18.28	14.96	0

ulaC (948715)	L-ascorbate-specific enzyme IIA component of PTS	3.52	2.30	3.43E-12
Transcription regulators				
yjhI (949100)	DNA-binding transcriptional regulator (KpLE2 phage-like element)	4.03	2.90	2.17E-01
sgcR (946830)	DNA-binding transcriptional regulator (KpLE2 phage-like element)	2.20	24.56	3.03E-09
idnR (949058)	DNA-binding transcriptional repressor; 5-gluconate-binding	47.23	36.64	2.63E-11
yidL (948186)	predicted transcriptional regulator; AraC family	158.94	166.29	6.13E-12
yihW (948381)	predicted DNA binding transcriptional regulator	8.90	17.97	0
Restriction enzymes				
mcrC (948880)	subunit of 5-methylcytosine-specific restriction enzyme McrBC	3.74	127.24	8.35E-07
mcrB (949122)	subunit of 5-methylcytosine-specific restriction enzyme McrBC	3.69	115.64	2.08E-06
mcrF (948898)	methylated adenine and cytosine restriction protein	2.23	20.75	4.14E-11
Toxins				
yeeV (946534)	toxin of the YeeV-YeeU toxin-antitoxin system (CP4-44 prophage)	26.67	4.16	0
yafO (944916)	mRNA interferase toxin of the yafO-yafN toxin-antitoxin system	11.98	9.18	1.46E-11
Fimbriae or pilin related proteins				
yadK(944835)	predicted fimbriae-like adhesin protein	5.93	3.13	2.51E-02
yhcA (947741)	predicted periplasmic chaperone protein	3.50	2.01	0.371
yadL (944829)	predicted fimbrial-like adhesin protein	2.87	19.63	9.2E-08
yadM (944828)	predicted fimbrial-like adhesin protein	2.80	105.91	5.27E-06
htrE (944819)	probable outer membrane porin protein involved in fimbrial assembly	2.54	91.80	4.78E-07
ecpD (944859)	predicted outer membrane pilin chaperone	4.44	101.56	6.19E-06
yadN (944841)	predicted fimbrial-like adhesin protein	21.24	41.31	1.19E-12
pppA (947467)	bifunctional prepilin leader peptidase/methylase	17.01	5.20	0
fimA (948838)	major type 1 subunit fimbrin (pilin)	14.89	16.04	0
fimB (948832)	tyrosine recombinase inversion of on/off regulator *fimA*	24.28	25.39	1.37E-14
fimC (948843)	periplasmic chaperone	89.01	81.16	1.05E-14
fimD (948844)	outer membrane usher protein	24.14	24.88	0
fimE (948836)	tyrosine recombinase inversion of on/off regulator *fimA*	16.33	17.48	9.64E-12
fimH (948845)	minor component of type 1 fimbriae	14.66	14.96	0
fimI (948841)	fimbrial protein involved in type 1 pilus biosynthesis	15.41	17.02	0
Growth related proteins				
yjaZ (948495)	stationary phase growth adaptation protein	3.16	37.44	6.88E-08
nanS (948835)	9-O-acetylneuraminic acid esterase required for growth on glycerol	23.52	16.71	0

nanM (949106)	N-acetylneuraminic acid mutarotase supports the use of N-acetylneuraminate as the sole carbon source	30.07	23.77	3.13E-12
Membrane proteins				
ompL (948366)	outer membrane porin L	11.01	75.76	6.28E-05
ymfA (945684)	conserved inner membrane protein	24.92	25.83	9.27E-13
nanC (946843)	N-acetylneuraminic acid outer membrane channel protein	15.97	15.37	0
ycfZ (945685)	inner membrane protein	113.15	143.47	5.42E-11
yghJ (2847716)	predicted inner membrane lipoprotien	10.30	6.52	0
Others				
aslA (949015)	acrylsufatase like enzyme	15.36	14.02	0
idnO (947109)	5-keto-D-gluconate-5-reductase	22.76	20.01	0
idnD (944769)	L-idonate 5-dehydrogenase, NAD-binding	37.68	35.55	0
idnK (946066)	D-gluconate kinase	16.84	18.78	0
yfeS (946887)	conserved protein	7.38	185.49	2.91E-06
yjhC (948808)	predicted oxidoreductase involved in metabolism of sialic acid	3.67	127.74	1.29E-06
sgcQ (948834)	predicted nucleoside triphosphatase; KpLE2 phage-like element	2.61	27.58	2.84E-09
sgcX (948840)	predicted endoglucanase; KpLE2 phage-like element	2.71	25.05	7.37E-09
ythA (5625581)	expressed protein	4.31	506.93	1.55E-06
rhsD (945116)	rhsD element protein	4.33	370.31	1.77E-06
ybbC (945115)	probable false positive lipoprotein prediction	3.79	305.60	1.47E-06
ybiU (945439)	predicted protein	14.68	15.69	0
yafP (944912)	predicted acyltransferase	14.72	11.06	1.08E-08
astB (946259)	succinylarginine dihydrolase	24.46	13.00	0
pphB (947196)	serine/threonine protein phosphatase 2	9.27	11.89	1.04E-14
yghD (947025)	predicted secretion pathway M-type protein	10.89	8.93	3.97E-14
yghG (947470)	predicted protein	226.01	157.01	0
yidJ (948188)	predicted sulfatase/phosphatase	14.80	16.51	0
yihQ (948376)	alpha-glucosidase	18.67	17.64	0
yihR (948375)	predicted aldose-1-epimerase	78.42	65.16	4.22E-14
yihS (948374)	D-mannose isomerase	11.03	15.27	0
yihT (948373)	predicted aldolase	11.59	16.73	0
yihU (948372)	Gamma-hydroxybutyrate dehydrogenase	29.36	17.99	0
yihV (948382)	predicted sugar kinase	9.84	17.64	0

