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Effects of mlc Gene Modulation on Acetate Accumulation in Escherichia Coli Culture

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EFFECTS OF *mlc* GENE MODULATION ON ACETATE ACCUMULATION IN
ESCHERICHIA COLI CULTURE

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by

Melvin F. Chen

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APPROVED FOR THE DEPARTMENT OF CHEMICAL ENGINEERING

SAN JOSE STATE UNIVERSITY

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ABSTRACT

EFFECTS OF *mlc* GENE MODULATION ON ACETATE ACCUMULATION IN
ESCHERICHIA COLI CULTURE

by Melvin F. Chen

When *Escherichia coli* (*E. coli*) is grown in the presence of excess glucose,

acetate is produced, oftentimes as an undesired by-product. Mlc is a global repressor for

sugar transporters in *E. coli*, including glucose. This body of work examines the

overexpression of Mlc via expression vectors in *E. coli* cultures under constitutive and

inducible promoters. Sequence changes to the translational start codon and codon 52 of

the *mlc* sequence inserted in the expression vectors were introduced. These changes were

evaluated for their impact on glucose uptake rates, acetate production, and overall cell

growth when Mlc was overexpressed in *E. coli* cultures in the presence of excess glucose.

Furthermore, expression vectors carrying the *mlc* gene versions were co-transformed with

a plasmid encoding for a therapeutic protein in order to study the impact of Mlc

overexpression on the production of the therapeutic protein. Results showed varied

levels of Mlc overexpression; however a correlation was drawn between increased Mlc

expression and decreased acetate production as a result of slower glucose uptake into the

cell. This characteristic resulted in improved cell growth in the form of higher density

cultures. In addition to growing to higher cell densities, a 1.7-fold increase of therapeutic

protein production was observed in cultures overexpressing Mlc, compared to the control.

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CHAPTER ONE: INTRODUCTION

Cohen *et al.* showed in 1973 that it is possible to successfully transform biologically functional recombinant plasmids into *Escherichia coli* (*E. coli*). This event initiated the field of biotechnology [1] and *E. coli* will be forever linked to the birth of biotechnology. In 1978, scientists at Genentech, Inc. and the City of Hope National Medical Center successfully synthesized human insulin utilizing recombinant DNA technology and the machinery of *E. coli*. In 1982 the Food and Drug Administration (FDA) approved for the first time a recombinant protein (human insulin) to be used as a therapeutic [2] and through 2008 there have been 151 FDA and/or European Medicines Agency (EMEA) approved recombinant therapeutics produced from bacterial or yeast cells [3, 4]. *E. coli* is a gram-negative, rod-shaped bacterium that has been utilized in industry because of its "ease of manipulation," "long history in laboratory cultures," and known genome [5]. *E. coli* cultures have been shown to achieve high volumetric recombinant protein productivities at high cell densities and a major effort has been made in industry to improve production cell densities to maximize product yield [6].

1.1   Glucose Metabolism in *E. coli* cultures

The general assumption is that *E. coli* cells will continue to grow as long the nutrients required for growth are present. One major requirement for cell growth is a carbon source and oftentimes glucose is provided to fill this need [6]. To maximize cell growth and density, glucose is often provided in excess; the consequence, however, is the formation of acetate. It has been suggested that acetate production is the result of an imbalance between glucose metabolism and respiration [7]. Within *E. coli*, glucose is

metabolized through glycolysis and one of the products from this process, pyruvate, is oxidized and shuttled into the tricarboxylic acid cycle (TCA) also known as the citric acid cycle. The latter stage is cellular respiration and it is an imbalance between this and glycolysis that leads to excess acetate formation, as depicted below in Figure 1. Under aerobic conditions acetate is formed as a byproduct of pyruvate and acetyl CoA metabolism [8]. A major contributor of acetate formation is the metabolism of acetyl-CoA through the Pta-Ack pathway, which becomes activated from overloading the TCA cycle [10]. The Pta-Ack pathway is responsible for the catabolism and anabolism of acetate. *E. coli*, however, will metabolize glucose solely as its carbon source if it is present; therefore, acetate continues to accumulate, as a result of glucose-rich media.
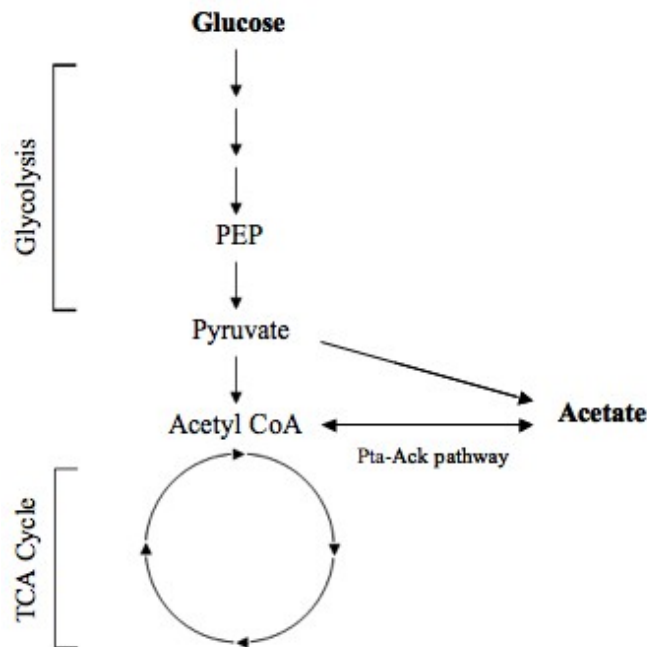
Figure 1. Acetate is formed as a result of glucose metabolism in *E. coli* culture [8]. Overloading the TCA cycle can lead to acetate accumulation.

The products from the TCA cycle are then passed to the membrane bound electron transport chain (ETC) where ATP is produced. Under aerobic conditions,

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oxygen serves as an electron acceptor at the end of the ETC. *E. coli* is metabolically versatile and can sustain growth under anaerobic conditions. The absence of oxygen results in the reduced utilization of the TCA cycle leading to the incomplete oxidation of glucose or other sugars. As a result, acetate is formed as the primary byproduct [9].

1.2   The Acetate Problem

The accumulation of acetate within *E. coli* has a toxic effect and has been studied for decades dating back to the early 1980s [6]. Within the cell, acetate can be found in two forms, as an anion salt (CH_3COO^-) and as a protonated form (CH_3COOH) depending on pH; these are diagrammed below in Figure 2. It is generally understood that acetate is produced in its protonated acidic form under aerobic conditions in a medium of relatively neutral pH [11]. As a result, acetate lowers the pH of the culture thereby creating an unfavorable environment for the cells to grow. It has been shown to have the greatest negative effects at concentrations greater than 2 g/L [6]. Studies have shown a direct impact of acetate accumulation on recombinant protein production as well as a reduction in cellular growth rate, biomass yield, and cell densities [10].
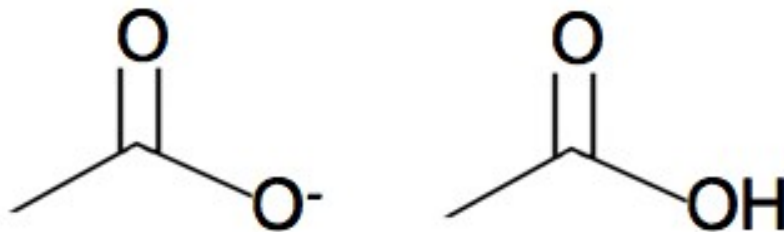
Figure 2. The ion and protonated form of acetate (from left to right). Both forms are found at neutral pH [5].

As a result, several efforts have been made to eliminate or minimize the effect acetate has on fermentation cultures including the use of fed-batch cultures to control the

level of glucose uptake, two-stage cultures, *in situ* removal of acetate, and buffered media to control the pH of the culture. Each has resulted in limited or minimal success [11].

1.3   Background

1.3.1   Transport of sugar across cell membrane

For glucose and other sugars to be utilized by *E. coli*, they must be transported from the medium across the cell membrane into the cell where it undergoes glycolysis. The mechanism responsible for the transport of sugar utilizes a complex network of enzymes and proteins known as the phosphotransferase system (PTS). There is a specific PTS responsible for the transport of glucose; however, there are others responsible for transporting other sugars that also have the capability of transporting glucose. The role of the PTS, as the name suggests, is to transfer a phosphate group from phosphoenolpyruvate (PEP) to a sugar. In the glucose specific PTS, the phosphate group is passed from PEP to Enzyme I (EI) to histidine protein (HPr) to Enzyme IIA (EIIA^{Glc}) to the inner membrane bound Enzyme IIB and integral membrane protein Enzyme IIC (referred to jointly as EIICB^{Glc}) and lastly to glucose upon transfer across the cell membrane forming glucose-6-phospate (G6P), the first step of glycolysis [12]. This is illustrated in Figure 3. This initial step in glycolysis prevents glucose from escaping from the cell back into the medium.

Enzymes EI and HPr are generic transporters, while EIIA^{Glc} and EIICB^{Glc} are specific to the transport of glucose. Each is encoded by the *ptsI*, *ptsH*, *crr*, and *ptsG* gene, respectively where *ptsI*, *ptsH* and *crr* are a part of the *pts* operon and *ptsG* is part of the *ptsG* operon. An operon describes the collection of genes (called structural genes)

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generally under the control of a single promoter (which initiates transcription).

Additionally, this group of genes may be under the control of an operator, a region of

DNA in which a repressor may bind to negatively regulate transcription.
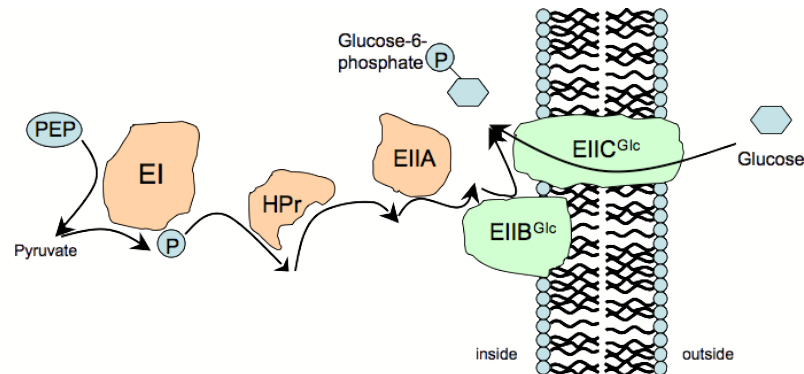
Figure 3. The phosphotransferase system (PTS) transfers glucose from outside the cell across the cell membrane into the cytoplasm by process of transferring a phosphate group originating from phosphoenolpyruvate (PEP) [12].

1.3.2   Gene Regulation and Expression

The regulation of transcription generally occurs in regions just upstream of the

transcriptional start site [13]. These regions are called the promoter and the operator.

The promoter is the sequence of DNA recognized and bound by RNA polymerase (the

enzyme responsible for transcription). Some promoters are always "on," also known as

constitutively active, meaning RNA polymerase is always able to bind, and transcription

can continuously occur. There are also instances of positive and negative regulation,

where promoters need to be turned on or can be turned off with an inducer or repressor.

There are proteins that recognize and bind to the operator region, leading to a

conformational change, which alters the interaction between RNA polymerase and

promoter. Figure 4 illustrates the relationship of the promoter and operator in relation to

the transcribed region of a gene.
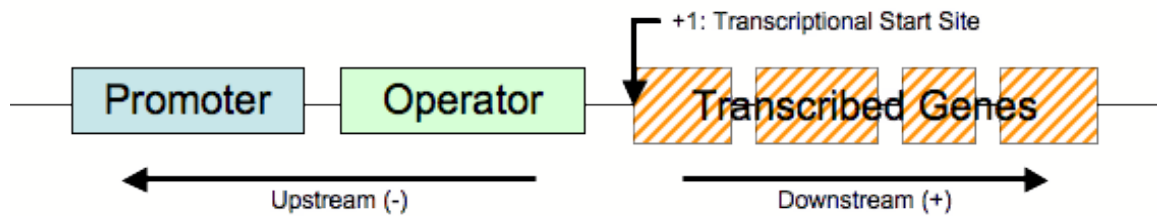
Figure 4. A generalized illustration of the regions of a gene responsible for regulation and expression. The transcriptional start site is referred to position +1; all other sites are given in relation to this site [13].

1.3.3 Positive Regulation by CRP-cAMP

An example of positive regulation is the CRP-cAMP complex [13]. Cyclic adenosine monophosphate (cAMP) binds to the cAMP repressor protein (CRP) forming a CRP-cAMP complex that is able to bind a specific site in DNA (neither protein is able to bind DNA by itself). The bound complex leads to a conformational change in the DNA, which then allows for RNA polymerase to bind and transcribe the downstream genes. The expression of the *pts* and *ptsG* operons are both regulated by this complex.

1.4 Discovery of the *mlc* gene

In 1995, Hosono *et al.* created a gene library to identify genes that would overcome acetate inhibition [10]. They created a mixture of plasmids by ligating expression vector pUC19 (digested with *BamHI*) with segments of chromosomal *E. coli* DNA. A wild-type strain of *E. coli* was transformed with the resulting plasmid mixture. The transformants were then plated on agar plates and the resultant colonies were observed. Most were small; however, one was much larger. It was postulated that the larger colony was the direct result of the acquired ability to overcome the toxic effects of acetate. The plasmid DNA from that colony was isolated and through a DNA deletion technique, the DNA sequence responsible for forming the larger colony was isolated.

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This gene was named *mlc* short for "making large colonies" (convention states that when using all lower case, italicized letters, *mlc* refers to the gene, whereas capitalizing the first character, [e.g. Mlc] describes to the protein). These steps are illustrated below in Figure 5. Subsequent studies have shown that the introduction of an Mlc expressing plasmid in to *E. coli* can overcome the inhibitory effects of acetate [14].
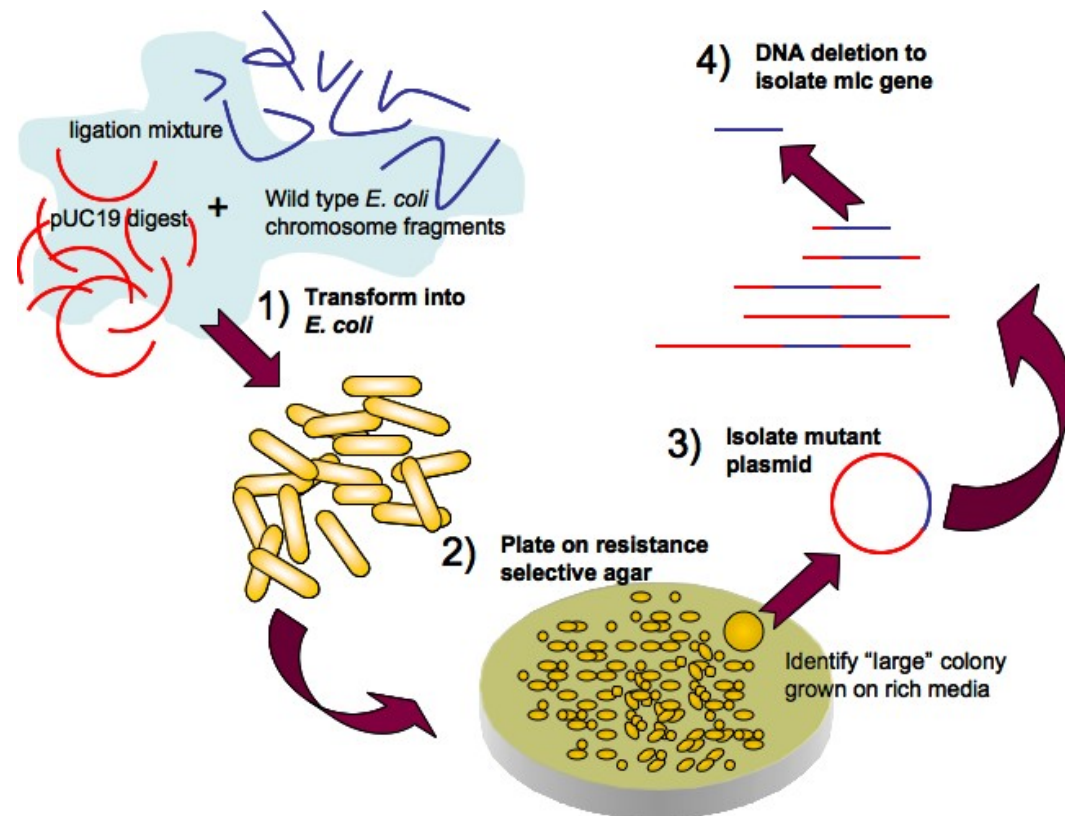
Figure 5. The *mlc* gene was discovered be transforming recombinant plasmids into *E. coli* and identifying those plasmids that allowed for the growth of large colonies [10].

Mlc is a 44.5 kDA protein that has been shown to be a transcriptional regulator, which controls glucose uptake in *E. coli* by repressing the expression of genes associated with the phosphotransferase system (PTS) and transport of glucose across the cell membrane. Overexpression of Mlc slows the uptake of glucose into the cell significantly,

such that acetate is formed as a by-product of glycolysis at a rate in which *E. coli* can process it before it accumulates to concentrations that inhibits cell growth [15].

It has been shown that Mlc is a global repressor for the genes encoding the transporters of sugar within *E. coli*, in particular, glucose [14]. By limiting the uptake of glucose within the cell, Mlc impacts the glycolytic pathway and TCA cycle. Reducing the rate at which glucose is introduced into the cell also lowers the rate at which pyruvate is produced through glycolysis. The decreased rate of pyruvate generation reduces the burden on the TCA cycle and allows for more pyruvate to be metabolized through the TCA cycle rather than through the Pta-Ack pathway, thus reducing the formation of acetate.

1.5   Significance

Overcoming acetate accumulation while continuing to grow in the presence of excess glucose could allow for *E. coli* cultures to achieve higher cell growth rates and fully utilize their carbon source. With recombinant protein production, higher growth rates and longer culture runs could lead to increased product yields. Limiting the rate of glucose uptake by overexpressing Mlc may reduce acetate production such that there is no accumulation or it is reduced significantly, minimizing any toxic effects.

This project studied the overexpression of Mlc and its effect on cell density and recombinant protein production. Modulations to the *mlc* gene and its expression were incorporated based on results reported in the literature where several sequence changes improved the expression of Mlc. These included sequence variations to the *mlc* gene,

induced and constitutive expression, and use of *E. coli* host strains of with different

genotypes.

CHAPTER TWO: LITERATURE REVIEW

2.1   Discovery of the *mlc* gene

In 1995, Hosono *et al.* attempted to identify the gene(s) that were linked to the inhibition of *E. coli* growth in glucose-rich media caused by the accumulation of acetate [10]. A ligation mixture of the plasmid pUC19 digested with *Bam*H1 and chromosome fragments from wild-type *E. coli* strain W3110 partially digested with *Sau*AI was transformed into the streptomycin resistant *E. coli* strain JM103. From the resulting transformants, one colony (out of 3,000) grew noticeably larger than the others. The DNA inserted into the plasmid was shortened until the gene that gave the host strain the ability to grow larger colonies was isolated. This work is illustrated in Figure 3. Because this gene was speculated as being responsible for <u>m</u>aking <u>l</u>arge <u>c</u>olonies, it was named after the acronym: *mlc*. The nucleotide sequence that was identified as the gene *mlc* is depicted in Figure 6.

The researchers continued to study the growth of *E. coli* with the *mlc* containing plasmid compared to the wild-type strain [10]. In the presence of glucose, the wild-type strain accumulated acetate, which lowered the pH and caused growth to stop. This occurred prior to the exhaustion of the glucose supply. A similar culture was grown using a sodium phosphate buffer (pH 7.2) to help control the pH. In this case, acetate was produced and initially accumulated in culture, however once the glucose was fully metabolized the acetate was depleted from the media as well, supporting the theory that the drop in pH due to acetate accumulation renders a toxic effect. A culture of the mutant strain consumed glucose at a slower rate compared to the aforementioned wild-type

cultures (regardless of buffered media), however nearly half the amount of acetate was

accumulated and similar to the buffered cultures, they continued to grow until both

glucose and acetate were completely consumed.

Figure 6. The nucleotide sequence for *mlc* gene was shown to be responsible for overcoming acetate inhibition in glucose-rich media. The boxed region starting at 110 bp indicates the CRP-cAMP binding site responsible for positive regulation. The amino acids associated with the codons in the coding region of the gene are listed below in sequence (in single character abbreviations) [10].

Lastly, Hosono *et al.* (1995) showed that the Mlc protein was a sequence homolog to NagC, a protein involved in the uptake and metabolism of N-acetylglucosamine (a glucose derivative) [10]. Amino acid sequence comparison showed 40.0% identity and 81.3% similarity between the two proteins.