yjiN (948860)	zinc-type alcohol-dehydrogenase like protein	6.56	6.29	7.38E-15

Discussion

Cellular growth rate regulates gene expression and is known as growth rate-dependent regulation (Pedersen et al., 1978; Pearse and Wolf, 1994; Tao et al., 1999; Pease et al., 2002). Growth rate changes affect the rates of transcription or mRNA turnover, leading to changes in gene expression levels and protein synthesis. Pedersen et al. (1978) studied concentrations of 140 individual *E. coli* proteins as a function of growth rates in five different media. 102 of 140 proteins analyzed showed linear variation with changes in growth rate. In another study, Pease et al. (2002) demonstrated that expression levels of pyridoxal 5´- phosphate synthesis genes in *E. coli* K12 increased with increase in growth rates over a wide range. The expression levels were measured as the amount of mRNA synthesized per gene and the rate of protein accumulation per gene.

Gene expressions of ATCC8739, SMS-3-5 and BL21 were compared to address why ATCC8739 grow faster on acetate. *folK* gene was one of the 71 genes that were upregulated by more than 2-fold in ATCC8739. 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (HPPK), the protein product of *folK* gene catalyzes the transfer of pyrophosphate from ATP to 7,8-dihydro-6-hydroxymethylpterin, leading to the biosynthesis of folate cofactors. Folates are required for the syntheses of a variety of essential nutrients, such as thymidylate, methionine, purines, serine, glycine and pantothenic acid and hence crucial for cellular replication and growth (Harvey, 1973; Hjortmo et al., 2008). Harvey (1973) observed that addition of 0.1 µg/mL of trimethoprim (TMP; 2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine) reduced the growth rate of *E. coli* by 50 %. TMP inhibited dihydrofolate reductase which in turn blocked the synthesis of thymidine, methionine, glycine and pantothenate which are products of folate pathway. 50 or 100 µg/mL TMP reduced the flow of one-carbon units through the folate

pathway by more than 99.9 %, and cells could sustain growth at a reduced growth rate only by supplementing the medium with the products of this pathway. In another study, highest folate levels were observed during a batch fermentation of glucose to ethanol by *Saccharomyces cerevisiae* at a high growth rate (μ_{max} = 0.37 h^{-1}). Hjortmo et al. (2008) hypothesized that at high growth rates, the requirements for nucleotides for DNA replication and mRNA synthesis and the requirements for amino acids for protein synthesis become large which in turn result in a large folate pool (Hjortmo et al., 2008). Gene *yafP* was more than 5-fold downregulated in ATCC8739 (Table 2.5). Gutierrez at al. (2011) hypothesized that YafP and PolIV proteins form a toxin-antitoxin pair and modulate the balance between mutagenitcity and cytotoxicity. In the absence of PolIV, YafP, the putative acetyltransferase increased the cytotoxicity of two DNA damaging nitroaromatic compounds, 4-nitroquinoline-1-oxide (NQO) and nitrofurazone (Gutierrez et al., 2011). Lower expression of the *yafP* gene in ATCC8739 might indicate towards lower cytotoxicity and in turn an improved growth than the other strains. Gene *yidL* (araC family) is a predicted transcriptional regulator of sugar catabolism whereas genes *yihO* and *yihP* are uncharacterized members of galactose-pentose-hexuronide transporters. However, it is not clear as to how these genes might play a role in growth on acetate.

As stated earlier, AMP-ACS pathway (catalyzed by enzyme acetyl-CoA synthetase ACS) and/or PTA-ACK pathway (catalyzed by acetate kinase ACK and phosphotransacetylase PTA enzymes) mediate the conversion of acetate to acetyl-CoA. However, there were no significant differences in the expression of *acs*, *ackA* and *pta* genes among the strains during the growth on acetate. Interestingly, 13 genes of phenylacetate degradation pathway were more than 2-fold upregulated in ATCC8739 when compared with two other non K-12 strains SMS-3-5 and BL21. The expression level differences of these 13 genes were also positively correlated to growth rate

differences between the strains. These genes are organized into two divergent catabolic operons, *paaABCDEFGHIJK* and *paaZ* and a regulatory operon *paaXY*. PaaX protein acts as a transcriptional repressor, and it is inactivated by phenylacetyl-coenzyme A (Ferrandez et al., 2000; Fernandez et al., 2006). These observations might explain why ATCC8739 grows fast on acetate and indicate towards the possibility that acetate is degraded through the phenylacetate degradation pathway in *E. coli*.

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CHAPTER 3

A NOVEL ADAPTIVE EVOLUTION METHOD TO INCREASE THE GROWTH RATE OF *ESCHERICHIA COLI* ATCC8739 ON ACETATE[1]

[1] Rajaraman, E. and Eiteman, M.A. To be submitted to *Biotechnol. Letters*

Abstract

Among eighteen *E. coli* strains, the C strain ATCC8739 has previously been observed to have the greatest growth rate (0.405 h^{-1}) on acetate. ATCC8739 was evolved in an A-stat fed with 2 g/L acetate for 250 hours. When the growth rate reached 0.5 h^{-1}, three colonies were isolated and compared for growth on acetate. All three isolates grew faster than the parent strain ATCC8739 on acetate, with isolate MEC136 achieving a growth rate of 0.51 h^{-1}. A gene expression comparison between ATCC8739 and MEC136 revealed that gene *yihO* is expressed X-fold less in MEC136.

Introduction and Literature Review

Degradation products like furaldehydes, weak acids and phenolic compounds are formed in pretreatment steps in the process of biological conversion of lignocellulose to biofuels (Parawira and Tekere, 2011). These degradation products are microbial inhibitors (Klinke et al., 2002; Klinke et al., 2004). Acetic acid (acetate) is the most abundant organic acid found in lignocellulosic hydrolysate, and it is generated as an unavoidable degradation product from acetylated xylose in hemicellose (Pettersen, 1984; Sakai et al., 2007b). Presence of acetate can decrease the specific growth rate and biomass yield of *E. coli* on glucose or xylose (Luli and Strohl, 1990; Roe et al., 1998; Takahashi et al., 1999; Roe et al., 2002), and decrease the rate of fermentation of Baker's yeast (Maesen and Lako, 1952). In the presence of other inhibitors like furfural, acetate can synergistically impact the ethanol yield (Palmqvist et al., 1999).

Several methods have been proposed to remove acetate prior to microbial fermentation. These methods include overliming, evaporation (Converti et al., 2000), adsorption with activated charcoal (Carvalho et al., 2006), liquid-liquid extraction (Um et al., 2011), electrodialysis and bipolar electrodialysis (Wong et al., 2009; Wong et al., 2010). All these methods have the disadvantage of involving an additional processing step. Recently a novel method was reported to remove acetate selectively from a mixture of sugars and acetate (Lakshmanaswamy et al., 2011). This method is based on engineering a strain of *E. coli* which will consume only acetate in the presence of multiple sugars. The strain MG1655 *ptsG manZ glk xylA* consumed 10 g/L acetate during 30 h of aerobic batch fermentation in a medium containing 20 g/L glucose and 10 g/L xylose, with less than 1 g/L sugar had been consumed at the time that acetate was exhausted (Lakshmanaswamy et al., 2011).

Microbial "detoxification" of acetate-containing lignocellulosic hydrolysates has a few important advantages. First, some of the acetate carbon is retained as biomass which could serve as microbial nutrients in a subsequent process. Second, as a microbial process itself, selective removal of acetate is very compatible with existing conversion steps (e.g., pH, temperature). However, growth on acetate is comparatively slower than growth on sugars, and *E. coli* strains with knockouts in numerous sugar-consumption genes grow even slower than wild-type *E. coli* on acetate, particularly in the presence of the unmetabolizable sugars. For example, MG1655 *ptsG manZ glk crr xylA* attained a μ_{MAX} of 0.23 h^{-1} in the absence of glucose and xylose and at 0.12 h^{-1} in the presence of sugars in batch fermentations (Lakshmanaswamy et al., 2011). Understanding and improving the growth of *E. coli* on acetate generally would therefore improve the microbial acetate-removal process.