2.2   The function of Mlc as a repressor

Due to its high homology to NagC, Mlc was studied with regards to its role in sugar transport within *E. coli*. In 1998, Plumbridge and Decker *et al.* showed that Mlc affected the manXYZ operon and maltose regulon, respectively [16, 17]. Each encodes for transporters responsible for bringing specific sugars into the cell. The manXYZ operon is responsible for several sugars including glucose and mannose. The maltose regulon consists of four operons that produce the transporters for maltose and maltodextrins and is regulated by a single regulator protein, MalT [18]. Using DNase footprinting, a technique that identifies DNA-protein interactions with radioactively labeled DNA templates and DNase (an enzyme that randomly cleaves DNA), regions of DNA that Mlc bind could be identified.

In the case of the *manXYZ* operon, Mlc was found to bind to two binding sites for the repressor NagC. Mlc in fact, was shown to bind more tightly to NagC operators than NagC itself. Plumbridge [16] conducted two similar sets of experiments on NagC and Mlc to study their impact on the manXYZ operon. Each repressor gene was knocked out and overexpressed (in separate experiments) and the expression of the first gene of the operon, *manX*, was observed. For NagC, the null mutation resulted in minimal effects whereas the Mlc null yielded a three-fold increase in *manX* expression. Overexpression

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of NagC led to an eight-fold decrease in *manX*. Similarly, overexpression of Mlc through

a plasmid utilizing the endogenous Mlc promoter reduced expression by 10-fold.

Another experiment performed by Plumbridge [16] overexpressed Mlc using the *lac*

promoter and yielded greater than 100-fold decrease in *manX* expression.

The effect of Mlc on the maltose regulon is different, but yields similar results.

The regulon is under the positive control of a single transcriptional activator, MalT [18].

Experiments show that Mlc does not bind directly to regions of the maltose regulon but

rather binds to the regulating region of *malT*, thus repressing the expression of the

positive activator. The expression of Mlc from a multicopy plasmid reduced the

expression of MalT, while the null mutation of Mlc led to a three-fold increase in

expression. Additionally, using the same DNase footprinting technique, Decker *et al.*

(1998) showed that Mlc binds to its own regulating region, thus repressing its own

expression [17]. Having been shown to repress the expression of various sugar

transporters, suggests that Mlc may regulate other operons as well and may be a global

regulator of carbohydrate metabolism.

Later in 1998, Plumbridge and Kimata *et al.* [14, 19] conducted separate studies

in which they each linked Mlc to the repression of a gene that is part of the

phosphotransferase system (PTS). That gene, *ptsG*, encodes one component ($EIICB^{Glc}$)

of the two part glucose-specific transporter EII^{Glc}. They showed that Mlc binds to two

different sites in the *ptsG* promoter region, which inhibits the transcription of *ptsG* by

prohibiting the binding of RNA polymerase. Kimata *et al.* (1998) showed that although

Mlc affects the transport of glucose and other sugars, the sugars did not directly affect

Mlc activity [14]. Figure 7 shows a gel mobility shift assay, where several sugars were run with Mlc and DNA fragments containing the *ptsG* promoter. The results show that glucose, mannose and xylose did not affect the binding of Mlc to the *ptsG* promoter.
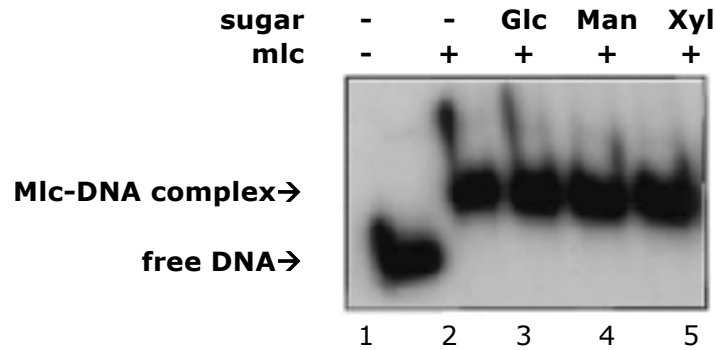
Figure 7. A gel mobility assay show that the presence of sugar (Glc = glucose, Man = mannose, Xyl = xylose) does not affect the activity of Mlc. "+" denotes the presence of while "-" denotes the absence. Adapted with permission from ref. [14] © 1998 Molecular Microbiology.

Several studies were conducted to further investigate the effect Mlc has on the PTS, specifically its role in regulating *ptsHI*, the operon that codes for two PTS proteins, enzyme I (EI) and HPr. Each is a soluble protein that functions to transfer phosphate groups to membrane-bound sugar transporters such as the glucose transporter EII^{Glc} [20-22]. PtsHI has been shown to be under the control of two promoters, P0 and P1 [23]. Examination of the mRNA through S1 analysis showed that Mlc enhances expression from the P0 promoter, however only a negligible difference in expression is observed with the P1 promoter. Additionally, similar to the previous findings, the researchers found that Mlc represses the *ptsHI* operon, while glucose induces its expression. It was shown that a mutation in the *mlc* gene led a four-fold increase in *ptsH* expression, while the presence of *mlc* on a multicopy plasmid repressed expression by three-fold. Thus it

was concluded that Mlc negatively regulates the pts operon. The regulation of the *pts* operon is depicted in Figure 8.
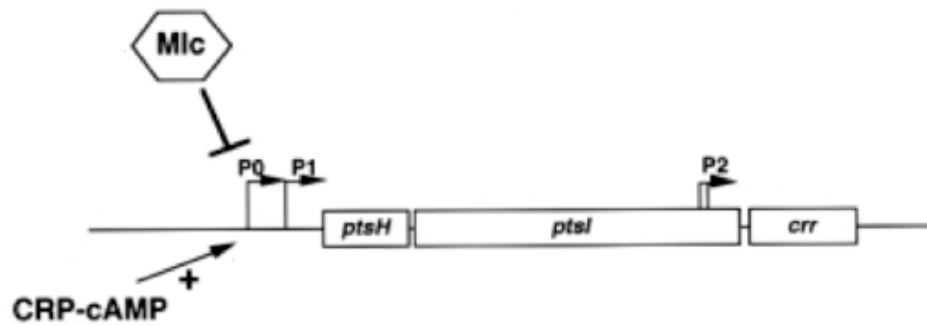
Figure 8. The *pts* operon consists of three genes: *ptsH*, *ptsI* and *crr*. It is positively and negatively regulated by CRP-cAMP and Mlc, respectively at the P0 promoter site. Reprinted with permission from ref. [23] © 2001 EMBO Journal [23].

It has been shown through repeated experiments that Mlc represses several PTS associated operons and that in wild-type cells, glucose relieves the Mlc-mediated repression and induces the expression of the targeted genes. The mechanism, however, by which glucose directly or indirectly modulates Mlc activity had not yet been determined. In 2000, Tanaka *et al.* showed that an interaction exists between non-phosphorylated EII^{Glc} and Mlc [23]. Without a glucose source present, EII^{Glc} is in the phosphorylated form and does not bind Mlc. As a result, the repressor freely moves throughout the cytoplasm regulating the transcription of the pts operon. In the presence of glucose, EII^{Glc} is generally found in the dephosphorylated state because of PEP-dependent phosphorylation of glucose. In the phosphorylated form, Tanaka *et al.* (2000) showed EII^{Glc} sequesters Mlc to the membrane, physically removing Mlc from DNA access in the cytoplasm thereby removing the repressor and inducing transcription of the

pts operon. Figure 9 depicts the proposed model of EII^{Glc}-Mlc interaction in the presence

and absence of glucose. Nam *et al.* (2001) later confirmed these findings [24].

A -glucose

IICB^{Glc}

-P

IIA^{Glc}-P

HPr

Mlc

EI

IICB^{Glc} is low and phosphorylated

PEP

Mlc is in the cytoplasm

B +glucose

Glucose

IICB^{Glc}

IIA^{Glc}

Mlc

G-6-P

HPr

EI

IICB^{Glc} is high and dephosphorylated

PEP

Mlc is sequestered at the membrane
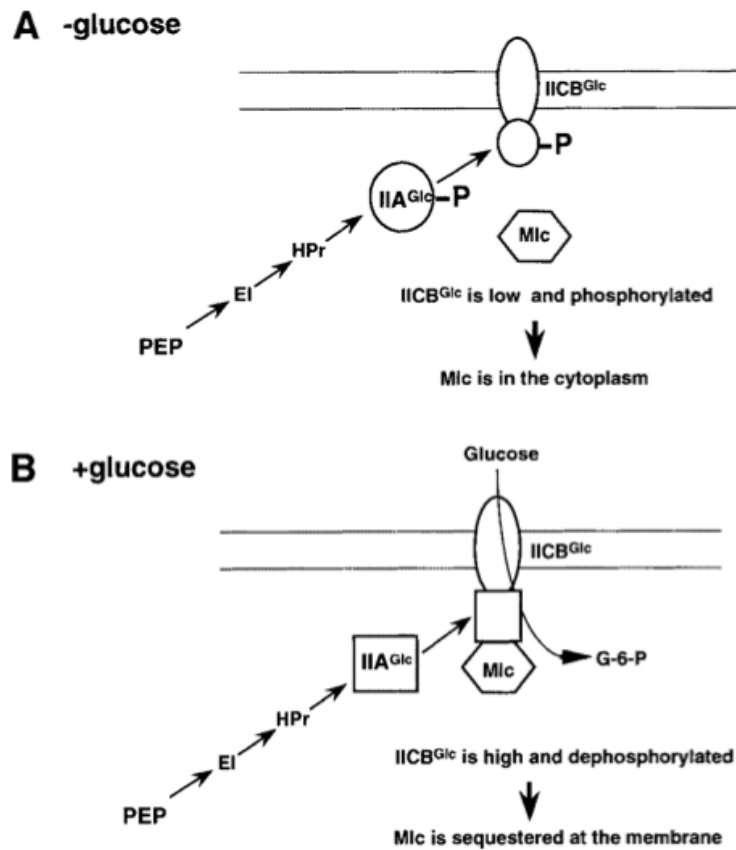
Figure 9. The membrane bound component of the glucose transporter, IICB^{Glc} sequesters Mlc when it is in the dephosphorylated state limiting the Mlc available to repress the expression of *pts* related operons. Senarios A and B depicts the situations where glucose is absent and present, respectively. In the absence of glucose, IICB^{Glc} is phosphorylated and is unable to bind Mlc. Reprinted with permission from ref. [23] © 2001 EMBO Journal [23].

2.3   Modulation of Mlc expression

With its function as a repressor for the *pts* operon better understood, Cho *et al.*

(2005) sought to utilize the ability for Mlc to regulate glucose metabolism within *E. coli*

and study how it could affect recombinant protein expression [11]. The researchers

created strains of *E. coli* that overexpressed Mlc with specific mutations in the regulatory

region of the repressor gene. Three mutations were designed, the first was a mutation in

the start codon replacing GUG with AUG; the second involved an alteration to the Mlc

binding site to prevent autoregulation; and the third mutation was an optimization of the

Shine-Dalgarno sequence and a mutation in the -10 region from CACCAT to TATAAT.

The first mutation was introduced into a host strain of *E. coli*, MC4100, and named

SR752. Two additional Mlc overexpressing strains were constructed, one a combination

of the first and second mutations, named SR753, and another combining all three, SR754.

The modifications to the promoter region of the Mlc gene are shown in Figure 10. Only

mutant SR754 produced enough Mlc to be detected through Western blot analysis

(including the wild-type MC4100 strain) suggesting SR752 and SR753 did not modulate

Mlc expression significantly.

```
MC4100   CACCATAGCCTACAGATTATTTCGGAGCGCGAAAATATAGGGAGTATGCGaTG
SR752    CACCATAGCCTACAGATTATTTCGGAGCGCGAAAATATAGGGAGTATGCGaTG
SR753    CACCATAGCCTACAGgccggTTCGctttatacccAgcgAGGGAGTATGCGaTG
SR754    tAtaATAGCCTACAGgccggTcaatgttaatccgtaAgAGGGAGTATGCGaTG
```

Figure 10. Specific mutations were made in the regulatory region of the Mlc gene in
order to maximize the affect of Mlc on acetate accumulations. Changes from the wild-
type MC4100 strain are shown in lowercase red letters. Adapted with permission from
ref. [11] © 2005 Journal of Biotechnology.

The effect of Mlc overexpression on cell growth was compared between SR754

and wild-type MC4100 in the presence glucose. It was shown that although higher initial

specific growth rates were observed with MC4100, SR7554 continued to grow after

MC4100 had entered the stationary phase. The measured specific growth rates of

MC4100 and SR754 at various concentrations are shown in Table 1. These data shows

that the growth rate of the SR754 mutant is not as significantly impacted by an increase

in available glucose as is the wild-type strain. Additionally, it was observed that there

was a continuous accumulation of acetate in the MC4100 culture through the 15 hours of

measured growth, whereas the SR754 culture initially saw a rise in acetate concentration

in the first six hours followed by a steady decrease. A comparison of MC4100 and

SR754 cell growth as well as glucose consumption and acetate production are displayed

in Figures 11 and 12, respectively.

Table 1. A comparison of specific growth rates between MC4100 (wild-type) and SR754
(mutated Mlc regulatory region) at varying glucose concentrations. Adapted with
permission from ref. [11] © 2005 Journal of Biotechnology.

Glucose concentration (%)	Specific growth rate $[h^{-1}]$	
	MC4100	SR754
0.0	0.54	0.55
0.1	0.84	0.74
0.3	1.20	0.75
0.5	1.31	0.82
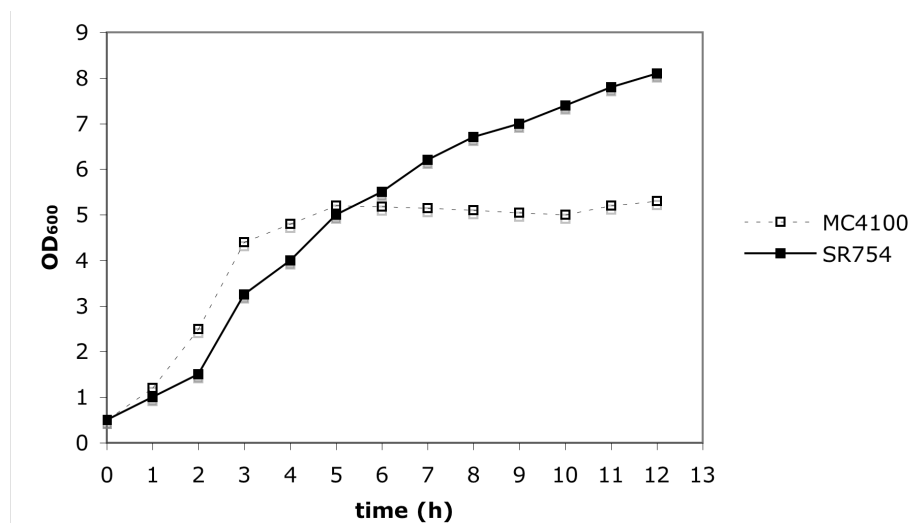
Figure 11. Approximate growth profiles based on data from Cho *et al.* of wild-type MC4100 and mutant SR754 strains were compared. Although SR754 growth initially lagged behind MC4100, it continued to be productive long after MC4100 reached its stationary phase. Adapted with permission from ref. [11] © 2005 Journal of Biotechnology.
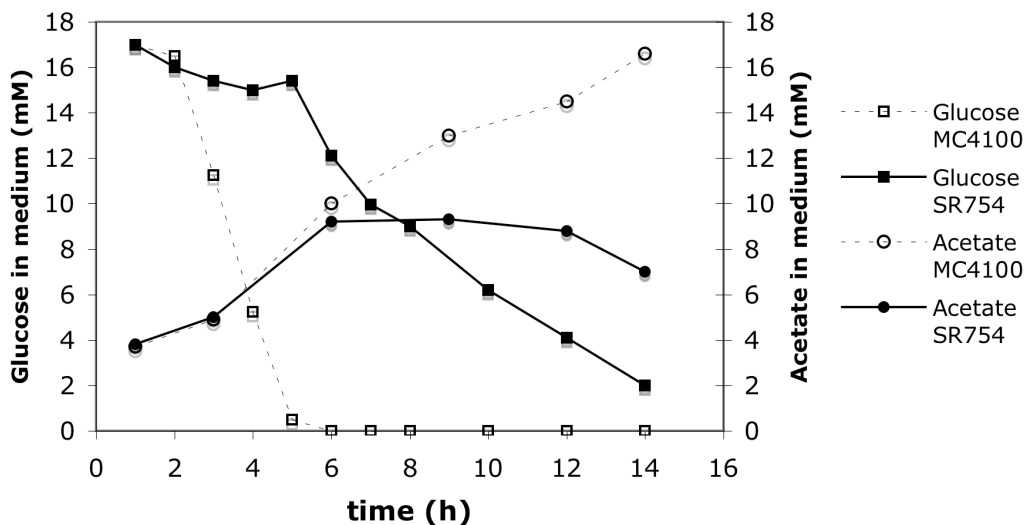
Figure 12. Comparison of approximate glucose consumption and acetate accumulation between wild-type MC4100 and mutant SR754 based on data collected by Hosono *et al.* While glucose is consumed more rapidly in MC4100, acetate continues to accumulate throughout the culture, whereas with SR754, a slower rate of consumption of glucose leads to a plateau in acetate accumulation. Adapted with permission from ref. [11] © 2005 Journal of Biotechnology.

Both MC4100 and SR754 were transformed with plasmid, pGAL and pGFPuv separately. The first encodes for the ß-galactosidase gene and the latter for the green fluorescent protein. Studies showed that under similar growth conditions with 0.3% glucose, SR754 produced four times more ß-galactosidase activity and 10 times greater fluorescence as compared to MC4100. This suggests that the reduced acetate accumulation may lead to better recombinant protein production in addition to prolonged cell growth.

Later in 2005, Gerber *et al.* isolated and identified a point mutation in the *mlc* gene, R52H, while expressing and purifying recombinant Mlc for crystallization and X-ray analysis [15]. This discovery was accidental and they found that this point mutation allows for higher concentrations of Mlc expression as compared to the wild-type; it was likely selected for analysis due to the larger colonies that formed.

2.4   Summary

The protein Mlc has been found to be a global regulator of sugar transport in *E. coli*. It represses the genes encoding PTS enzymes and proteins. In the example of glucose related transport, Mlc represses both the *pts* and *ptsG* operons when glucose is absent. In the presence of glucose, Mlc is sequestered by the PTS enzyme EII^{Glc}, preventing the regulator from repressing the PTS operons. Since Mlc represses its own expression, it was found that certain mutations within its own regulatory region lead to increased expression. Additionally, a point mutation (R52H) within the coding region of Mlc also leads to increased expression.

CHAPTER THREE: OBJECTIVES

The objectives of this study were threefold. The first was to overexpress Mlc via high and low copy expression plasmids in *E. coli*. The second objective was to study the effect of the Mlc overexpression on acetate accumulation in *E. coli* culture, and lastly, to examine the impact the overexpression of Mlc has on the production of a therapeutic protein.

Unlike the gene modulation of *mlc* proposed by Cho *et al.* [2005, 11] which included changes to the endogenous promoter and regulation regions, this series of experiments utilized both inducible and constitutive promoters in pBR322 and pACYC177 based expression plasmids, respectively to overexpress Mlc. Additionally, variations in the *mlc* sequence that have been identified in the literature, which have been shown to increase Mlc expression used with the inducible and constitutive systems to determine the most effective combination.

Correlation of the overexpression of Mlc to decreased acetate accumulation and improved therapeutic protein production may have industrial significance. The potential to create a more robust *E. coli* production host that can tolerate fluctuations in the concentration of glucose could reduce the complexity in at least one stage of recombinant protein production. Additionally, lower concentrations of acetate accumulation may lead to increased cell densities and subsequent protein production.

CHAPTER FOUR: MATERIALS AND METHODS

4.1   Materials

A complete list of materials used can be found in Appendix A.

4.1.1   Expression Plasmids

Two plasmids were used to evaluate Mlc overexpression, pLMG17 and

pACYC177. These were chosen because they have been shown to be compatible with

the production plasmid that was later co-transformed to express the therapeutic protein of

interest in conjunction with Mlc. The pLMG17 plasmid was pBR322 based and included

the inducible *tac* promoter. The backbone of the second plasmid was pACYC177 [26,

27], which contained a constitutive promoter associated with the beta-lactamase (*bla*)

gene; however, additional modifications to the plasmid were also made in-house at

Genentech Inc. The plasmids confer resistance to chloramphenicol and kanamycin,

respectively. Additional properties of these plasmids can be found in Table 2.

Table 2. Properties of both expression plasmids used to study the overexpression of Mlc
in *E. coli*.