Continuous culture systems like chemostats, auxotats and turbidostats provide a constant environment and are frequently used for studying evolution (Dykhuizen and Hartl, 1983; Sauer, 2001). Crecy et al. (2007) designed a continuous culture system called evolugator to adapt a crippled mutant to higher growth rates. An *Acenitobacter baylyi* strain carrying an engineered deletion is *efp*, a gene involved in translation was evolved for 200 generations and variants with higher growth rates were selected (de Crecy et al., 2007). In another study, Blaby et al. (2012) demonstrated the use of adaptive evolution in an evolugator to increase the growth of *E. coli* MG1655 at higher temperatures. The resulting thermophile was able to grow at temperatures as high as 48 °C and the optimum growth temperature of the strain was increased from 37 °C to over 46 °C (Blaby et al., 2012).

The chemostat is a type of continuous culture method that has been widely used for studying microorganisms under steady-state conditions at a constant specific growth rate (μ).

Under these well-defined conditions, growth parameters such as growth rate or concentration of limiting nutrients can be varied independently (Sauer, 2001). In an accelerostat (A-stat the dilution rate is changed slowly at a constant rate to maintain the culture in a quasi steady-state (Nahku et al., 2010). Therefore, the A-stat is a convenient means for increasing the specific growth rate of a culture slowly.

Comparison of the maximum specific growth attained by eighteen *E. coli* strains on acetate demonstrated that a C strain (ATCC8739) had the greatest maximum specific growth rate (0.405 h^{-1}) (Chapter 2, Table 2.2). The objective of this study is to evolve *E. coli* ATCC8739 for faster growth on acetate using an A-stat and compare gene expression of the initial wild-type and the evolved strains in order to understand characteristics which might impact growth on acetate.

Materials and Methods

Bacterial Strain and Growth Conditions

E. coli C (ATCC8739) was used for the adaptive evolution process. Defined medium used (per liter): 0.425 g citric acid, 3.325 g KH_2PO_4, 1.125 g $(NH_4)_2HPO_4$, 0.3 g $MgSO_4 \cdot 7H_2O$, 3.25 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 0.375 mg $CuCl_2 \cdot 2H_2O$, 3.75 mg $MnCl_2 \cdot 4H_2O$, 0.625 mg $CoCl_2 \cdot 6H_2O$, 0.75 mg H_3BO_3, 0.625 mg $Na_2MoO_4 \cdot 2H_2O$, 25 mg Fe(III) citrate, 1.125 mg thiamine·HCl, 2.1 mg $Na_2(EDTA) \cdot 2H_2O$. Shake flasks and the initial batch phase contained 5 g/L (84.7 mM) acetate, while the feed of the A-stat contained 2 g/L (33.9 mM) acetate. The A-stat was conducted using 800 mL defined medium in a 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific Co. Edison, NJ, USA). To prevent wall growth during the process, the glass vessel and the stainless steel parts of the system were coated with PEG-Silane. First, the fermenter was cleaned and hydroxyl groups were created on surfaces by exposure to hot piranha solution (7:3 1.2 M H_2SO_4:3.0% H_2O_2). Silane was used to attach anti-biofouling polyethylene

glycol (PEG) to the surface as previously described (Papra et al., 2001; Lan et al., 2005). The PEG-silane was coated onto the all surfaces by three days of exposure using 30 mM 2-[methoxy(polyethyleneoxy)propyl] trimethoxysilane (PEG-silane, Gelest SIM6492.7) in toluene. The coating on the stainless steel was confirmed by contact angle analysis, with an average contact angle of 34° indicating the surface presence of the hydrophilic PEG-silane.

To initiate the A-stat, a 5 mL culture of *E. coli* cells was grown in a 30-mL shaking test tube containing 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl and 2.5 g/L acetate, then transferred to a baffled 250 mL shake flask containing 50 mL defined medium incubated at 37°C and 250 RPM, and finally to the bioreactor. Air was sparged into the bioreactor at a flow rate of 1.0 L/min, the agitation was 500 rpm, the temperature was maintained at 37°C, and the pH controlled at 7.0 using 20 % (w/v) NaOH and 20% (v/v) H_2SO_4. When the OD surpassed 2.0 in the batch phase, the A-stat feed was initiated at a dilution rate of 0.25 h^{-1}. After about 4 residence times, the dilution rate was increased by 0.01 h^{-1} about every 4 - 7 hours. When the dilution rate attained 0.35 h^{-1}, the dilution rate was increased by 0.01 h^{-1} about every 6 -12 hours until the dilution rate was 0.5 h^{-1}.

Defined medium was used for the growth rate comparison of evolved strains (per liter): 1.70 g citric acid, 13.30 g KH_2PO_4, 4.50 g $(NH_4)_2HPO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$, 13 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 1.5 mg $CuCl_2 \cdot 2H_2O$, 15 mg $MnCl_2 \cdot 4H_2O$, 2.5 mg $CoCl_2 \cdot 6H_2O$, 3.0 mg H_3BO_3, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 100 mg Fe(III) citrate, 4.5 mg thiamine·HCl, 8.4 mg $Na_2(EDTA) \cdot 2H_2O$, and 5 g (84.7 mM) acetate using $Na(CH_3COO) \cdot 3H_2O$ (concentrations of acetate ion are reported). Acetate solutions were sterilized separately and later combined with other components. The pH was adjusted to 7.0 with a 20% (w/v) NaOH solution. Cells were routinely stored on Luria-Bertani agar plates and then transferred to 50 mL medium in 250 mL

shake flasks, incubated at 37 °C and agitated at 350 RPM. When the OD reached 2.0, 5 mL were transferred to 3 identical flasks for growth rate measurement.

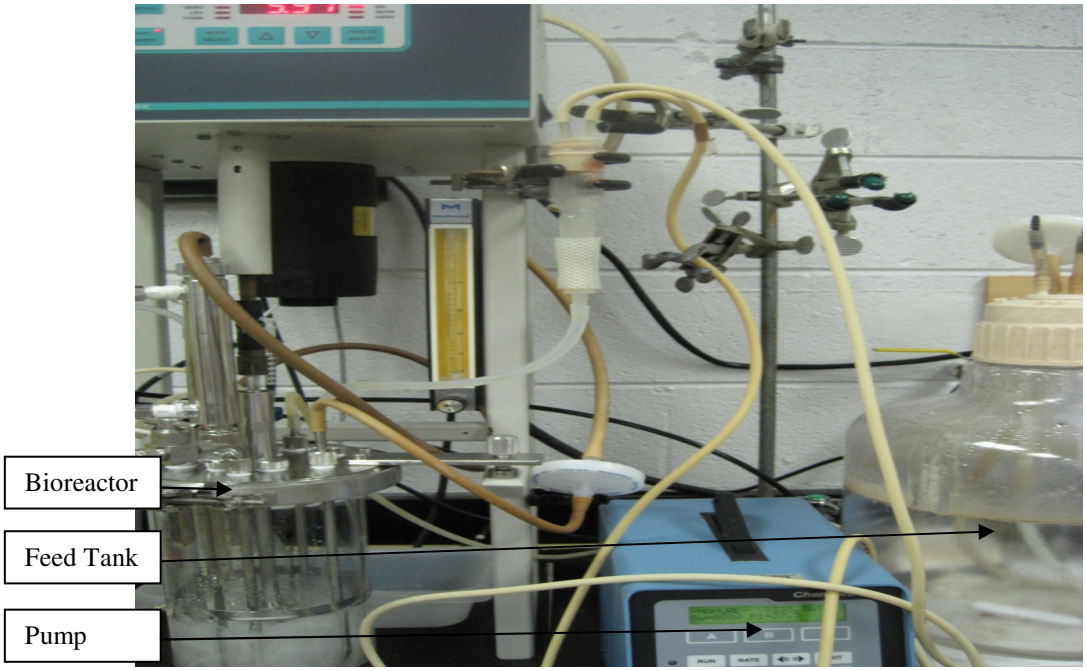

Figure 3.1: Experimental set-up of an A-stat process

Transcriptome Analysis

Escherichia coli K12 (4 × 72K format) microarrays were used in this study (Roche NimbleGen catalog #A6697-00-01.The cDNA samples were shipped to a core facility at Florida State University for microarray analysis.

Cell lysis and RNA Extraction

Samples having OD of 0.5 to 0.6 were mixed with the QIAGEN RNA protect reagent in the volumetric ratio of 1:2, incubated for 5 min at room temperature (15 – 25°C) and centrifuged (5000 × g for 10 min). Supernatant was discarded, and the pellet was stored at -20°C. 200 µL of TE buffer containing 10 mg/mL lysozyme and 15 µL proteinase K was used as the lysis reagent. Addition of lysis reagent was followed by incubation in a water bath at 37°C for 10 min and three freeze-thaw cycles (Lahtvee et al., 2009). Total RNA was extracted using QIAGEN RNeasy Mini kit and genomic DNA was removed using QIAGEN RNase-free DNase set.

cDNA synthesis

Single stranded cDNA was synthesized from total RNA using superscript III first strand synthesis system (Invitrogen, Carlsbad, CA). 5 µg of total RNA was combined with the following components on ice (per sample): 2 µL of 50 ng/µL random hexamers, 2 µL of 10 mM dNTP Mix, and Diethylpyrocarbonate (DEPC)-treated water to make up the total volume to 26 µL. The samples were incubated at 65 °C for 5 min in a thermocycler (Finnzymes, Vantaa, Finland). The first strand cDNA synthesis mix consisted of the following reagents (per reaction): 8 µL 5× 1^{st} strand buffer, 2 µL 0.1 M DTT, 2 µL RNase OUT, 2 µL Superscript reverse transcriptase III, and 14 µL of first strand cDNA synthesis master mix. After mixing thoroughly, samples were incubated at 50°C for 4 hours and at 70°C for 15 minutes in the thermocycler. 2 µL of RNase H was added to the PCR product to remove RNA complementary to the cDNA and was followed by incubation at 37°C for 20 min in the thermocycler. cDNA cleanup was carried out using QIAGEN Qiaquick PCR purification kit.