Plasmid	pACYC177	pLMG17
Promoter	constitutive	inducible
Plasmid Size (kbp)	3.9	2.8
5' restriction site	KpnI	NcoI
3' restriction site	XbaI	HindIII
Antibiotic Resistance	Kanamycin	Chloramphenicol
Concentration of antibiotic used for selection (ug/mL)	5.0	12.5

The expression plasmids (pLMG17 and pACYC177) were digested with the

appropriate restriction enzymes that are listed in Table 2 to generate digested vectors.

The digested vectors were then run on a 1% agarose BioRad gel to verify digestion and

approximate product size. The product was purified using the Qiagen QIAquick Gel

Extraction kit.

4.1.2   *mlc* Expression Inserts

The *mlc* expression inserts were generated by amplifying the *mlc* sequence

through polymerase chain reaction (PCR) using a set of restriction site specific primers.

These primers were designed to include the DNA sequence encoding the restriction site

corresponding to the targeted expression vector as well as a short 15-20 base pair (bp)

mlc sequence. For example, the forward primer for the *mlc* insert to be used with the

pLMG17 expression vector begins with the sequence for the NcoI restriction site

followed by the first 15-20 bp of the *mlc* sequence; this is illustrated in Figure 13. A

complete list of primer sequences used can be found in Appendix B.

pLMG17 sequence surrounding ncoI restriction site

ncoI

5' A T A A C A A T T T   C A C A C A G G A A   A C A G A C C A T G   G A A T T C C A G C

First 40 bp of *mlc* sequence

5' G T G G T T G C T G   A A A A C C A G C C   T G G G C A C A T T   G A T C A A A T A A

Forward primer sequence for *mlc* amplification and insertion in pLMG17 cloning plasmid

ncoI

5' A G G A A A C A G A   C C A T G G G T G G   T T G C T G

pLMG17 sequence
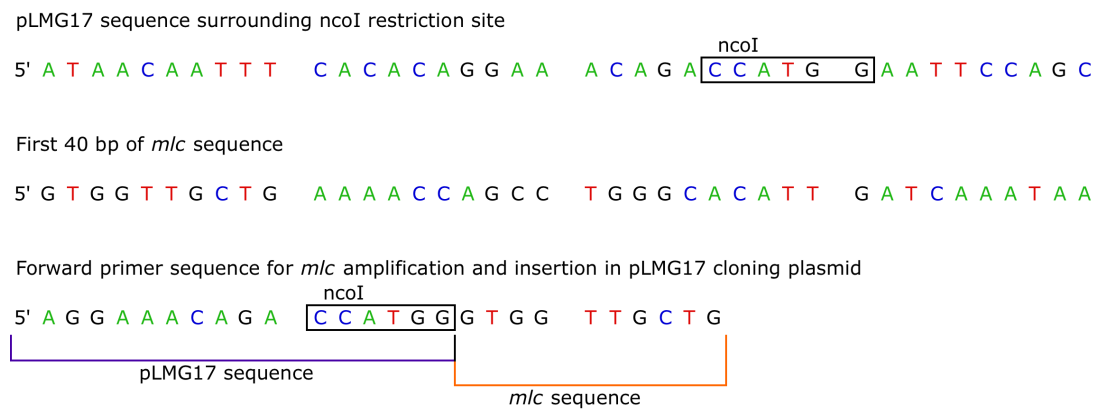
mlc sequence

Figure 13. An example of a primer sequence used for *mlc* amplification via PCR.

The source for the DNA template used for amplification was plasmid DNA

containing the *mlc* sequence insert, provided by Genentech, Inc. In order to have a stock

of this plasmid, it was transformed in to DH5α competent cells (Invitrogen) via heat

shock transformation and streaked on to an LB plate with 12.5 ug/mL chloramphenicol

(the antibiotic resistance conferred by the plasmid) and incubated overnight at 37°C.

Next a single colony from this plate was re-streaked and incubated overnight at 37°C. A

single colony from the second plate was used to inoculate 50 mL of liquid LB media and

grown overnight at 37°C in an incubated shaker. The plasmid DNA was isolated and

purified from this culture using the Qiagen QIAprep miniprep kit.

The PCR protocol used can be found in Appendix C. After amplification, the

PCR products were run on 1% agarose BioRad gels to verify amplification and

approximate product size and then purified using the Qiagen QIAquick Gel Extraction

kit. The gel-purified product was digested using the appropriate restriction enzymes.

Digestions were carried out at 37°C for two hours in New England Biolabs (NEB) buffer

2 solution. Calf intestinal alkaline phosphatase (CIP) was added for the last 30 minutes

of digestion of the expression plasmids to prevent re-ligation by removing the

phosphorylated ends of DNA. The digestion products were gel purified using the same

procedures that were used for the PCR products.

4.1.3   *mlc* Sequence Variants

Several *mlc* sequence variants were created, including changes to the start codon

sequence (from GTG to ATG), and a point mutation at codon 52 replacing an arginine

(R) to a histidine (H), also referred to a R52H. A histidine tag (a chain of six histidine

amino acids added to the end of the *mlc* sequence) was used in some constructs to

perform analysis on the protein level [25]. A complete list of the sequence variants is

shown in Table 3.

Table 3. Eight *mlc* constructs were designed and evaluated in each of the expression
plasmids. Changes to the start codon and codon 52 were studied along with the addition
of a histidine tag.

Start Codon	Codon 52	Histidine Tag	Alias
GTG	Arginine Histidine	No	GTG GTG-R52H
	Arginine Histidine	Yes	GTG-His GTG-R52H-His
ATG	Arginine Histidine	No	ATG ATG-R52H
	Arginine Histidine	Yes	ATG-His ATG-R52H-His

The change in the start codon sequence from GTG to ATG was created by

incorporating a single base pair change (from G to A) in the *mlc* start sequence in the

forward primer used for *mlc* amplification. The point mutation in codon 52 was

generated using the Stratagene QuikChange Site-Directed Mutagenesis Kit. This kit

required a pair of primers (forward and reverse) centered on the targeted codon. The

constructed primers were 51 bp in length and included a sequence change from an

arginine to histidine at codon 52. The histidine tag was added by including the sequence

for a six histidine chain to the 3' reverse primer after the *mlc* sequence but before the

restriction site.

The digested and purified expression vectors and *mlc* inserts were ligated using a

buffered NEB T4 DNA ligase mixture. Ligation was carried out overnight at 16°C. The

ligation protocol can be found in Appendix D.

DNA sequencing was performed in-house at Genentech to verify the sequence for each of the *mlc* constructs inserted into the expression vectors.

4.1.4 Therapeutic Protein Plasmid

A Genentech, Inc. product was used to study the effect of Mlc on the production of a therapeutic protein. The product will be referred to as Therapeutic Protein A (TPA) and was produced using an *E. coli* host strain. The plasmid encoding for TPA (referred to as pTPA) confers tetracycline resistance; 5 ug/mL of tetracycline was used throughout the course of experiments for pTPA selectivity.

4.1.5 Host Strains

Two *E. coli* host strains were used throughout the course of this study, 1A2 (*ΔfhuA (ΔtonA)*) and16C9 (*ΔfhuA phoAΔE15 Δ(argF-lac)169 deoC*). Both were derived from W3110 *E. coli*. 1A2 contained only a single mutation for *fhuA*, an undesired phage receptor [28] and is otherwise treated as wild-type. 1A2 was used to determine baseline wild-type expression of Mlc. The host strain 16C9 has been established as a suitable production host for TPA [29].

4.1.6 Media

Three types of media were used throughout the shake flask portion of this study, Luria Broth (LB), City Broth, and CRAP media. LB is a nutrient rich media that has been used throughout industry as a standard for growing *E. coli* [30]. Variable amounts of additional glucose were added to provide a better understanding of the effect the overexpression of Mlc had on glucose uptake and acetate accumulation. City Broth is a more robust media, containing more nutrients than LB, and was used to evaluate the

overexpression of Mlc in higher density cultures. Lastly, CRAP media has been
established as a suitable media for the production of therapeutic proteins [31] under the
control of the *phoA* promoter and was used in this study to evaluate the effect the
overexpression of Mlc had on the production of TPA.

A media optimized for use in higher cell density cultures and the production of
TPA was used in the DASGIP bioreactors in order to provide the sufficient nutrients to
sustain growth and higher cell densities throughout the 36-hour fermentation runs. The
media included yeast extract, salts, peptones, $MgSO_4$, glucose and trace elements.

4.2   Methods

4.2.1   Transformation

Transformations were carried out by heat shock treatment. After the initial
construction of the plasmids encoding Mlc was completed, DH5α cells (Invitrogen) were
used for their high transformation efficiency to generate a plasmid stock via miniprep
(Qiagen QIAprep Spin Miniprep Kit). Competent 1A2 and 16C9 cells were used for
subsequent transformations. A more detailed procedure can be found in Appendix E.

4.2.2   Shake Flask Cultures

The growth of *E. coli* cells transformed with plasmids encoding Mlc was studied
in shake flask cultures where a mixture of media, glucose, antibiotic, and inoculum
totaling a volume of 25 mL was added to each shake flask. Erlenmeyer flasks of 125 mL
volume were used to maintain a 1:5 volume ratio between culture and air. The
concentration of the appropriate antibiotic(s) used is listed in Table 2. Each flask was

inoculated to an initial optical density OD_{550} of 0.1. Cultures were grown overnight in a

37°C shaking incubator operated at 250 RPM.

Plasmids constitutively expressing Mlc were grown in 1A2 host cells in both LB

and City Broth under varying glucose concentrations. Similarly, induced Mlc expression

was studied in 1A2 cells with City Broth. These growth conditions are listed in Table 4.

A subset of this group was co-transformed with pTPA in 16C9 host cells. Additional

experiments were conducted to study the concentration of isopropyl β-D-1-

thiogalactopyranoside (IPTG) required to induce a sufficient *mlc* response.

Table 4. Each of the eight *mlc* constructs was studied in pACYC177 and pLMG17 in
1A2 host cells.

Host Strain	Plasmid/ Promoter	Media	Glucose (g/L)
1A2	pACYC177 (constitutive)	LB	0 and 10
		City Broth	10
	pLMG17 (inducible)	City Broth	10

During the initial round of shake flask experiments, samples and measurements

were taken at approximately four, seven, and 24 hours post inoculation. This consisted of

OD_{550} readings and the collection of supernatant samples and 1 OD_{550} pellets. For

subsequent shake flask culture experiments, sampling was performed every two to four

hours for the first 16 hours, and final sample point after 24 hours.

4.2.3   Bioreactor Fermentation

The DASGIP SR1000DLS parallel bioreactor system was used as it provided a

representative environment for the production of TPA in conjunction with the

overexpression of Mlc. The system allowed for one sided pH control; 6M NH₃OH was

fed as needed based on the pH 7.3 set point. Glucose was initially batched into the

system at a concentration of 1.67 g/L and then fed based on a glucose feed algorithm. The

algorithm ultimately controlled the cultures under micro-aerobic conditions (limited

oxygen).   This algorithm was selected as it had previously been established as an

appropriate feed algorithm for the production of TPA. The reactors operated at a

temperature set point of 37°C, agitation of 1355 RPM, and an airflow rate of 60 slpm for

36 hours. Samples were taken approximately every four hours to measure OD_{550},

osmolality, and gross glucose concentrations. Supernatant, whole broth and 1 OD_{550}

pellet samples were also collected for further metabolite, RNA, and protein analysis.

Four different fermentation experiments were run on the DASGIP system (two of

which were run in duplicate). Two reactors were inoculated with cultures of 16C9 cells

co-transformed with plasmids encoding for TPA and Mlc. The other two reactors were

inoculated with 16C9 cells co-transformed with pTPA and plasmid pLMG17 without the

mlc insert as control cultures for TPA expression. The reactors were inoculated with 55

mL of inoculum measuring approximately 1.5 OD_{550}. To test the impact of Mlc on the

overfeeding of glucose in culture, a glucose excursion was executed that increased the

glucose feed rate by approximately 20% for 10 minutes once the culture had reached an

oxygen limiting state and stable glucose feed rate. The conditions for the DASGIP

fermentation runs are outlined in Table 5.

Table 5. A total of six cultures were run using the DASGIP bioreactor system. Four overexpressed Mlc in conjunction with TPA, while two only produced TPA.

Host Strain	Condition	Transformed Plasmids	Expressed Protein	Glucose Excursion	Run ID
16C9	1	pTPA and pLMG17-mlc (GTG-R52H-His)	TPA and Mlc		JB017, JB024
	2			X	JB018, JB026
	3	pTPA and pLMG17-no insert	TPA		JB023
	4			X	JB025

4.2.4   RNA Preparation

RNA was isolated and purified from the 1 OD_{550} pellets collected from the cultures using the Qiagen RNeasy Mini kit. RNA samples were diluted a concentration of 10 ng/µL in RNase free water for analysis.

4.2.5   Protein Detection

Three methods were used to detect the histidine tagged Mlc protein, Western blot with use of the Anti-His (C-term)-HRP Antibody (Invitrogen), InVision His-Tag In-Gel Stain (Invitrogen), and the Universal His Western Blot Kit 2.0 produced by Clontech.

4.3   Instrumentation

4.3.1   Thermal Cycler

An Applied Biosystems Veriti® 96-well thermal cycler was used for the amplification of DNA sequences through polymerase chain reaction (PCR). The PCR protocol that was used can be found in Appendix C. The PCR products were used both for cloning and analysis.

4.3.2   Visible (Vis) Spectrophotometer

A Thermo Scientific Genesys 20 visible spectrophotometer was used for the quantification of cells in culture. Cells in suspension scatter light such that the more

turbid the suspension, the greater the scattering. The spectrophotometer measures the amount of scattering at a fixed wavelength of light (in this case $\lambda=550$) and determines the optical density (OD_{550}). The readings obtained at OD_{550} are a surrogate reading for cell mass.

4.3.3 Ultraviolet-Visible (UV-Vis) Spectrophotometer

The quantification of nucleic acids (both DNA and RNA) was required for other analytical steps such as real time quantitative PCR (RT-qPCR) and DNA sequencing; the Thermo Scientific NanoDrop 2000c was used for this purpose.

4.3.4 RT-qPCR

The Agilent Stratagene Mx3005P quantitative PCR (qPCR) system was used to detect the level of *mlc* RNA. The amplification of *mlc* RNA was performed using gene specific primers and a 6-carboxyfluorescein (FAM) labeled probe and TaqMan One-Step RT-PCR Master Mix Reagents produced by Applied Biosystems. Primer sequences can be found in Appendix B. The amplification of two endogenous genes, *cyaA* and *murA* were used as a reference in order to quantify the level of *mlc* RNA detected.

4.3.5 Metabolite Analysis

The Cobas Integra 400 plus (Roche Diagnostics) was used to measure concentrations of acetate and glucose found in supernatant samples. Glucose was quantified using Cobas Substrates Glocose HK. Acetate was quantified using acetic acid standards from Randox Laboratories Limited. Samples were diluted 1:5 in ultrapure water (18.2 MΩ·cm) for analysis.

4.4   Laboratory Safety

It is of grave importance that laboratory procedures are carried out safely, and that precautions are taken to minimize the danger from potential hazards around the work area. There should be a clear understanding of the equipment and personnel available in case of an accident.

4.4.5   Personal Protective Equipment

A laboratory coat, gloves and protective eyewear are required at all times while working in the laboratory.

4.4.6   Chemical Hazards

Listed on Table 6 are the chemicals with known hazards that were used throughout the course of these experiments and the precautions taken to minimize the risk of injury. In the case of an accident, sinks were available on either bench top and an eyewash and shower were located immediately outside of the laboratory. If assistance was required, for personal injury, chemical spill, etc the Genentech Inc. security line could be dialed for immediate response.

Table 6. A list of chemical and their known hazards that were used throughout the course of this study.

Chemical Compound	Health Risk/Hazard	Precaution
Ethidium bromide[4]	mutagen, intercalates double stranded DNA	use of PPE
β-mercaptoethanol[4]	toxin, respiratory tract irritant	use within fume hood
Ethanol	flammable	stored within flammable cabinet
Guanidine hydrochloride[1,2]	irritant	use of PPE, follow provided SOP
Acetic acid[1]	irritant	use of PPE, follow provided SOP
Sodium hydroxide[1]	irritant	use of PPE, follow provided SOP
Isopropanol[1, 2]	flammable	use of PPE, follow provided SOP
Guanidine thiocyanate[2,3]	irritant	use of PPE, follow provided SOP

[1] Components of buffers included in Qiagen QIAprep Miniprep kit
[2] Components of buffers included in Qiagen QIAquick Spin kit
[3] Components of buffers included in Qiagen RNeasy Plus Mini kit
[4] Ethidium bromide and β-mercaptoethanol will be disposed of in dedicated waste receptacles

CHAPTER FIVE: RESULTS

Wild-type 1A2 cells were grown in overnight shake flask cultures with City Broth
and glucose concentrations varying from 0 to 20 g/L (0% to 2% w/v). An accumulation
of acetate was observed in cultures batched with 10 g/L and 20 g/L glucose. These
results can be found in Table 7 and Figures 14 and 15. Table 7 also shows a peak optical
density (OD) of 14.60 was achieved after 25 hours in the culture charged with 5 g/L
glucose. Peak ODs fell to 9.85 and 7.35 for the cultures charged with 10 and 20 g/L
glucose, respectively.

Table 7. Wild-type 1A2 cells were grown in City Broth with various glucose
concentrations. Acetate accumulation was observed in cultures batched with 10 g/L and
20 g/L glucose.

			Time (hr)					
			3	4	6	8	10	25
		OD_{550}	1.73	3.40	5.64	7.95	7.95	10.20
	0	Acetate (mM)	8.86	7.84	3.46	0.02	0.07	0.11
		Glucose (g/L)	0.00	0.00	0.06	0.00	0.00	0.00
		OD_{550}	2.11	5.86	9.64	12.70	12.70	14.60
Glucose (g/L)	5	Acetate (mM)	11.26	38.37	38.98	14.70	0.12	0.00
		Glucose (g/L)	4.60	0.02	0.00	0.00	0.00	0.00
		OD_{550}	2.20	6.32	8.56	9.75	9.80	9.85
	10	Acetate (mM)	9.99	28.81	55.82	79.16	99.89	99.89
		Glucose (g/L)	7.93	4.20	2.10	0.40	0.00	0.00
		OD_{550}	1.94	4.51	5.38	5.75	5.70	7.35
	20	Acetate (mM)	9.35	21.13	44.00	58.44	82.30	106.70
		Glucose (g/L)	16.26	11.65	12.35	11.55	10.55	8.05

Figure 14 illustrates the inverse relationship observed between acetate
concentration in the media and OD. The greater the concentration of glucose batched
upfront for each culture led to a greater concentration of acetate in the media, which
resulted in lower overall cell growth. Additionally, it was determined that 20 g/L glucose

was more than the culture could metabolize over 25 hours. Conversely, the culture

batched with 10 g/L glucose had fully metabolized the sugar source over the same period

of time. This is shown in Figure 15.
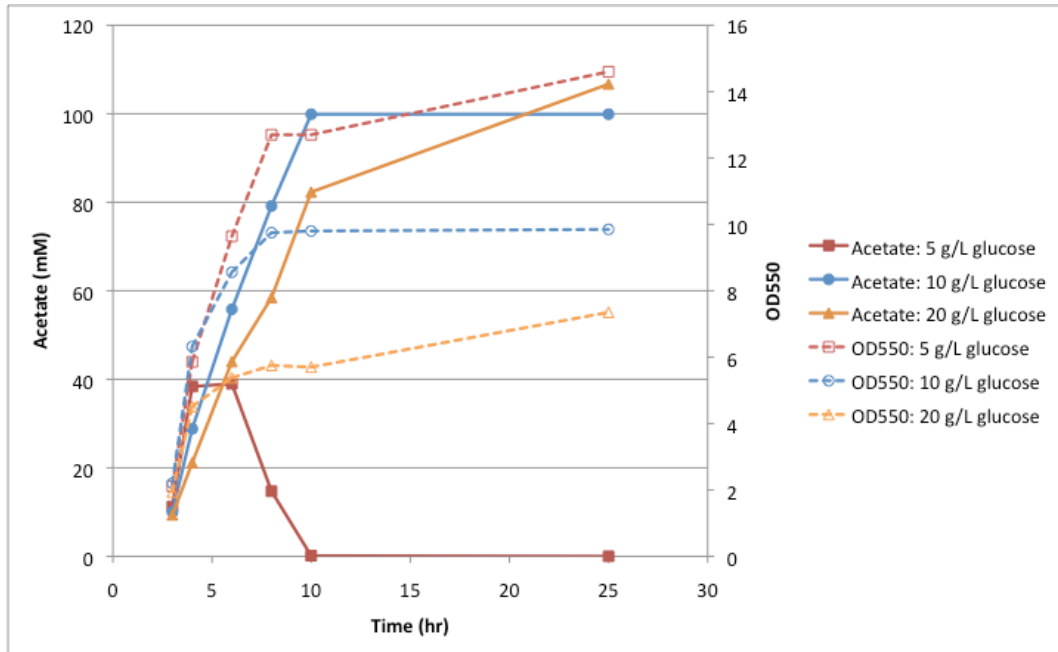
Figure 14. Acetate and growth profiles of 1A2 cultures grown in City Broth with 5, 10, and 20 g/L glucose. Acetate accumulation was observed in the media with 10 and 20 g/L glucose (shown in blue and orange, respectively).
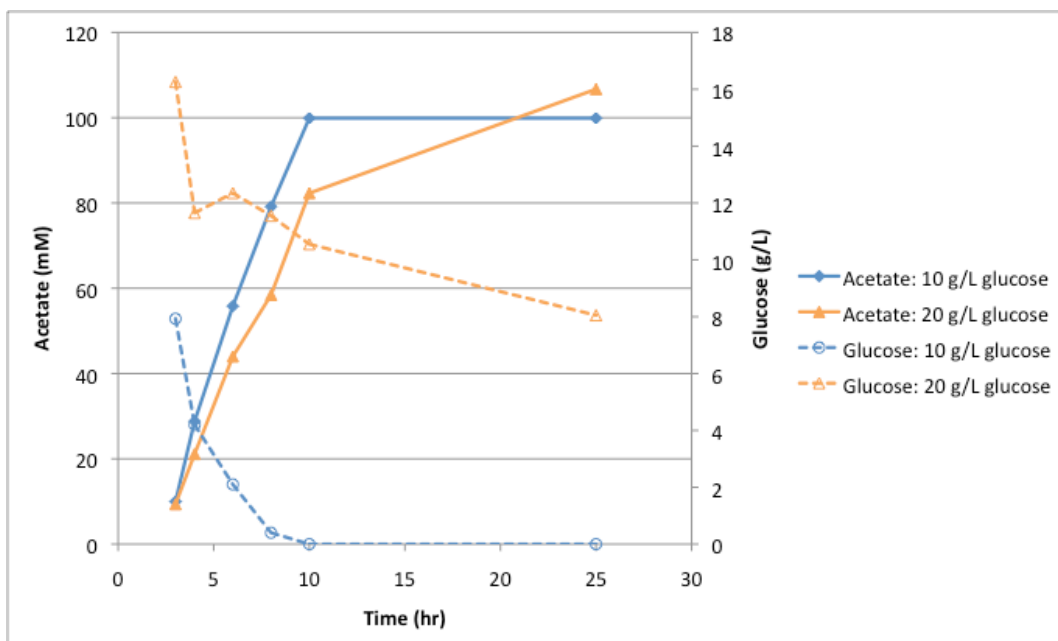
Figure 15. Acetate and glucose profiles of 1A2 cultures grown in City Broth which were batched upfront with 10 and 20 g/L glucose.

Based on this preliminary experiment it was determined that the starting concentration of glucose for experiments moving forward would be 10 g/L (1% w/v). This concentration was sufficient to lead to acetate accumulation in the wild-type cells, however did not overwhelm the system such that the glucose could not be fully metabolized.

5.1   Constitutive arm (pACYC177)

Each of the eight *mlc* constructs was evaluated under constitutive expression in the pACYC177 plasmid transformed in the 1A2 host strain. Shake flask cultures were carried out in LB with 0 and 10 g/L glucose. Additionally, overnight cultures were conducted in City Broth with 10 g/L glucose. OD_{550} and acetate concentration from these experiments can be found in Tables 8, 9, and 10. No acetate accumulation was observed in the cultures in which no glucose was initially charged. However, in both cohorts

batched with 10 g/L glucose, significant acetate accumulation was observed by the endpoint for all *mlc* variants. Greater acetate accumulation was observed in the cultures grown in City Broth compared to those grown in LB.

Table 8. Overnight shake flask cultures of pACYC177 containing an *mlc* insert (constitutively expressed) transformed into 1A2 cells. Cells were grown in strictly LB media (no glucose). A, B, and C refer to sampling time points. A was taken between 3-4 hours post inoculation, B 6-7 hours, and C 23-29 hours.

	OD_{550}			Acetate (mM)		
	A	B	C	A	B	C
GTG	1.46	3.72	3.28	1.62	0.25	0.40
GTG-His	1.50	3.73	3.52	1.82	0.00	0.30
GTG-R52H	1.50	3.73	3.14	1.69	0.00	0.25
GTG-R52H-His	1.48	3.65	3.28	2.25	0.00	0.35
ATG	1.89	2.77	2.38	2.62	0.00	0.13
ATG-His	1.46	3.06	3.40	1.65	0.29	0.32
ATG-R52H	1.85	---	---	2.70	---	---
ATG-R52H-His	1.91	2.58	2.32	3.05	0.29	0.12
wild-type 1A2	2.82	3.86	3.24	0.00	0.00	0.00

Table 9. Overnight shake flask cultures of pACYC177 containing an *mlc* insert (constitutively expressed) transformed into 1A2 cells. Cells were grown in LB and 10 g/L glucose. A, B, and C refer to sampling time points. A was taken between 3-4 hours post inoculation, B 6-7 hours, and C 23-29 hours.

	OD_{550}			Acetate (mM)		
	A	B	C	A	B	C
GTG	1.54	2.47	2.78	10.65	17.90	38.96
GTG-His	1.53	2.71	2.74	11.02	18.56	38.91
GTG-R52H	1.75	2.41	2.78	9.81	13.92	39.46
GTG-R52H-His	1.78	2.19	2.46	10.34	15.19	45.60
ATG	1.65	2.38	2.66	11.32	17.94	39.63
ATG-His	1.45	3.45	4.36	9.62	9.16	29.68
ATG-R52H	1.82	2.13	2.18	10.87	14.54	41.73
ATG-R52H-His	1.90	2.51	2.90	10.53	12.96	45.24
wild-type 1A2	2.64	2.77	3.40	6.82	16.24	44.24

Table 10. Overnight shake flask cultures of pACYC177 containing an *mlc* insert (constitutively expressed) transformed into 1A2 cells. Cells were grown in City Broth and 10 g/L glucose. A, B, and C refer to sampling time points. A was taken 4 hours post inoculation, B 7-8 hours, and C 25-30 hours. GTR: Greater than reportable, concentration greater than 103 mM.

	OD_{550}			Acetate (mM)		
	A	B	C	A	B	C
GTG	4.76	11.36	14.12	27.09	86.98	GTR
GTG-His	5.00	9.96	12.80	26.65	92.28	GTR
GTG-R52H	4.85	9.96	12.08	29.96	82.65	GTR
GTG-R52H-His	5.00	10.60	13.52	31.45	83.13	GTR
ATG	4.40	6.54	7.64	27.14	62.04	GTR
ATG-His	4.26	7.16	8.08	24.39	66.69	GTR
ATG-R52H	4.81	8.88	10.56	32.05	85.57	GTR
ATG-R52H-His	5.10	12.02	15.44	34.87	81.66	GTR
wild-type 1A2	6.32	9.75	9.85	28.81	79.16	99.89

5.2   Inducible arm (pLMG17)

A series of shake flasks cultures was conducted to determine the time point in which the inducer, IPTG should be added to maximize the effect Mlc overexpression has on the culture. The wild-type *mlc* sequence (GTG) was used in the pLMG17 plasmid with host 1A2 and grown in City Broth and 10 g/L glucose. Three different conditions were evaluated: no IPTG was added, 3 mM IPTG was added four hours post-inoculation, and 3 mM IPTG was added eight hours post-inoculation. A culture of wild-type 1A2 cells was grown as a control. Table 11 shows the OD_{550}, acetate, and glucose concentrations measured over the course of these overnight cultures.

Table 11. 3 mM IPTG was added to cultures of *E. coli* that had been transformed with the inducible plasmid pLMG17 containing the GTG *mlc* construct. The 1A2 host strain was used and grown with City Broth and 10 g/L glucose. No IPTG was added to the wild-type control culture.

			Time (hr)						
			4	8	10	12	14	16	24
3 mM IPTG added at time point	n/a	OD_{550}	2.46	11.20	15.95	16.15	16.10	15.60	15.50
		Acetate (mM)	9.40	32.44	55.87	28.20	0.54	0.31	0.00
		Glucose (g/L)	9.40	3.75	0.00	0.00	0.00	0.00	0.00
	4 hr	OD_{550}	2.30	9.90	14.40	14.75	15.20	14.95	14.15
		Acetate (mM)	9.60	29.41	64.35	27.84	0.00	0.00	0.00
		Glucose (g/L)	9.55	3.40	0.00	0.00	0.00	0.00	0.00
	8 hr	OD_{550}	2.20	11.80	14.60	15.75	15.90	16.20	14.40
		Acetate (mM)	10.14	26.10	54.61	26.62	0.28	0.00	0.00
		Glucose (g/L)	9.65	4.00	0.10	0.00	0.00	0.00	0.00
1A2 Wild-type control		OD_{550}	6.40	5.65	8.00	8.45	8.65	8.95	8.50
		Acetate (mM)	51.10	106.3	136.7	140.2	133.3	132.0	137.9
		Glucose (g/L)	4.30	0.50	0.50	0.00	0.00	0.00	0.00

In each of the cultures transformed with the pLMG17 plasmid, no acetate accumulation was observed after 24 hours, although a moderate peak was observed at 10 hours. Conversely, the wild-type 1A2 culture showed an immediate rise in acetate concentration in the media at the 4 hour mark and continued to remain high throughout the duration of the culture. This is illustrated in Figure 16. Although there was greater growth in the control group initially, its growth stalled out after 10 hours, never reaching above 9 OD_{550}, whereas the entire pLMG17 group measured above 15 OD_{550} after 14 hours.
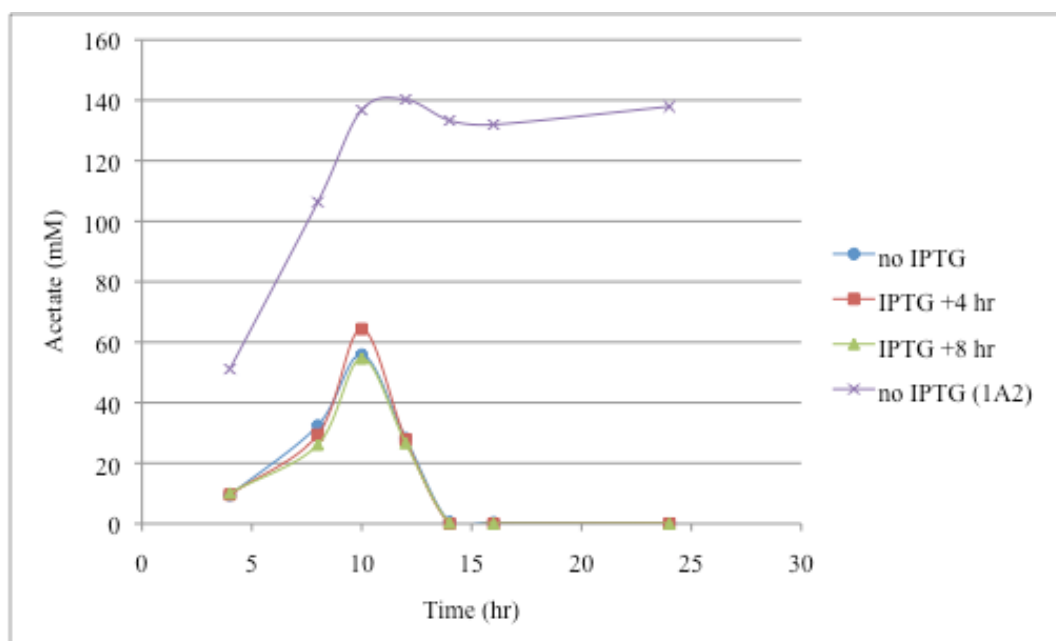
Figure 16. Acetate profiles from pLMG17 *mlc* induction experiment.

Next, each of the eight *mlc* constructs was evaluated in the pLMG17 plasmid with

1A2 as the host stain. Overnight shake flask cultures were run in duplicate for each

construct; City Broth media was used and 10 g/L glucose batched upfront. Acetate and

growth profiles from these experiments are shown in Figures 17 through 19. A culture of

wild-type 1A2 was grown as a control. Data shown in the figures is presented as an

average of the replicates unless otherwise stated. A complete listing of all the growth,

acetate and glucose data collected can be found on Table 14 in Appendix F.

During this first set of experiments in which *mlc* constructs were expressed via

the pLMG17 vector in City Broth, significant variability was observed in the acetate

profiles over the course of the 24 hour cultures, as shown in Figure 17. Even though each

of the cultures overexpressing Mlc showed lower levels of acetate accumulation in the

media compared to the 1A2 control culture, some only showed reduced levels of acetate,

40

whereas others showed near elimination; the cultures representing the latter group are

highlighted in Figure 18.
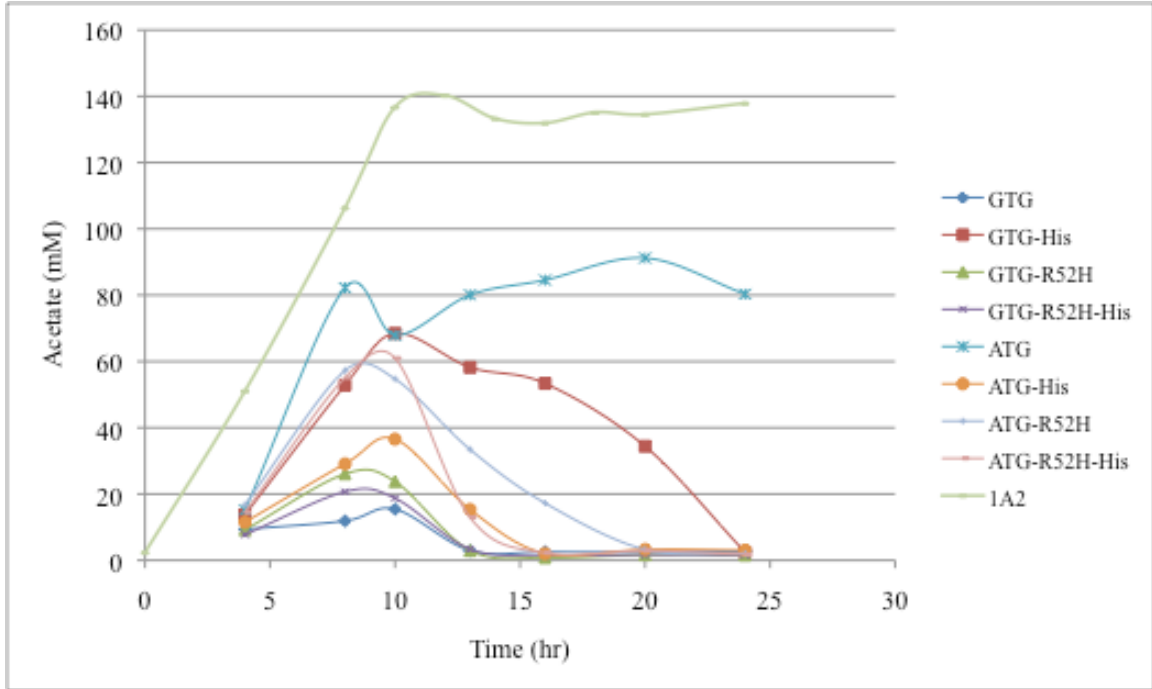
Figure 17. The average acetate profiles for each *mlc* constructs expressed through
pLMG17 in City Broth and 10 g/L glucose. 1A2 was used as the host strain.

The GTG, GTG-R52H, GTG-R52H-His, and ATG-His *mlc* constructs showed

minimal acetate production and accumulation when overexpressed via the pLMG17

expression vector and transformed in the 1A2 host strain. Figure 18 shows the acetate

profiles of both replicates for these four *mlc* constructs. Though minimal levels of

acetate are reached by the 16 hours mark, variability in the profiles can be seen for each

pair.
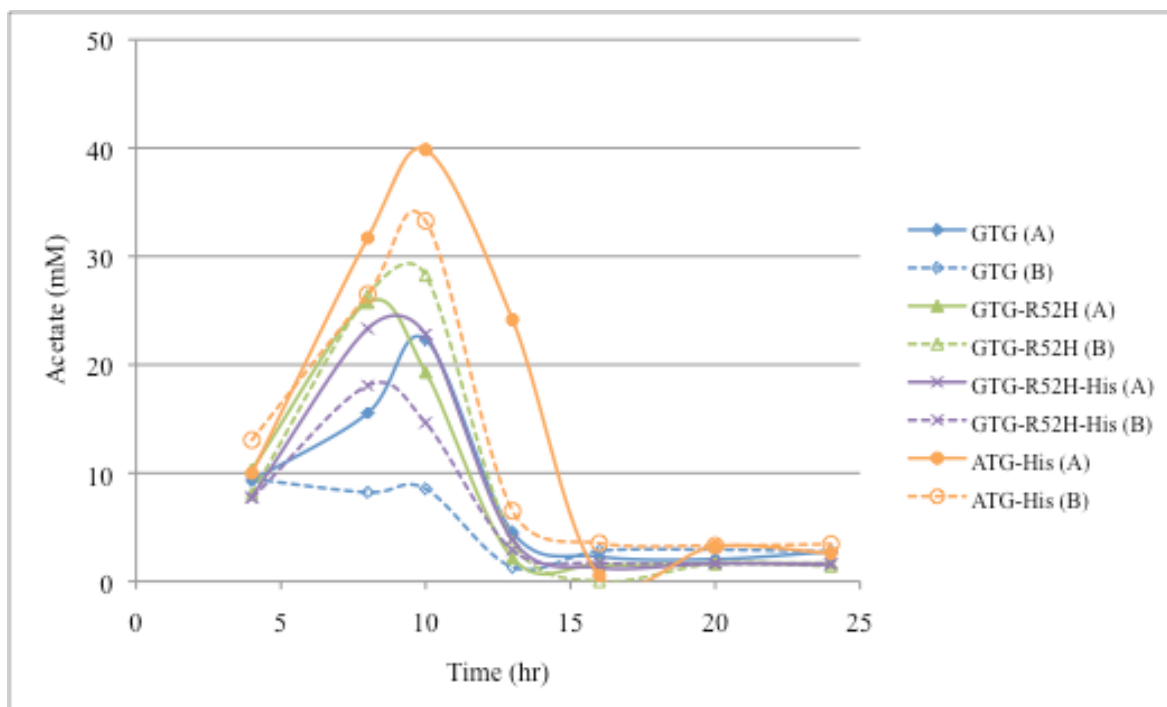
Figure 18. A closer look of the acetate profiles of the *mlc* constructs which yielded the lowest amounts of acetate accumulation when grown in City Broth and 10 g/L glucose. Data shown is both of the replicate shake flask cultures grown for each construct.

Each of the cultures overexpressing Mlc grew to a greater OD than the 1A2 control; this is illustrated in Figure 19. In general the growth profiles show an inverse relationship with the acetate concentration in the media. The four *mlc* constructs highlighted in Figure 18 for the lowest acetate concentrations also reached the greatest OD.
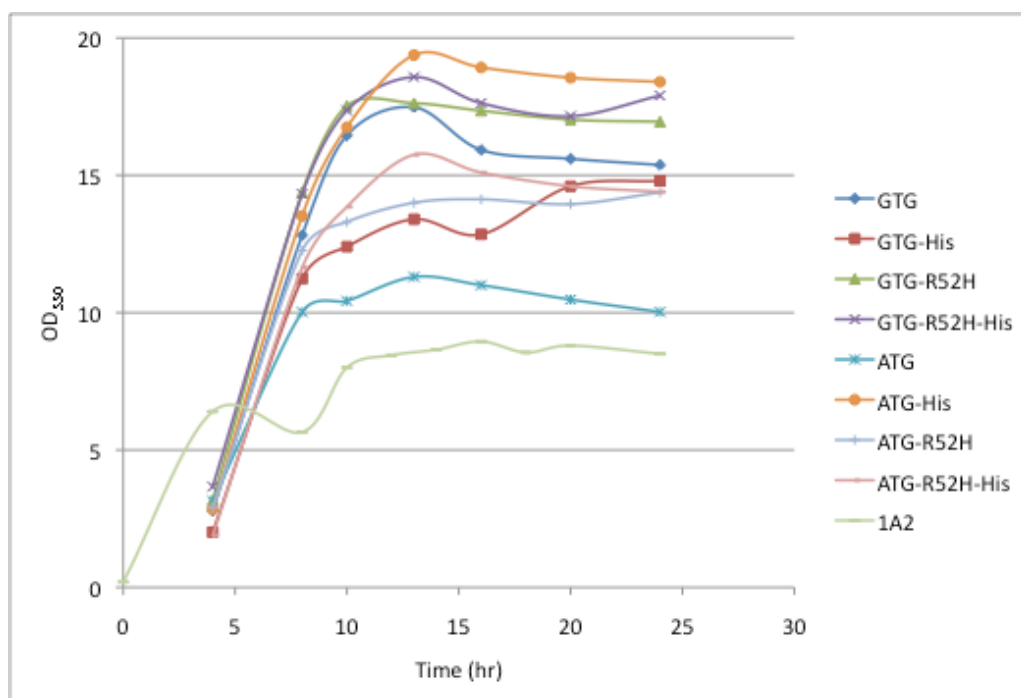
Figure 19. Growth profiles of overnight shake flask cultures overexpressing Mlc in 1A2 grown in City Broth and 10 g/L glucose.