Microarray analysis

The labeling, hybridization, washing and image scanning steps were performed at a core facility in Florida State University. The samples were labeled with cy3 random nonamers (dye) on an Applied Biosystems Veriti, hybed on a 4 bay NimbleGen hybridization (Applied Biosystems, Foster City, CA). Scanning of the array was performed using a NimbleGen MS200 2 µm scanner (Roche NimbleGen, Madison, Wisconsin). Raw data extraction, image analysis and normalization steps were performed using NimbleGen's DEVA software. ArrayStar software was used for data analysis (DNASTAR, Madison, WI).

Analytical Methods

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth.

All fermentation samples were stored at -20°C for analyses. These samples were centrifuged (8400×g for 10 minutes), and the supernatant solution used for HPLC measurement of acetate concentrations. A Shimadzu HPLC (LC-6A pump, SCL-6B system controller and auto-injector) with a refractive index detector (Waters Model 2410), a Coregel 64-H ion-exclusion column (transgenic) and a mobile phase of 4 mN H_2SO_4 was used for the analyses (Eiteman and Chastain, 1997).

Results

A-stat growth on acetate

ATCC8739 has previously been reported to have a growth rate of 0.405 h^{-1} on acetate medium (Chapter 2, Table 2.1). In this current study ATCC8739 was evolved for growth on acetate by exposure to acetate at progressively greater growth rates in A-stat culture (Fig. 3.1). After achieving steady-state at a growth rate of 0.25 h^{-1}, the D was increased by 0.01 h^{-1} periodically, slowing the rate as the D approached the reported μ_{MAX} of for ATCC8739 (0.405 h^{-1}). After 2.5 h at a dilution rate of 0.47 h^{-1}, the OD had decreased by 12%, so the dilution rate was reduced to 0.45 h^{-1}. After an additional 9.5 h, the OD had decreased by an additional 50%, so the feed was paused for 30 min. After this time, the feed was resumed at 0.45 h^{-1}, and the process continued until the dilution rate attained 0.5 h^{-1}. The A-stat method was carried out for about 250 h.

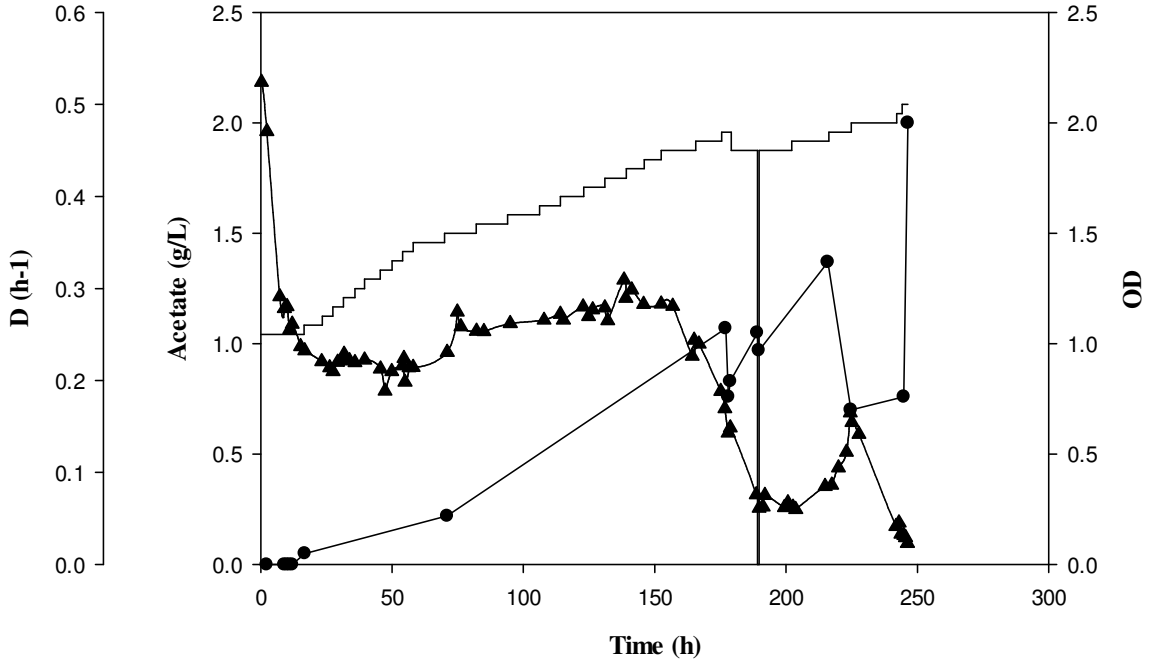

Figure 3.2: Growth characteristics of ATCC8739 during the A-stat process. The graph represents stepwise change of D, change in OD (▲) and acetate concentrations (●) over the course of 250 hours.