In each of the eight experimental cases, a decrease in acetate accumulation in the media was observed compared to the wild-type 1A2 culture. This decrease was particularly significant in four cases, highligted in Figure 18; the wild-type *mlc* sequence (GTG), the R52H point mutation with and without the histidine tag (GTG-R52H and GTG-R52H-His), and the change in start codon paired with the histidine tag (ATG-His). In each of these cultures, the peak acetate found in the media did not exceed 40 mM, and any acetate produced was nearly fully metabolized by the 16 hour mark. For the control culture, acetate concentration plateaued around 130 mM by the 12 hour mark and was relatively steady for the remainder of the experiment.

Prior to running cultures on the DASGIP bioreactor system, each of the four *mlc* constructs identified to best limit acetate accumulation was co-transformed with the

plasmid encoding for TPA in 16C9 host cells, pTPA.   Each of the four co-transformations were grown in shake flask cultures with CRAP media and batched upfront with 10 g/L glucose. Each was run in duplicate. A culture of 16C9 cells co-transformed with pTPA and a blank pLMG17 plasmid (no *mlc* insert) was used as a control. The growth and acetate profiles of these runs are shown in Figures 20 and 21, respectively. The figures shown represent an average of the replicate shake flask cultures, unless otherwise stated. A complete table of growth, acetate, and glucose data can be found on Table 15 in Appendix F.

The four cultures co-expressing Mlc appear to reach the exponential growth phase about two hours after the control culture as shown in Figure 20. Additionally, three of the four Mlc cultures reached greater OD than the control. The culture expressing the GTG *mlc* construct appeared to reach a similar growth plateau as the control culture, approximately 4 OD.
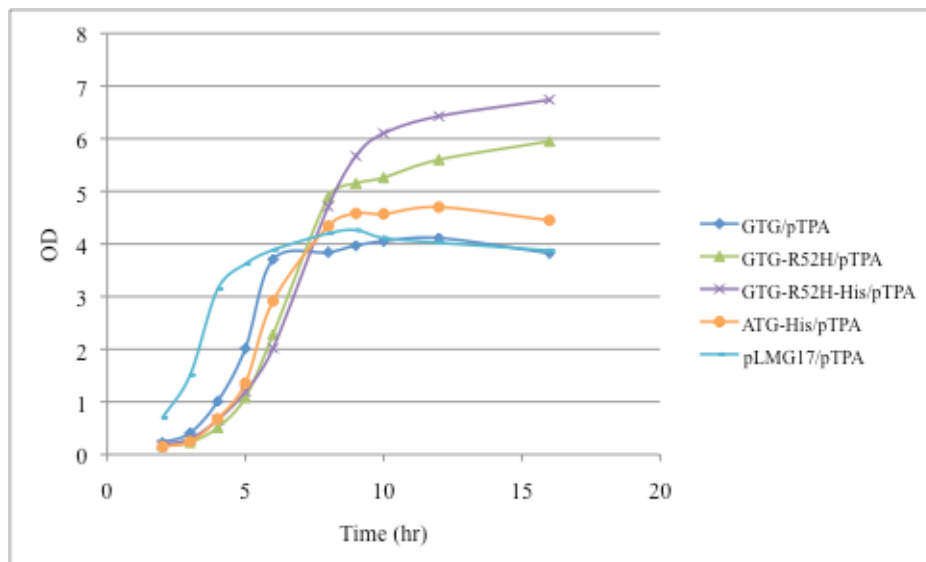
Figure 20. Growth profile of shake flask cultures producing Mlc and TPA in CRAP media and 10 g/L glucose.

Similar to the previous set of experiments, an inverse relationship was observed between the growth and acetate profiles; the greater the acetate concentration in the media, the lower the overall density of the culture. Figure 21 shows the acetate profiles of each of the cultures co-expressing Mlc with TPA along with the control culture. The data is shown as an average of the two replicate cultures with exception to the culture containing the GTG-R52H-His *mlc* construct (shown in purple), in which acetate profiles for both replicates are shown. Significant variance in the acetate profile was observed in this particular set of replicates after the eighth hour.
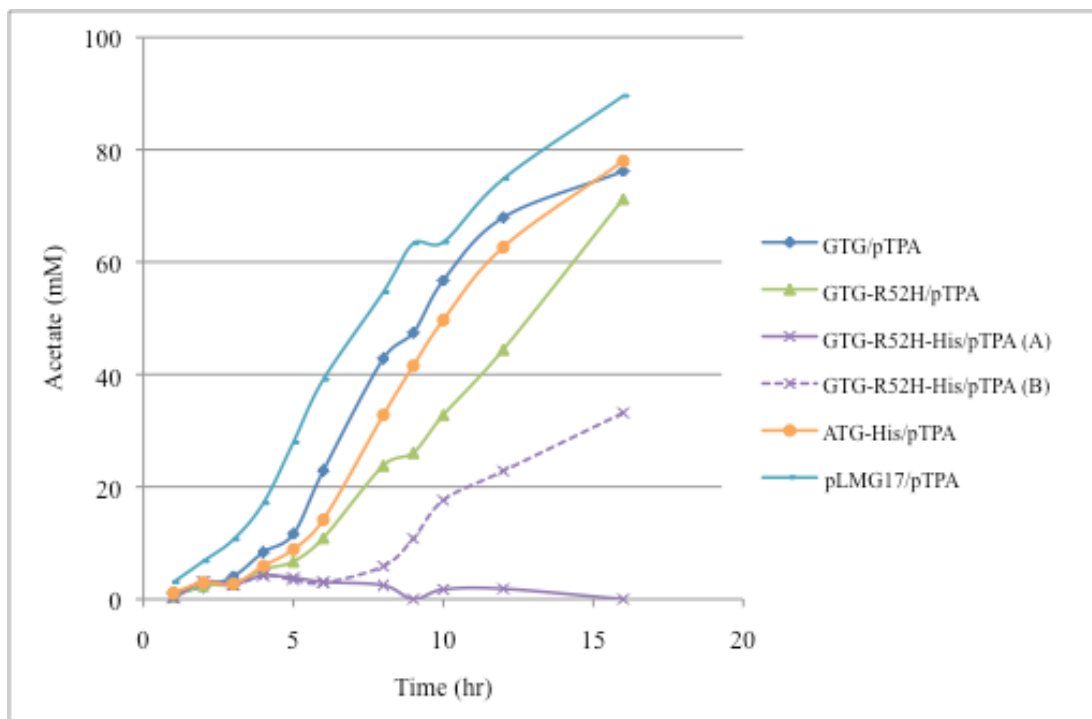
Figure 21. The GTG-R52H-His *mlc* construct yielded the least acetate accumulation over a 16 hour culture when co-expressed with TPA in C.R.A.P media and 10 g/L glucose. Individual data sets from each GTG-R52H-His/pTPA replicate are shown.

RT-qPCR analysis was performed to compare levels of *mlc* transcription during

the course of the cultures. Samples were collected 6, 8 and 10 hours post inoculation. At

a minimum a tenfold increase of *mlc* RNA was observed in the cultures overexpressing

Mlc compared to the control, however greater increases were certainly observed as seen

in Figure 22. Once again, the data sets for both cultures containing the GTG-R52H-His

mlc construct are shown due to significant variation in the replicate pair. The decrease in

mlc RNA content in the "B" replicate at the 8 hour time point corresponds with the

increase in acetate concentration in the media, as shown in Figure 21.
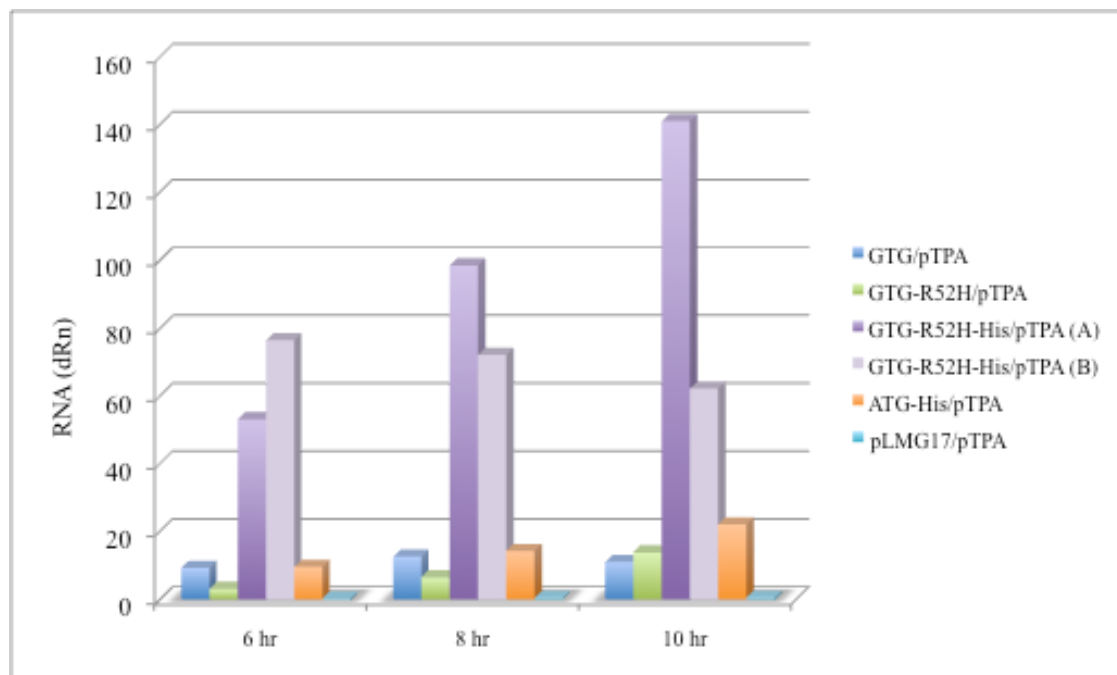
Figure 22. RT-qPCR results comparing *mlc* expression relative to two housekeeping
genes 6, 8 and 10 hours post inoculation. Individual data sets from each of the GTG-
R52H-His/pTPA replicates are shown.

Based on the data collected from the shake flask experiments the co-

transformation containing the GTG-R52H-His *mlc* construct was selected to be run in the

DASGIP bioreactor system. In addition to consistently exhibiting the greatest effect on minimizing acetate accumulation in culture, the histindine tag would allow for protein detection via Western blot or in-gel staining.

A total of six fermentation runs were conducted as listed on Table 5. Four runs expressed both Mlc and TPA (JB017, JB018, JB024, and JB026), and two were part of the control group (JB023 and JB025). Each run was carried out for 36 hours and a glucose excursion was executed after a consistant glucose feed rate was established (between 18-24 hours post-inoculation) for runs JB018, JB025, and JB026. Glucose, acetate and growth profles from this set of experiments are presented in Figures 23 through 28. Each profile is shown twice, the first over the course of the entire 36 hour run, and a second focusing on the initial 16 hours. A complete list of all growth, acetate, glucose, and RNA data from the DASGIP experiments can be found on Table 16 of Appendix F.

The glucose profiles of the six DASGIP runs show an accumulation of glucose in those cultures co-expressing Mlc. This is illustrated in Figures 23 and 24. The maximum glucose concentrations were observed between 8 and 12 hours post-inocullation, with the greatest concentration of 26 g/L occuring during run JB024. The other cultures co-expressing Mlc experienced glucose peaks around 10-15 g/L. Each of the Mlc culutres required manual adjustments to the glucose feeding algorithm (GFA) to prevent further glucose accumulation. It is important to note, that although the concentration of glucose in the control cultures (JB023 and JB025) appear to be low, glucose was fed into the cultures, however instead of accumulating in the media, it was rapidly being consumed.

Over the initial several hours of the runs more glucose was fed into the control cultures, than in those co-expressing Mlc (data shown in Appendix G).
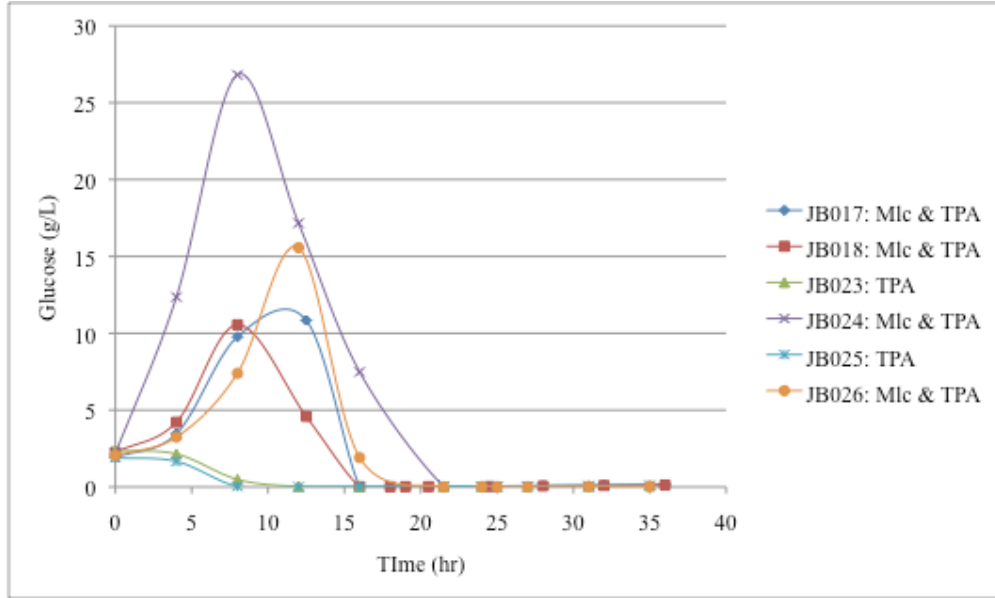
Figure 23. Profile of glucose present in the media over the entire 36 hours of the fermentation. An accumulation of glucose was observed in each of the runs co-expressing Mlc, whereas no such accumulation was observed in the controls.
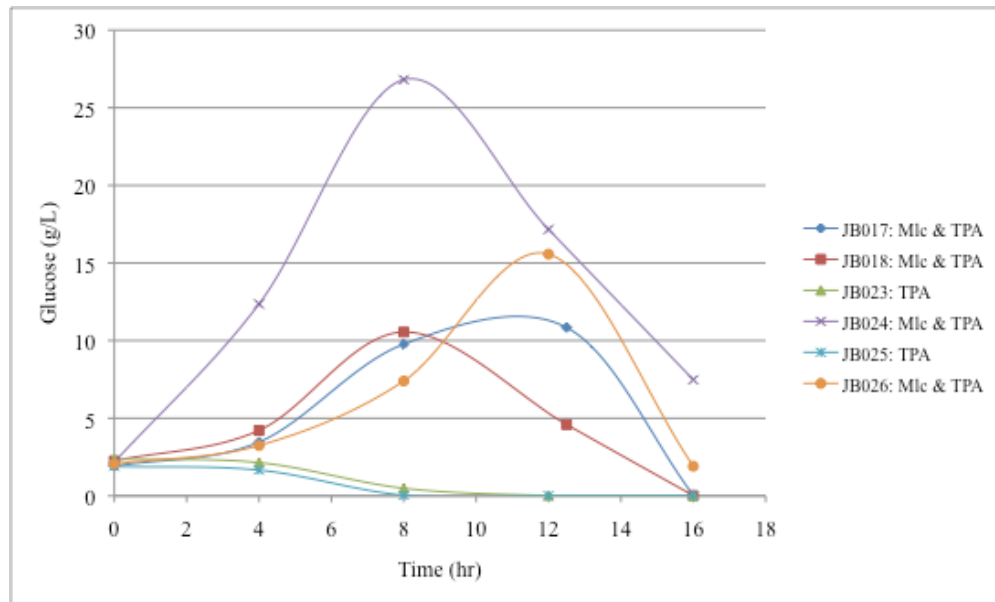
Figure 24. Profile of glucose present in the media over the first 16 hours of the fermentation.

Significant acetate accumulation was observed in the two control cultures (JB023 and JB025) as well as in one of the cultures co-expressing Mlc (JB024), as illustrated in Figures 25 and 26. The onset of acetate accumulation during the JB024 run was delayed by approximately 12 hours compared to the controls. A rise in acetate concentration was also observed after 30 hours; this was most prominent in the control cultures.

Runs JB017 and JB018 each had peak glucose concentrations in the media of close to 10 g/L (similar to the shake flask cultures), and no appreciable rise in acetate concentration in the media was observed. Run JB026, had a slightly higher peak glucose concentration in the media of 15 g/L, which resulted in a small spike in acetate (20 mM) at the 16 hour mark (similar to what was seen in the shake flask cultures).
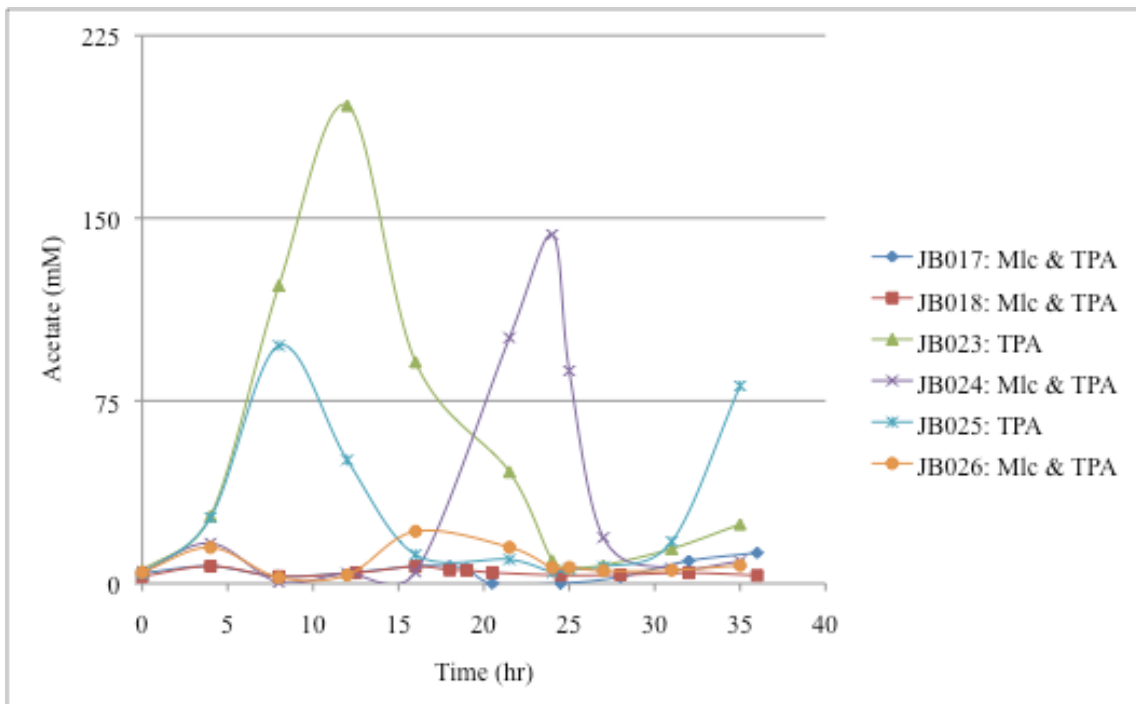
Figure 25. Profile of acetate present in the media over the course of the 36 hour fermentation.
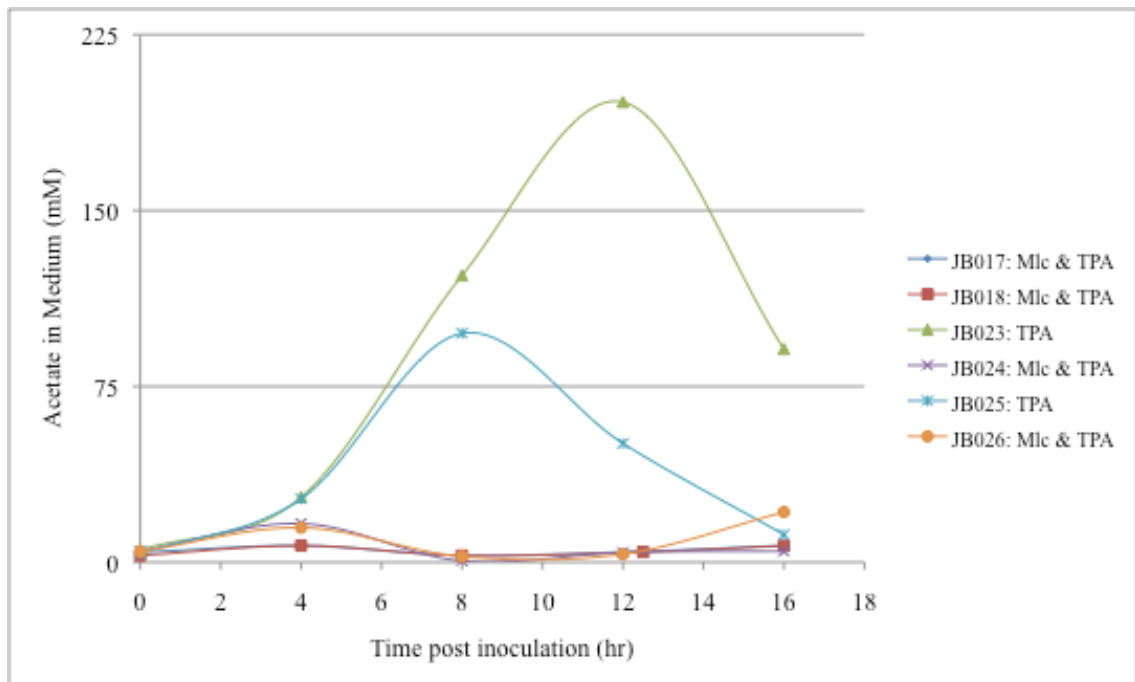
Figure 26. Acetate levels remained low in cultures overexpressing Mlc during the first 16 hours (JB017, JB018, JB024, and JB026).

The growth profiles for the six DASGIP bioreactor runs are shown in Figures 27 and 28. The two control cultures (JB023 and JB025) initially out grow those cultures over-expressing Mlc, presumably as a result of the rapid consumption of all available glucose. However after eight hours, the growth rates of the control cultures slow down; the timing coincides with the accumulation of acetate in the media. Although the growth of the cultures overexpressing Mlc initially lag behind the controls, all of the runs achieve comparable OD by the completion of the 36 hour experiment; each surpassing 100 OD after 27 hours.
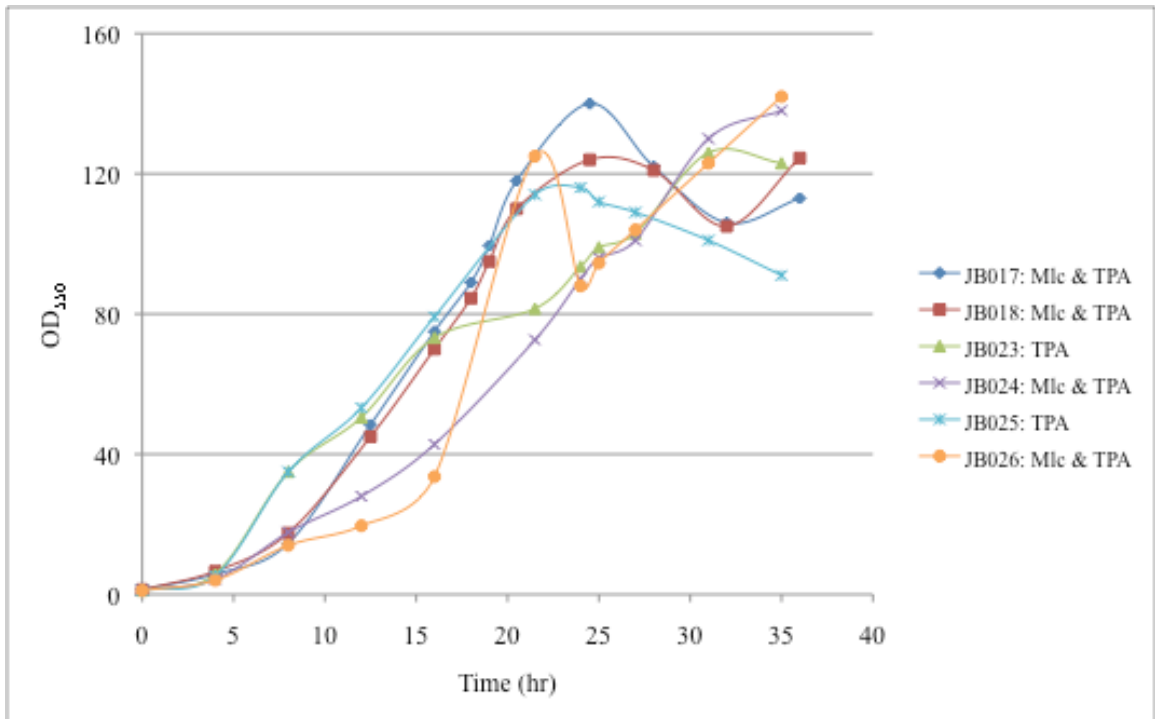
Figure 27. Growth profiles from cultures run on DASGIP bioreactor system.
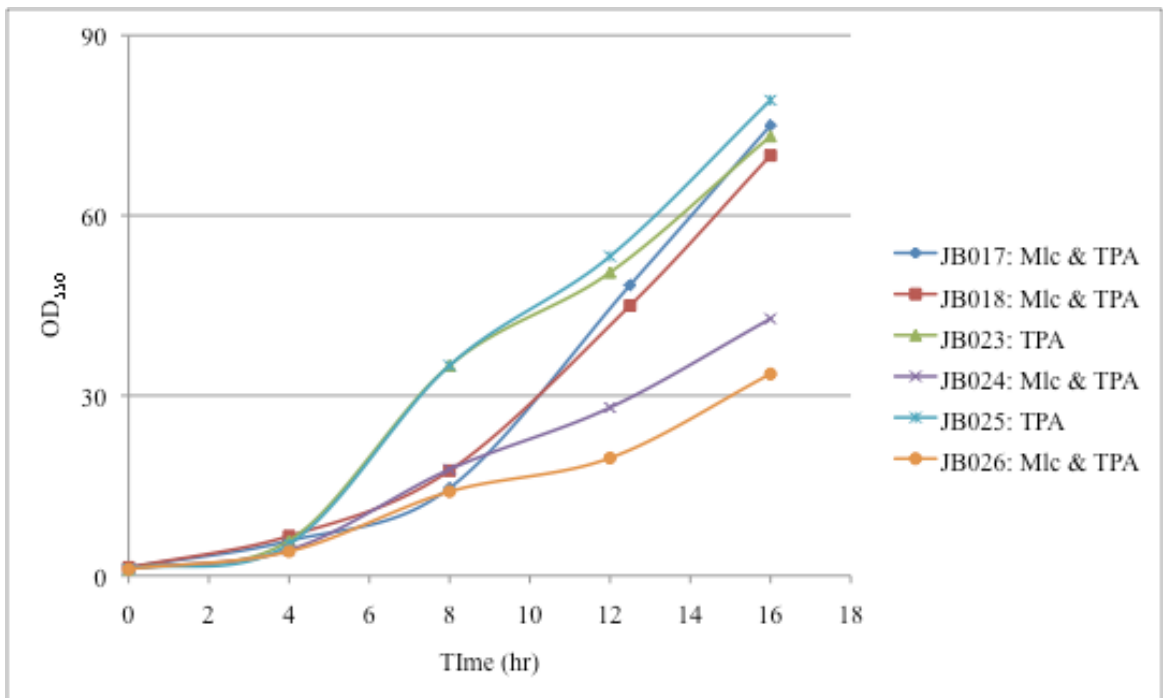
Figure 28. Growth profiles of the cultures during the initial 16 hours.

RT-qPCR was performed on samples collected 8 and 12 hours post-inoculation to quantify the level of *mlc* transcription. These results are shown in Figure 29. Each of the cultures co-expressing Mlc showed a significant increase in *mlc* RNA compared to the controls. At a minimum, a nearly 100-fold increase was observed in the Mlc cultures.

An assay was also run to determine the concentration of TPA produced throughout the course of the fermentation. Samples were collected 24 and 36 hours post-inoculation. These results are shown in Figure 30. After 24 hours, there were no clear trends separating the cultures co-expressing Mlc from the controls, however after 36 hours, an average 1.7-fold increase in total TPA production was measured in the cultures expressing Mlc compared to the control.
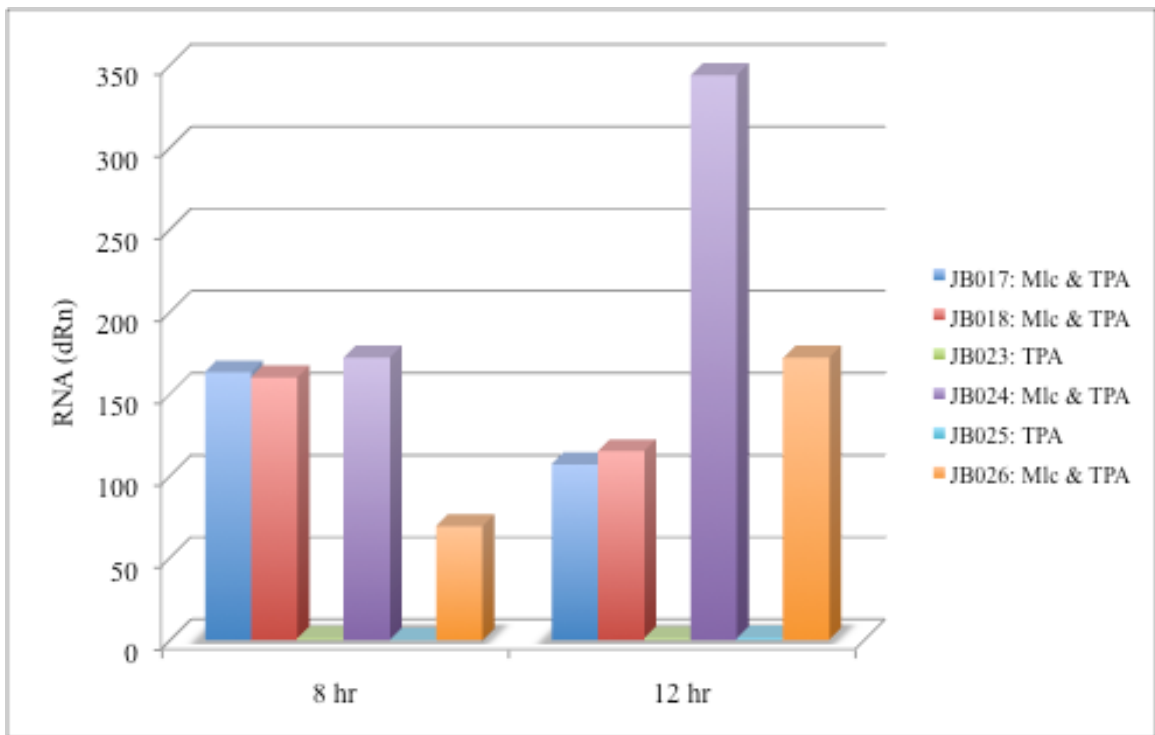
Figure 29. Relative expression of *mlc* normalized to two house keeping genes at 8 and 12 hours post inoculation.
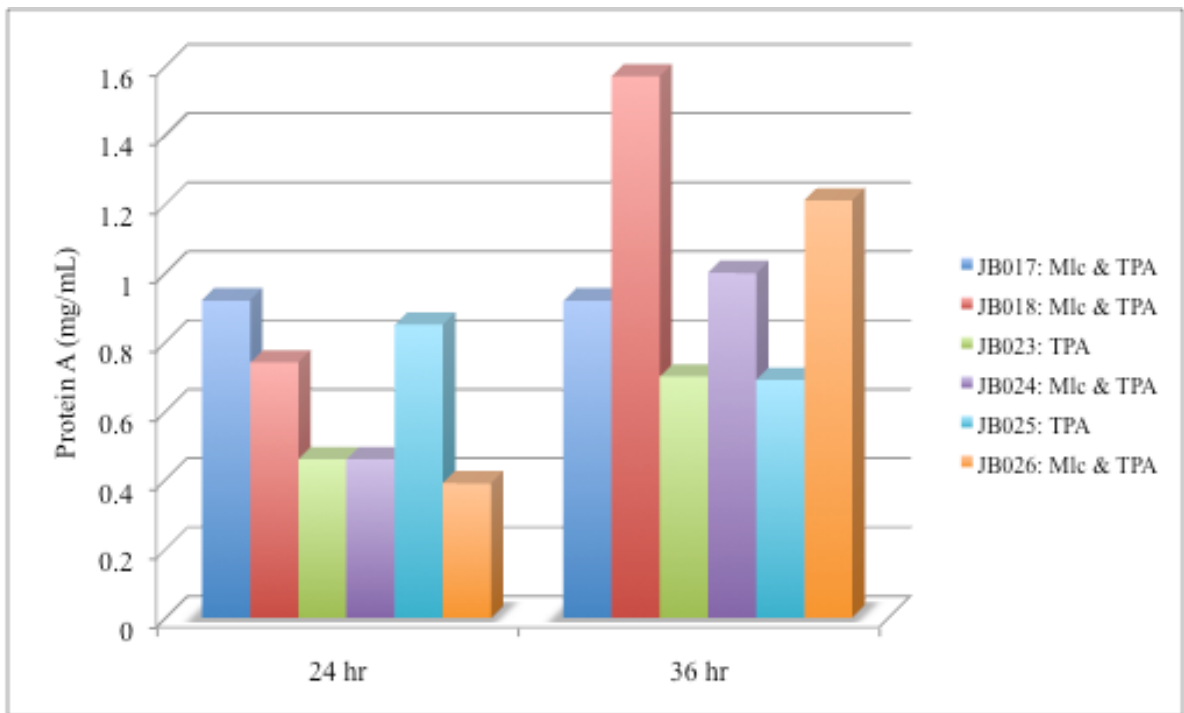
Figure 30. Therapeutic Protein A titer from DASGIP bioreactor cultures.

CHAPTER SIX: DISCUSSION

6.1   Constitutive Arm (pACYC177)

The growth and acetate data from the experiments constitutively expressing Mlc showed no appreciable difference between the wild-type culture and those cultures overexpressing Mlc. The control experiment in which no glucose was added showed similar growth between both groups after 24 hours and no significant acetate accumulation, as seen in Table 8. In the two sets of experiments in which 10 g/L glucose was batched up front, shown in Tables 9 and 10, respectively, acetate accumulation was observed after 4 hours in all cases and continued to rise through the course of the experiments.

The experimental data suggest that there was not enough Mlc being produced through the pACYC177 expression vector to positively affect acetate accumulation in culture. Although constitutively expressed, pACYC177 is a low copy number plasmid and in these instances was not able to provide a sufficient Mlc response to mitigate acetate accumulation due to the overfeeding of glucose, thus closing this arm of experiments.

6.2   Inducible Arm (pLMG17)

6.2.1   Inducer Evaluation

Data from the induction experiment, presented in Table 11 and illustrated below in Figure 31, show similar glucose and acetate profiles from each of the cultures carrying the pLMG17 plasmid regardless of when IPTG was added, if at all. This induction experiment did show however, a slower depletion of glucose and significant acetate

reduction from the media compared to the 1A2 control culture. The control culture did

not achieve a measured optical density greater than 9 (OD_{550}) and the acetate

concentration in the media reached a plateau around 130 mM. This suggested that Mlc

was expressed from the pLMG17 expression plasmid regardless of the addition of the

inducer, and minimized acetate accumulation while positively affecting overall cell

growth.
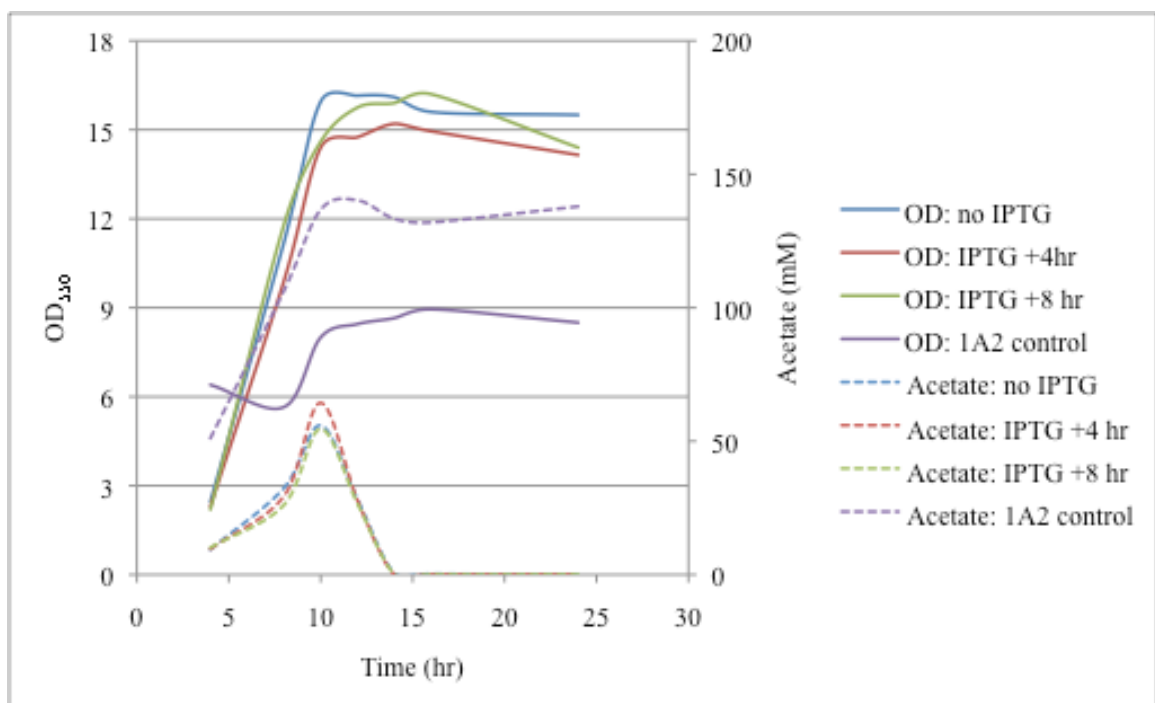
Figure 31. The addition of IPTG to the culture did not appear to affect the growth or acetate profiles of the cultures. The control culture reached an OD550 of 9, and acetate concentration reached a plateau around 130 mM.

The *tac* promoter, which regulates transcription of *mlc* in the pLMG17 expression

vector, is not completely repressed and does allow for some residual basal levels of

expression [32]. The leaky nature of the *tac* promoter appeared to allow sufficient

transcription of *mlc* without an inducer, which positively affected the glucose, acetate and

growth profiles of the cultures. Based on this finding, IPTG was not used in subsequent experiments with the pLMG17 expression plasmid.

6.2.2 Mlc Overexpression in Overnight Cultures

The first set of experiments in which the eight *mlc* constructs were overexpressed via the pLMG17 expression vector yielded varying concentrations of acetate accumulating in the media as seen in Figure 17. Each culture overexpressing Mlc showed lower concentrations of acetate in the media compared to the 1A2 control culture, suggesting that Mlc, when overexpressed, can limit the glucose uptake into the cell and mitigate the formation of acetate. The variability in the acetate profiles between each of the Mlc cultures, however, intimates variability in the level of overexpression of Mlc. This may have been the result of basing the transcription of *mlc* on the leakiness of the *tac* promoter. Although no significant variation was observed in the growth and acetate profiles during the induction experiment, the dynamic nature of *E. coli* culture may have resulted in varying levels of "leaky" transcription.

The general trends in the growth and acetate profiles are in line with what Cho *et al.* observed when overexpressing Mlc in culture [11]. In both experiments the cultures overexpressing Mlc reached greater cell density compared to the wild-type, as illustrated in Figures 11 and 19. Additionally, the acetate concentration in the media continued to rise in the wild-type culture while they trended downward (and in some cases were nearly eliminated) in the Mlc cohort, as shown in Figure 17.