Growth rate study of evolved strains

Three colonies isolated from the A-stat operating at 0.5 h^{-1} were selected to determine specific growth rate on a defined medium containing 5 g/L acetate. The maximum specific growth rates (μ_{max}) of all 3 isolates MEC134, MEC135 and MEC136 were greater than that of the parent strain ATCC8739 (Table 3.1). Compared to the wild-type, MEC134 and MEC136 attained a much faster growth rate 0.50-0.51 h^{-1}, while isolate MEC135 had a μ_{max} of 0.432 h^{-1}. A comparison of plots of ln(OD) versus time clearly illustrates the difference in growth rates between the evolved strains and the parent strain (Figure 3.3).

Table 3.1: Maximum specific growth rates (μ_{max}) of the evolved strains in comparison with the parent strain

E. coli Strains	μ_{max} (h^{-1})
MEC134	0.50±0.01
MEC135	0.43±0.01
MEC136	0.51±0.01
ATCC8739	0.41±0.01

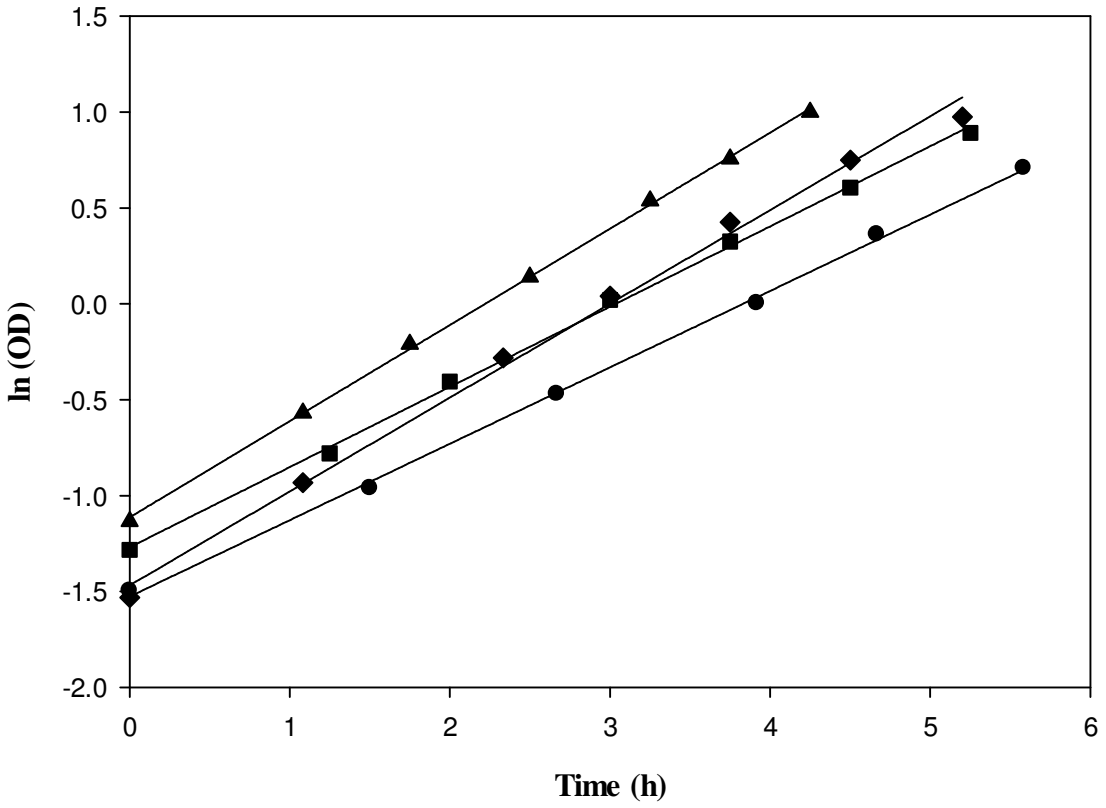

Figure 3.3: Growth rate comparison of MEC134 (♦), MEC135 (■), MEC136 (▲) and the parent strain ATCC8739 (●) on a defined medium containing 5 g/L acetate.

Transcriptome analysis

MEC136 was selected for transcriptome comparison with ATCC8739 to determine what genes were preferentially expressed in MEC136 and may be correlated to the latter's elevated growth rate on acetate. Only one gene named *ylcG* was more than upregulated 2-fold or more in MEC136 than ATCC8739. On the other hand, 10 genes were downregulated 2-fold or more in MEC136. Among the 10 genes, *rhsE yfdL* and *yjdQ* are pseudo genes. *ymfM* and *rnlA* are prophage proteins.