Through the first series of shake flask experiments grown in City Broth media, cultures overexpressing Mlc with the GTG-R52H and GTG-R52H-His constructs closely

paralleled one another. As shown in Figure 32, the growth profiles from these two

constructs approached 18 OD_{550} and acetate found in the media peaked prior to the 10

hour mark and was mostly depleted three hours later. These results were expected, the

histidine tag was only added to the end of the *mlc* sequence for analysis. It was not

intended to affect Mlc expression [25] nor appears that is has.
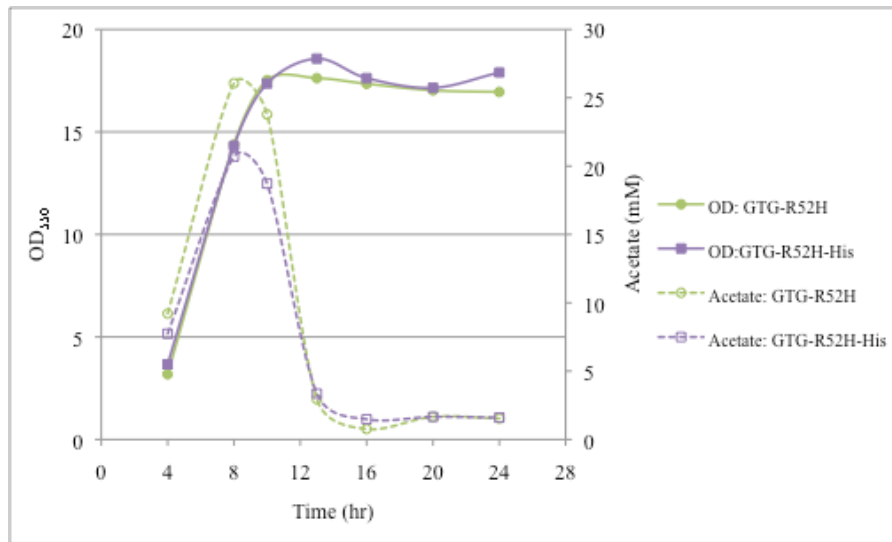
Figure 32. Growth and acetate profiles from cultures overexpressing Mlc containing the R52H point mutation with and without a histidine tag.

6.2.3 Co-Expression of Mlc with TPA

A couple of differences were observed when comparing the growth and acetate

profiles between the cultures solely expressing Mlc, illustrated in Figures 17-19, and

cultures co-expressing Mlc along with TPA, shown in Figures 20 and 21. Firstly, the

latter group only reached half the OD as the cultures growing in City Broth. This is

likely the result of the combination of several factors: media, (CRAP media versus City

Broth), host strain (16C9 versus 1A2), and lastly the extra burden of expressing TPA

along with Mlc. The selection of media and host strain were based on past experiments targeting TPA production.

It is important to note that although the first set of experiments reached a higher OD, both groups exhibited similar growth, reaching stationary phase after 12 hours. Conversely, the acetate profiles for the two experiment groups differ, for the group co-expressing TPA, the acetate concentration in the media was still on the rise after 16 hours, whereas when Mlc was solely overexpressed in culture, the acetate concentrations had already diminished after 16 hours. This can be seen in Figures 18 and 21.

The deviation in acetate profiles may be explained by the slower growth rate of the group co-expressing Mlc and TPA. Due to the slower growth, glucose remained in the media for a much longer period of time. After 16 hours there was still glucose present in all but one of the cultures, whereas in the previous experiment (solely overexpressing Mlc), all of the glucose in the media has been consumed 13 hours post-inoculation. This is shown in Figure 33. Though the concentration of glucose that was batched upfront was the same for both groups of experiments (10 g/L), the slower growth rate for the cultures co-expressing Mlc and TPA resulted in a higher glucose burden for the cells (i.e. a higher glucose concentration when normalized for cell density). Based on the trend lines, glucose levels should have likely been depleted after 20-24 hours post inoculation; it is after this point that a direct comparison between acetate levels should be made.

The glucose profiles across both sets of experiments illustrated in Figure 33, show a significant change in the glucose consumption rate in four of the cultures: 1A2

(control), pLMG17/pTPA (control), GTG/pTPA, and ATG-His. The timing of this

change in glucose consumption rate corresponds to the cultures transitioning to the

stationary phase of growth, which can be seen in Figures 19 and 20. Additionally, each

of these four cultures exhibited the highest concentration of acetate in the media,

suggesting that the change in glucose consumption rate may correspond to the point in

which the acetate accumulation in the media was significant enough to stunt overall cell

growth.
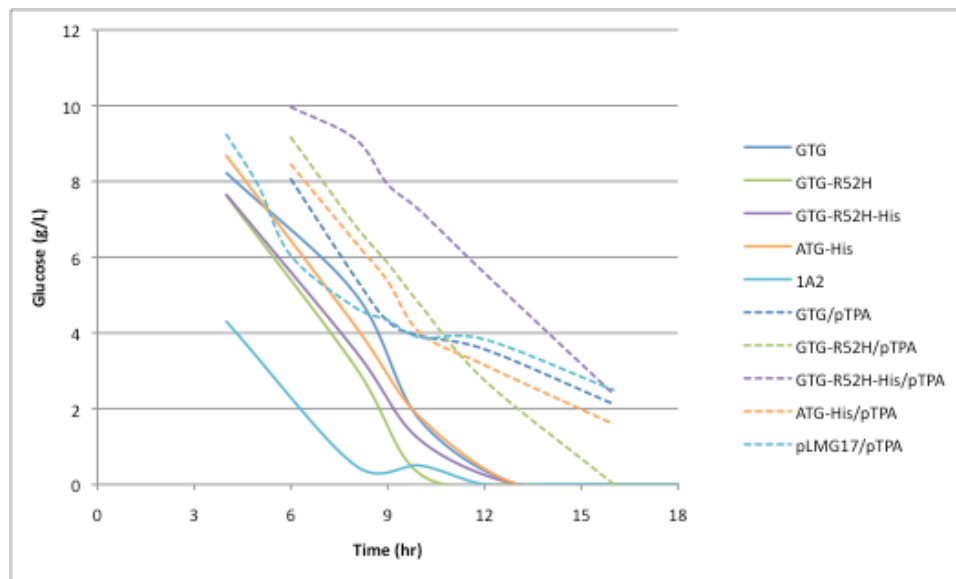
Figure 33. A comparison of the concentration of glucose in the media between the two
sets of experiments. The data presented in dotted lines represents the glucose profiles for
those cultures co-expressing Mlc and TPA, the solid lines are only producing Mlc.

Despite the differences in growth rate and glucose concentration in the media, the

GTG-R52H-His *mlc* construct performed well both when solely overexpressed and co-

expressed with TPA in culture. In the second experiment (when co-expressed with TPA)

there was appreciable variability in the data between the two replicates, as seen in Figure

21. Replicate A only showed baseline levels of acetate, while acetate levels in the media

for replicate B climbed after 6 hours, but still did not surpass 40 mM. Though after 16

hours, the acetate levels were on the rise, the glucose concentration in the media was

already approaching 2 g/L, which suggests that the acetate concentration in the media

would soon peak at a maximum concentration that was nearly half of what was measured

in the other three *mlc* constructs tested.

Unlike the previous experiment in which Mlc was solely overexpressed in culture,

the GTG-R52H-His and GTG-R52H *mlc* constructs did not share similar growth and

acetate profiles when transformed via the pLMG17 plasmid and co-expressed with TPA.

The untagged construct performed similarly to the other two constructs tested in this set

of experiments, with a rapidly rising acetate profile, exceeding 70 mM after 16 hours. It

was also the first of the cohort to completely consume all of the glucose in the media,

shown in Figure 33 (green dotted line). The differences in the growth and acetate profiles

of the histidine tagged and untagged constructs are illustrated in Figure 34.
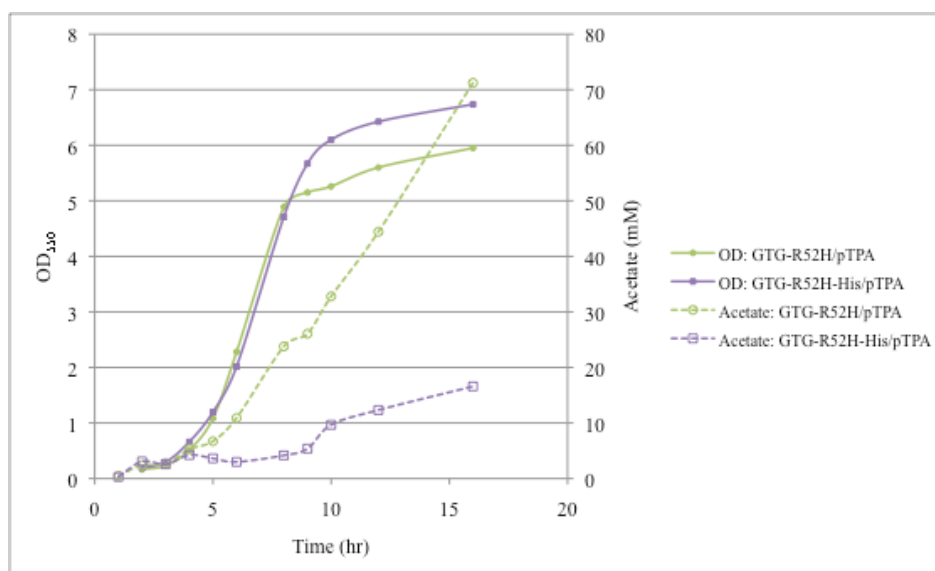
Figure 34. Growth and acetate profiles for cultures co-expressing Mlc and TPA. The
mlc constructs include the R52H point mutation with and without a histidine tag.

The expectation was that these two constructs would have behaved similarly such as they did in the first round of experiments, depicted in Figure 30, proving that the histidine tag did not affect Mlc expression or activity. Since the tagged construct outperformed the untagged, this deviation is likely not a result of the histidine tag, but rather related to the variation in acetate profiles that has been observed between the different *mlc* constructs throughout all of the experiments; which is hypothesized to be the result of *mlc* transcription based on the leaky regulation of the *tac* promoter.

Although the precise reason for the variation observed in the growth and acetate profiles is under investigation, it is important to note that in each of the experiments, those cultures overexpressing Mlc (either as a single or co-transformation) outperform the wild-type controls. To "outperform" in this context can be defined as achieving greater optical density and lower acetate accumulation in the media as a result of slower glucose uptake. This has been illustrated in Figure 17 and Figures 19-21 and is consistent with the results presented by Cho *et al.* [11].

Two methods were used to verify that the results obtained were caused by the overexpression of Mlc, protein detection via the histidine tag and RNA detection via RT-qPCR. Three different techniques were used to detect the histidine tag (two by Western blot and a third by in gel staining). The limit of detection of these three techniques was as low as 0.5 ng, however none were successful in resolving the Mlc band. It was concluded that although there was sufficient overexpression of Mlc to reduce acetate accumulation it was not enough to detect the attached histidine tag.

Although direct detection of the Mlc protein was not successful, the indirect measurement via RT-qPCR provided sufficient evidence that the observed effects on acetate accumulation and cell growth were the product of Mlc overexpression. RNA analysis, such as is shown in Figure 22, consistently showed increased levels of *mlc* RNA in the overexpressing cultures compared to nominal levels found in the control groups.

Additionally, increased *mlc* RNA levels corresponded with lower acetate concentrations in the media. This is most evident when comparing the GTG-R52H-His construct with the blank pLMG17 plasmid control as illustrated in Figure 35. The greatest presence of *mlc* RNA corresponds to the highest cell density, and lowest concentration of acetate. This general trend however, was not consistent in all cases. The ATG-His construct appears to have the second highest concentration of *mlc* RNA while only the third lowest acetate concentration. This may be the result of the state of growth the culture is in (exponential versus stationary), and while this deviation cannot be explained given the analysis performed, the results still demonstrates the relationship between the overexpression of Mlc and reduced acetate accumulation. Moreover, the GTG-R52H-His construct has consistently demonstrated the greatest impact on acetate accumulation in shake flask cultures and was selected as the best candidate to move forward for small scale bioreactor evaluation.
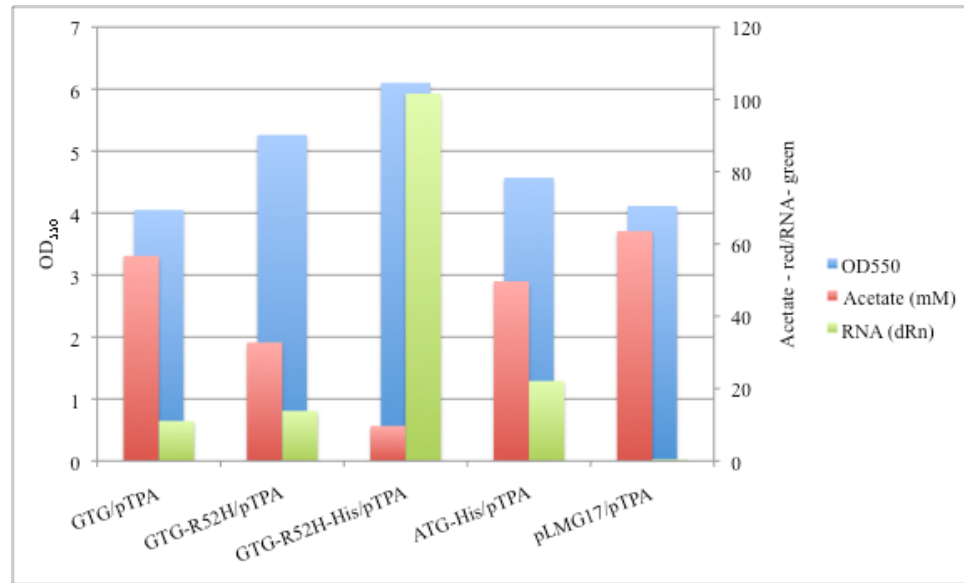
Figure 35. Measured growth, acetate and RNA levels after 10 hours in culture. 16C9 cells were co-transformed to overexpress Mlc and TPA.

6.2.4 Small Scale Fermentation in DASGIP Reactors

The transition from growing cultures in shake flasks to the DASGIP reactor system was made to better model the conditions in which therapeutic proteins are produced. The DASGIP reactor is inherently a different system and allows for much higher cell density cultures to grow compared to the shake flask. One significant difference is the availability of oxygen; where in the shake flasks it is in excess, the DASGIP systems is driven by a glucose feed algorithm (GFA) used to maximize cell densities and ultimately control the cultures under micro-aerobic conditions (dO_2 control).

Significant differences in the cell growth, acetate, and glucose profiles between the Mlc and control cultures were observed particularly during the first 16 hours of the DASGIP bioreactor runs. As the cultures entered into the exponential stage of growth,

the control cohort experienced more rapid growth rate represented by the steeper slope between hours 4 and 8 in Figure 28. This increased growth rate corresponded with the rapid depletion of glucose in the media shown in Figure 24, and the beginning of a rise in acetate in the media, shown in Figure 26.

Those cultures overexpressing Mlc experienced a much slower growth rate, which can be attributed to the slower uptake of glucose into the cells. This resulted in lower acetate accumulation in the media, and an unexpected rise in the glucose concentration. The rise in glucose was the result of the glucose feeding algorithm (GFA) attempting to establish an appropriate glucose feed rate (GFR) to support cell growth. Due to the slower glucose uptake in the Mlc cultures a manual intervention was required to reset the GFR because the GFA was overfeeding the cultures. Once manually reset, the GFA operated appropriately. Moving forward, the GFA should be optimized to better respond to the slower glucose uptake rate when Mlc is overexpressed in culture.

Despite the overfeeding caused by the GFA, three of the four cultures co-expressing Mlc presented a minimal acetate response in comparison to the two control cultures (JB023 and JB025). Only in one instance was an appreciable acetate response detected in a culture expressing Mlc, JB024, which had experienced a significant glucose overfeeding event due to the GFA. In each of these four cultures RT-qPCR data, shown in Figure 29, confirmed increased levels of *mlc* RNA compared to the controls. Similar to the results seen with the shake flask cultures, the overexpression of Mlc appears to mitigate acetate formation due to glucose overfeeding.

It is interesting to note that although JB024 underwent the overfeeding event from the onset of the culture, peaking at 8 hours, the acetate response was delayed, beginning after 15 hours and peaking at 24 hours. This was unlike the control cultures, which underwent an acetate response nearly immediately, peaking at 12 and 7 hours for JB023 and JB025, respectively. Upon review of the dissolved oxygen levels throughout these runs (data not shown), it appears that the acetate event observed during JB024 coincided with when the culture was reaching dO_2 control. This suggested that the acetate formed was not a direct result of overfeeding but may have been a consequence of the micro-aerobic conditions that impacted the aerobic respiration pathway and drove the formation of acetate. In addition, this culture condition may have exceeded the capacity of the overexpressed Mlc to slow the glucose uptake sufficiently.

This response is linked to the overexpression of Mlc and the overfeeding event, however is not a direct result of solely the overfeeding event, which can explain the lower peak concentration of acetate compared to those observed in the control runs. The overexpression of Mlc slowed the uptake of glucose into the cells resulting in a slower growth rate. However, due to the GFA a surplus of glucose had already been charged into the media, though it remained there as the culture slowly metabolized what it could. Close to the 15 hour mark the cell mass had increased enough that it was able to reach micro-aerobic conditions. Under typical operation the GFA would control the GFR to maintain a balance between growth and dO_2 content, however the glucose had already been charged in the media, forcing the culture into micro-aerobic conditions with excess glucose present which resulted in acetate production.

The acetate accumulation observed in the control cultures (JB023 and JB025) was a not a product of micro-aerobic conditions, but rather the overfeeding of glucose. There was no glucose accumulation in the media, though the GFA was feeding the cultures, which means cells were freely metabolizing any and all glucose available. Also the acetate events in the control cultures occurred before dO_2 control was achieved, which would mean the cultures were undergoing aerobic respiration as the TCA cycle became overwhelmed with pyruvate, driving the formation of acetate via the Pta-Ack pathway.

The objective of the glucose excursions conducted in the DASGIP bioreactor cultures was to produce a change in acetate concentration in the media caused by an overfeeding event. A 20% increase in the GFR was performed for 10 minutes once dO_2 control was established in the cultures. For run JB018 this occurred after 18 hours, for the remaining cultures (JB025 and JB026), after 24 hours. The results from the glucose excursions were inconclusive. Glucose concentrations in the media just before and after the excursions were near zero and no significant differences were observed between runs where an excursion was conducted and the controls (data not shown). This is most likely due to the glucose feed algorithm (GFA), which establishes the GFR to maintain an oxygen limited environment.. Under these conditions a 20% increase in the GFR for 10 minutes may have only created a momentary overfeeding event that the culture could withstand with minimal disruption. A prolonged excursion and/or greater increase in the GFR may have been required to fully evaluate the effect the overexpression of Mlc had once the culture was under dO_2 control.

Additionally, the acetate profiles after the glucose excursion was initiated, presented in Figure 36, show a rise in JB025 several hours after the glucose excursion. A similar but smaller rise is also observed in JB023, the corresponding control culture that did not undergo a glucose event. None of the cultures overexpressing Mlc experienced any significant rise in acetate levels. It is difficult to attribute the rise in acetate observed in JB025 with the glucose excursion, because a rise was observed in the control, however the increase in acetate may be a general indication of the health of the culture. Both of these cultures (JB023 and JB025) had significant acetate events within the first 16 hours due to glucose overfeeding and as a result may have been entering the end stage of growth as indicated by the accumulation of acetate. The rise in acetate also corresponded with a decrease in cell density from the 30 to 36 hour marks as shown in Figure 27. More experimentation is required to fully evaluate the effect overexpressing Mlc in culture can have on a glucose overfeeding event.
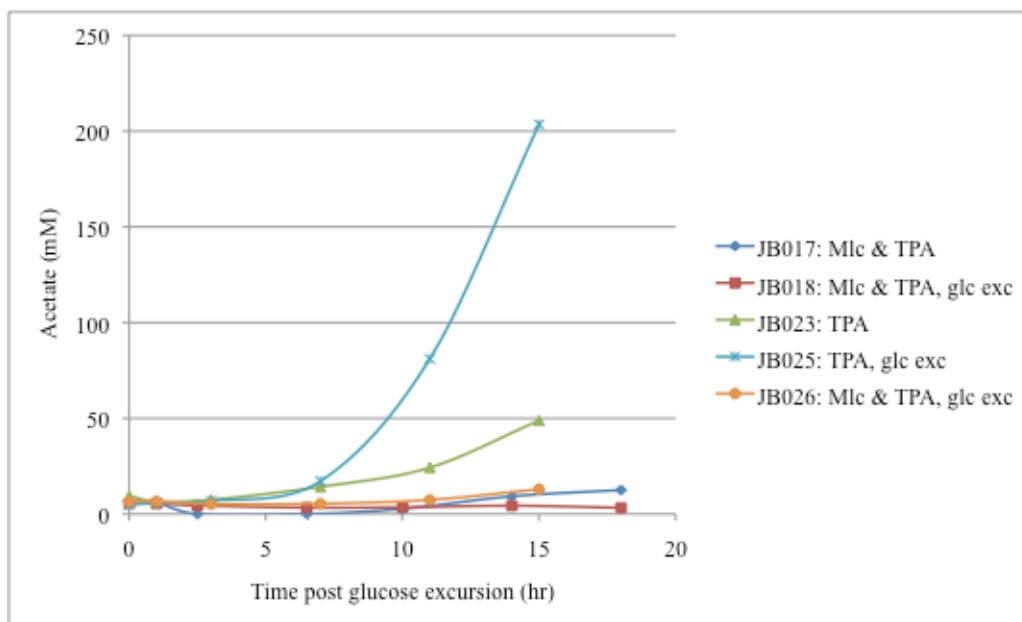
Figure 36. Acetate profiles for cultures after a 10 minute glucose excursion was executed for runs JB018, JB25, and JB026.