Table 3.2: Genes with atleast two-fold expression difference between MEC136 and ATCC8739

Gene(GeneID)	Function	Fold change	p-value
ylcG (1450240)	Expressed protein, DLP12 prophage	2.12 up	0.967
ymfM (945715)	e14 prophage; predicted protein	2.31 down	0.78
rhsE (946026)	Pseudo	3.75 down	1
yegJ (947201)	predicted protein	6.01 down	0.359
gtrB (949098)	Bactoprenol glucosyl transferase	3.91 down	1
yfdL (949099)	Pseudo	3.53 down	1
xapA (946878)	purine nucleoside phosphorylase II	2.23 down	0.921
rnlA (947107)	RNase LS	2.11 down	1
yihO (948377)*	predicted transporter	2.35 down	1
mcrB (949122)	5-methylcytosine-specific restriction enzyme	2.14 down	0.953
yjdQ (5625580)	Pseudo	2.33 down	1

***-** At least 2-fold downregulated in ATCC8739 in comparison to BL21 and SMS-3-5 (Chapter 2)

Discussion

Using an A-stat, *E. coli* ATCC8739 evolved into two isolates with 25% greater maximum specific growth rate. Gene expression comparison of the evolved isolate MEC136 with the parent strain ATCC8739 revealed that *ylcG* gene was more than 2-fold upregulated in MEC136. The *ylcG* gene encodes a DLP12 prophage protein classified as a small protein (length: 46 amino acids) with unknown function (Hemm et al., 2008). Of the 10 genes that were 2-fold or more downregulated in MEC136 in comparison to ATCC8739, *yihO* is the only gene that might be related to acetate metabolism. The YihO protein is an uncharacterized member of the GPH family of galactose-pentose-hexuronide transporters. Although the function of the protein is not known, it is believed to be involved in a proton or other ion-driven metabolite uptake system (Poolman et al., 1996). Interestingly, the expression of *yihO* is 100-fold lower in ATCC8739 compared to SMS-3-5 and BL21 (Chapter 2, Table 2.5). Therefore, this gene may serve an important role related to the uptake of acetate by ATCC8739 and MEC136.

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CHAPTER 4

CONCLUSIONS

The long term vision of this project is the complete removal of acetate present in lignocellulose hydrolysate using substrate-selective degradation (That is, selective consumption of acetate from a mixture of sugars and acetate). The motivation of this research is that substrate-selective degradation of acetate can be improved. Therefore, a major component of this research has been to understand and improve the growth of *E. coli* on acetate. The first goal of this research was to compare several strains of *E. coli* for growth on acetate and identify the fastest growing strain. Among the 18 strains that were compared, ATCC8739 was found to have the greatest maximum specific growth rate of 0.405 h^{-1}. Also, we observed that different strains of *E. coli* had different growth rates on acetate and they were widely distributed. Based on this observation, the second goal of this project was set to find out why ATCC8739 grow faster on acetate when compared to SMS-3-5 and BL21. Gene *folK* was more than 2-fold upregulated only in ATCC8739. The *folK* gene codes for a protein which is involved in folate biosynthesis. Products of folate pathway have been found to be vital for growth of *E. coli* (Harvey, 1973). Also, high levels of folate have been correlated with high growth rates in *Saccharomyces*

cerevisiae (Hjortmo et al., 2008). These observations suggest that overexpression of *folK* in ATCC8739 is correlated with the high growth rate of the strain. Surprisingly, no differential expression of acetate metabolic genes among the strains was found. However, we observed that 13 genes of the phenylacetate degradation were upregulated in ATCC8739. Additionally, the differences in these 13 genes were positively correlated with differences in growth rate of ATCC8739, SMS-3-5 and BL21 (Table 2.7). These observations might help understand the faster growth of ATCC8739 on acetate.

The third goal of this project was to evolve ATCC8739 for faster growth rate on acetate suing an A-stat. Three colonies isolated from this process at D = 0.5 h^{-1} and after 250 hours of evolution had maximum specific growth rates greater than the parent strain ATCC8739. Particularly, the isolate MEC136 had a μ_{MAX} of 0.51 h^{-1}. Gene expression comparison between ATCC8739 and MEC136 revealed that gene *yihO* is more than 2-fold underexpressed in MEC136. Interestingly, this gene is more than 100-fold underexpressed in ATCC8739 when compared with the other 6 strains. Although the function of this gene is not known, it is probably important to understand why ATCC8739 and MEC136 grow fast on acetate.

To conclude, the correlation between growth rate and gene expression differences is not clear. However, the fast growth rate of ATCC8739 on acetate could be explained by differences in expression of *folK* gene and genes of phenylacetate degradation pathway. This research also demonstrates that growth rate of *E. coli* ATCC8739 on acetate could be increased by a novel A-stat process.

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