The final TPA production from the DASGIP bioreactor cultures, shown in Figure 30, indicate that after 36 hours those cultures overexpressing Mlc produced greater concentrations of the therapeutic protein. On average, cultures co-expressing Mlc produced 1.7 times more TPA after 36 hours than cultures solely producing TPA; the best case (JB018) produced 2.3 times greater TPA than the control group. At the 24 hour mark, the cultures overexpressing Mlc were producing less TPA than the controls, likely due to the lower cell densities caused by the slower glucose uptake.

The production of TPA occurs over the course of the culture; taking the total TPA produced and normalizing to the integrated OD (iOD) of the culture, the group overexpressing Mlc shows a 1.7 times greater TPA production compared to the control group. This suggests that the overexpression of Mlc in culture may allow for greater cellular production of TPA, not just improved production as a result of higher cell

densities. In fact, two of the runs JB024 and JB026 had an overall iOD less than that of the control cultures, but yet still produced more overall TPA. The iOD for JB024 and JB026 are 2097 and 2196 versus 2413 and 2492 for JB023 and JB025, respectively. Due to variations in the GFR, including the initial overfeeding and glucose excursions, additional experiments would need to be conducted in order to draw any further conclusions directly correlating the affect the overexpression of Mlc has on the production of TPA.

The greater production of TPA in conjunction with overexpressing Mlc was consistent with the findings of Cho *et al.* [11]. Cho *et al.* found four times greater ß-galactosidase activity and 10 times greater fluorescents when overexpressing Mlc compared to the wild-type strain, while only approximately 1.5 times greater cell growth was observed.   This observation suggested greater recombinant protein production beyond an improvement in cell mass. The degree of improvement in recombinant protein productivity between this experiment and the results published by Cho *et al.* (1.7-fold compared to 4 and 10-fold) may be the result of the greater burden of expressing TPA via the co-transformation of two plasmids (one expressing TPA and the other Mlc), whereas Cho *et al.* had made changes to the endogenous *mlc* sequence thus requiring only the transformation of a single plasmid for recombinant protein expression. Additional experiments are required to correlate the overexpression of Mlc with the productivity of a recombinant therapeutic protein.

6.2.5 Further Experimentation

Based on the results from this series of work, several additional steps are required in order to better evaluate how the overexpression of different Mlc variants in *E. coli* culture affects production and accumulation of acetate. These steps include the selection of an appropriate expression plasmid, co-expression of alternate recombinant proteins, optimization of the glucose feed algorithm and glucose excursion used in the small scale fermentation, and procurement of an Mlc-specific anti-body.

First and foremost the selection of an appropriate expression plasmid is required for the proper evaluation of Mlc overexpression. In work completed, the constitutively expressed plasmid did not drive high enough Mlc expression, likely due to being a low-copy number plasmid. The inducible plasmid evaluated allowed for the transcription of *mlc* without the addition of an inducer caused by the leaky regulation of the *tac* promoter. Additionally, the experiments conducted showed that wild-type *E. coli* cultures rapidly consume any glucose available, leading to acetate formation and accumulation. Therefore, the plasmid selected to drive Mlc overexpression for future experiments should confer constitutive expression of Mlc via a high-copy number plasmid. The level of Mlc overexpression through this plasmid should remain consistent through the lifecycle of the culture.

After the selection of an appropriate expression plasmid, the evaluation of the four *mlc* constructs (GTG, GTG-R52H, ATG, and ATG-R52H) can be repeated. The plasmids should be transformed into 1A2 cells, and cultures grown in City Broth with 10 g/L glucose. A culture of 1A2 cells with a "blank" plasmid (no *mlc* insert) should be

used as a control. Cell growth, acetate, and glucose profiles should be analyzed to compare the effect each of the mutations has relative to that of the endogenous *mlc* sequence (GTG). Based on these results, at least one *mlc* construct should be selected to be co-transformed along with pTPA in order to study the effect the overexpression of Mlc has on the production on a therapeutic protein.

In addition to co-transforming cells with a plasmid encoding for TPA, another series of experiments in which either pGAL or pGFPuv is co-transformed along with the plasmid encoding for Mlc would bridge the work performed by Cho *et al* [11]. This effort would help determine if genomic modulation of *mlc* leads to greater recombinant protein production compared to overexpression of Mlc through an expression plasmid.

The glucose feed algorithm used in the small scale fermentation experiments needs to be modified to account for the slower glucose uptake rate by the cultures overexpressing Mlc. The current algorithm currently overshoots the initial glucose feed rate during the early stages of the culture such that dO_2 control cannot be achieved without manual intervention. A modified algorithm could minimize the overshoot thus creating a minimal overfeeding event.

Additionally for the glucose excursion test, preliminary experiments should be run to determine the percent increase in glucose feed rate and duration of the excursion that would be required to initiate an acetate response in a wild-type culture. More frequent sampling immediately following the glucose excursion should be conducted, starting at every 10-15 minutes for the first hour.

Lastly, acquiring purified Mlc protein and anti-Mlc mouse antibody for use with Western Blot analysis would help confirm the expression level of Mlc. This approach will allow for the direct detection of the Mlc protein and bypass the need for a histidine tag. Not only would this confirm successful translation of Mlc, but the ability to quantify the Mlc content would help determine if the concentration of Mlc was fluctuating throughout the course of the cultures. Variability in Mlc expression was a potential issue encountered in the inducible arm of experiments; leaky regulation of the *tac* promoter may have led to variable *mlc* transcription thereby affecting the levels of Mlc overexpression.

CHAPTER SEVEN: CONCLUSIONS

Leveraging the leaky regulation of the *tac* promoter, the inducible arm of this work demonstrated constitutive expression of Mlc. When transformed in 1A2 cells and grown overnight in City Broth, cultures expressing each of the eight *mlc* constructs grew to higher density and better limited acetate accumulation compared to the wild-type control. Variability was observed in the extent to how much each construct outperformed the control based on levels of acetate and glucose measured across the duration of the cultures. This trend may be the result of basing the overexpression of Mlc on the leaky regulation of the *tac* promoter, however the exact reason for the variability could not be determined.

Four of the *mlc* constructs were selected to be co-transformed with a plasmid encoding for therapeutic protein A (TPA) in 16C9 cells. These were grown in overnight shake flask cultures. Once again, each of the cultures overexpressing Mlc showed a positive impact on limiting acetate accumulation and overall cell growth compared to the control. However, as with the previous round of shake flask experiments, there was variability in the extent to which the cultures overexpressing Mlc outperformed the control. Ultimately, lower levels of acetate were observed in all test cases. RT-qPCR data also showed an inverse relationship between the level of *mlc* RNA and acetate accumulation. The GTG-R52H-His *mlc* construct was selected to be used the small scale fermentation experiments.

When grown in the DASGIP bioreactors, cultures overexpressing Mlc demonstrated a slower uptake of glucose resulting in minimal acetate accumulation over

the course of the experiment. These conditions allowed for the cultures to maintain a greater optical density, which resulted in the greater production of TPA compared to the control. Cultures overexpressing Mlc showed a 1.7-fold improvement in TPA production compare to the control when normalized to the integrated optical density. Glucose excursions were conducted in an attempt evaluate the how the cultures overexpressing Mlc could handle a glucose overfeeding event. The excursions however, were not significant enough (neither high enough feed rate or long enough in duration) to produce a noticeable disturbance in the acetate or glucose profiles.

Analysis of the data from the eight *mlc* constructs evaluated in the pLMG17 (inducible) expression plasmid showed inconsistent relationships between the change in the translational start codon (GTG to ATG) and point mutation at codon 52 (R52H) and their effect on acetate accumulation in culture. This was likely the result of varying levels of leaky transcription from the *tac* promoter. Though the data amongst the eight *mlc* constructs was variable, each construct did outperform the wild-type control, resulting in greater cell growth and lower acetate concentration in the media. Therefore, the overexpression of Mlc via high-copy expression plasmid in *E. coli* does limit the glucose uptake rate into the cell thereby limiting acetate accumulation and improving overall cell growth. Additionally, the construct selected for growth in the small scale fermentation, GTG-R52H-His, produced the most consistent and positive results throughout the course of experimentation. This construct incorporated the point mutation identified by Gerber *et al*. and suggests that the R52H mutation should be included in future studies focusing on optimizing of Mlc overexpression.

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APPENDIX A

Table 12. A list of the materials to be used throughout the course of this study.

Restriction enzymes (New England Biolabs)
   NcoI
   HindIII
   KpnI
   XbaI
pBR322 based plasmid cloning vector (modified in-house at Genentech Inc.)
pACYC177 based plasmid cloning vector (modified in-house at Genentech Inc.)
Qiagen reagents
   QIAquick Gel Extraction kit
   QIAprep Miniprep kit
   RNeasy Mini kit
Agarose gel running apparatus
   Invitrogen E-Gel cassette holder
   BioRad Mini Gel Sun GT
Agarose gels
   Bio-Rad Mini ReadyAgarose TBE Gel (variable %)
   Invitrogen E-Gel (variable %)
PCR (cloning) reagents
   Taq DNA Polymerase & reaction buffer (Roche)
   Mlc primers (Genentech Inc. in-house)
   T4 DNA Ligase & reaction buffer (New England Biolabs)
   QuikChange Site-Directed Mutagenesis kit (Stratagene)
QPCR reagents
   TaqMan® One-Step RT-PCR Master Mix Reagents (Applied Biosystems)
   Primers/probe (FAM/TAMRA) (Genentech Inc. in-house)
Bacterial cultures
   Growth media: LB, City Broth, CRAP (Genentech Inc. in-house)
   Antibiotic: chloramphenicol, kanamycin, tetracycline (Genentech Inc. in-house)

pACYC177 primers

Insertion Primers
Forward (~25 bp upstream of kpnI site): 5'-CCGCTCATGAGACAATAACCCTGATA
AATGC
Reverse (~25 bp downstream of xbaI site): 5'-CATCTTATTAAGGGGTCTGACGCTC
AGTGGAACG

Start codon change
Forward (from GTG to ATG): 5'-GGCCGGTACCATGGTTGCTGAAAACCAGCCTG
GGC

Histidine Tag
Reverse: 5'-CCGGTCTAGATTAGTGATGGTGAGGTGATGACCCTGCAACAGACG
AATCAACAAAGAACCG

pLMG17 primers

Insertion Primers
Forward (~25 bp upstream of ncoI site):
5'-GGCTCGTATAATGTGTGGAATTGTGAGCGG
Reverse (~30 bp downstream of hindIII site):
5'-GCTTCTGCGTTCTGATTTAATCTGTATCAGGCTG

Start codon change
Forward (ncoI site plus change from GTG to ATG): 5'-GGCCCCATGGATGGTTGCT
GAAAACCAGCCTGGGC

Histidine Tag
Reverse: 5'-CCGGAAGCTTTTAGTGATGGTGATGGTGATGACCCTGCAACAGAC
GAATCAACAAAGAACCG

R52H Point Mutation
Forward: 5'-CTGCCAGTATCACTAAAATTGTCCATGAGATGCTCGAAGCACACC
TGGTGC
Reverse: 5'-GCACCAGGTGTGCTTCGAGCATCTCATGGACAATTTTAGTGATAC
TGGCAG

RT-qPCR Primers
Forward: 5'-TCCCGGTCATCTCAGACA
Reverse: 5'-CTGATGTGCTGACTATACG
Probe: 5'-ATCCGTCAGCAGGCCCTTCC

Table 13. PCR protocol.

Step	Temperature (°C)	Time	
Initial Melting	94	3 minutes	
Denaturation	94	30 seconds	Repeated for 24 cycles
Annealing	50	30 seconds	
Extension/Elongation	72	4 minutes	
Final Elongation	72	1 minute	
Hold	4	∞	

Ligation Protocol

Perform over ice
20 uL total volume

Combine the following:
Buffer: 2 uL
Vector: 1 uL
Insert: 2 uL
Ligase: 1 uL
Water: 14 uL

Incubate at 16°C overnight

APPENDIX E

Transformations were carried out by heat shock. Approximately 100 μL of competent cells of the desired host strain were thawed in 1.5 mL Eppendorf tubes over ice. Next, 1 μL of the target plasmid was added and the mixture kept on ice for approximately 30 minutes. The tube was then placed at 45°C for 90 seconds and then placed back on ice for 60 seconds. 350 μL of super optimal broth with catabolite repression (SOC) media was added and the tube incubated in a 37°C shaker for one hour. The resulting culture was spread on LB plates with antibiotic added (antibiotic determined based on resistance conveyed by targeted plasmid), and placed in a 37°C incubator overnight.

One or two individual colonies were identified and re-streaked on antibiotic containing LB plates and allowed to grow overnight in a 37°C incubator. Individual colonies were be used to inoculate 5 mL cultures for either shake flask cultures or to make vial lots in which the cells were frozen down for later use.

Table 14. Raw growth, acetate, and growth data from single transformation of plMG17 (containing *mlc* insert) in 1A2.

	Host Strain	1A2									
	Expression Vector	pLMG17 (Inductive, no IPTG added)									
	Media	City Broth									
	Glucose	10 g/L batched upfront									
Growth OD$_{550}$	Time (hr)	4	8	10	13	16	20	24			
	GTG	2.79	12.48	16.70	17.45	15.15	15.40	15.45			
	GTG	2.79	13.16	16.20	17.50	16.70	15.80	15.30			
	GTG-His	1.95	10.12	10.35	11.05	10.95	10.40	10.10			
	GTG-His	2.01	11.24	12.40	13.40	12.85	14.60	14.80			
	GTG-R52H	3.21	14.60	17.75	17.75	17.20	17.15	17.15			
	GTG-R52H	3.15	14.16	17.30	17.50	17.50	16.90	16.75			
	GTG-R52H-His	3.65	13.88	16.73	18.35	18.05	16.75	17.90			
	GTG-R52H-His	3.68	14.76	18.00	18.80	17.20	17.55	17.90			
	ATG	3.15	10.28	10.75	11.50	11.30	10.60	10.10			
	ATG	3.14	9.76	10.10	11.10	10.70	10.35	9.95			
	ATG-His	2.85	13.32	16.10	19.10	18.60	18.60	18.15			
	ATG-His	2.85	13.72	17.40	19.65	19.25	18.50	18.65			
	ATG-R52H	2.87	11.40	11.40	12.40	13.45	13.80	13.95			
	ATG-R52H	2.94	13.12	15.20	15.60	14.80	14.10	14.80			
	ATG-R52H-His	1.89	11.64	13.85	15.75	15.10	14.60	14.40			
	ATG-R52H-His	1.93	11.36	11.50	12.10	12.05	11.45	11.85			
	Time (hr)	0	4	8	10	12	14	16	18	20	24
	1A2	0.20	6.40	5.65	8.00	8.45	8.65	8.95	8.55	8.80	8.50
Acetate (mM)	Time (hr)	4	8	10	13	16	20	24			
	GTG	9.29	15.53	22.31	4.54	2.29	2.05	2.75			
	GTG	9.45	8.21	8.54	1.31	2.82	2.93	2.74			
	GTG-His	13.97	66.32	82.32	90.87	82.40	84.16	84.81			
	GTG-His	13.73	52.83	68.46	58.21	53.37	34.41	2.43			
	GTG-R52H	10.35	25.73	19.30	2.15	1.54	1.66	1.67			
	GTG-R52H	8.07	26.40	28.26	3.73	0.00	1.65	1.41			
	GTG-R52H-His	7.79	23.33	22.80	3.84	1.26	1.67	1.55			
	GTG-R52H-His	7.68	18.07	14.64	2.88	1.71	1.65	1.66			
	ATG	14.54	61.11	59.94	77.83	88.44	86.84	81.37			
	ATG	15.83	103.31	75.78	82.28	80.65	95.58	79.26			
	ATG-His	9.99	31.70	39.85	24.15	0.56	3.25	2.61			
	ATG-His	13.04	26.52	33.27	6.51	3.53	3.30	3.45			
	ATG-R52H	16.66	65.17	66.43	61.58	32.00	3.37	3.25			
	ATG-R52H	16.90	49.38	43.00	5.29	2.50	3.12	0.90			
	ATG-R52H-His	14.54	55.12	60.99	12.94	2.23	2.92	1.77			
	ATG-R52H-His	13.98	63.35	72.02	87.83	94.57	89.51	73.34			
	Time (hr)	0	4	8	10	12	14	16	18	20	24
	1A2	2.36	51.10	106.35	136.74	140.24	133.30	131.96	135.14	134.56	137.91
Glucose (g/L)	Time (hr)	4	8	10	13	16	20	24			
	GTG	8.35	5.20	1.65	0.00	0.00	0.00	0.00			
	GTG	8.10	4.85	1.75	0.00	0.00	0.00	0.00			
	GTG-His	8.10	2.30	0.65	0.00	0.00	0.00	0.00			
	GTG-His	7.70	2.55	0.30	0.00	0.00	0.00	0.00			
	GTG-R52H	8.00	3.25	0.20	0.00	0.00	0.00	0.00			
	GTG-R52H	7.25	2.95	0.35	0.00	0.00	0.00	0.00			
	GTG-R52H-His	7.85	3.45	1.25	0.00	0.00	0.00	0.00			
	GTG-R52H-His	7.45	3.60	1.10	0.00	0.00	0.00	0.00			
	ATG	6.80	0.80	0.90	0.00	0.00	0.00	0.00			
	ATG	7.30	1.35	0.30	0.00	0.00	0.00	6.80			
	ATG-His	7.65	3.75	1.60	0.00	0.00	0.00	0.00			
	ATG-His	9.70	4.60	1.95	0.00	0.00	0.00	0.00			
	ATG-R52H	9.60	2.95	0.15	0.00	0.00	0.00	0.00			
	ATG-R52H	12.50	2.45	0.00	0.00	0.00	0.00	0.00			
	ATG-R52H-His	10.50	2.90	0.40	0.00	0.00	0.00	0.00			
	ATG-R52H-His	9.65	3.15	0.35	0.00	0.00	0.00	0.00			
	Time (hr)	0	4	8	10	12	14	16	18	20	24
	1A2	9.25	4.30	0.50	0.50	0.00	0.00	0.00	0.00	0.00	0.00

Table 15. Raw growth, acetate, and glucose data from double transformation of pLMG17 (containing *mlc* insert) and pTPA in 16C9.

	Host Strain	16C9										
	Expression Vector	pTPA & pLMG17 (Inductive, no IPTG added)										
	Media	C.R.A.P										
	Glucose	10 g/L batched upfront										
	Time (hr)	1	2	3	4	5	6	8	9	10	12	16
Growth OD$_{550}$	GTG/pTPA	---	0.24	0.40	1.03	2.09	3.70	3.78	4.04	4.04	4.10	3.98
	GTG/pTPA	---	0.21	0.42	1.00	1.94	3.71	3.90	3.90	4.06	4.13	3.68
	GTG-R52H/pTPA	---	0.17	0.22	0.54	1.13	2.36	5.00	5.04	5.26	5.65	5.90
	GTG-R52H/pTPA	---	0.16	0.25	0.49	1.04	2.20	4.78	5.26	5.26	5.55	6.00
	GTG-R52H-His/pTPA	---	0.21	0.31	0.69	1.19	2.07	4.70	5.62	5.94	6.23	6.50
	GTG-R52H-His/pTPA	---	0.17	0.29	0.64	1.20	1.96	4.72	5.72	6.26	6.63	6.98
	ATG-His/pTPA	---	0.15	0.25	0.62	1.35	2.83	4.16	4.46	4.40	4.65	4.38
	ATG-His/pTPA	---	0.16	0.24	0.74	1.37	3.00	4.52	4.70	4.74	4.75	4.53
	pLMG17/pTPA	---	0.66	1.49	3.12	3.62	3.87	4.15	4.20	4.10	4.03	3.88
	pLMG17/pTPA	---	0.74	1.53	3.17	3.62	3.90	4.25	4.33	4.13	4.03	3.88
Acetate (mM)	GTG/pTPA	1.07	2.22	4.08	8.23	11.84	22.66	41.93	47.17	58.24	64.64	72.07
	GTG/pTPA	0.88	2.23	3.99	8.50	11.37	23.11	43.74	47.68	55.21	71.21	80.27
	GTG-R52H/pTPA	0.75	1.73	2.63	5.18	6.83	10.80	23.24	25.18	31.82	45.62	70.19
	GTG-R52H/pTPA	0.13	3.11	2.73	5.34	6.58	11.01	24.34	26.96	33.76	43.25	72.28
	GTG-R52H-His/pTPA	0.14	3.13	2.71	4.17	3.84	3.08	2.48	0.00	1.74	1.86	0.00
	GTG-R52H-His/pTPA	0.45	3.04	2.45	4.28	3.42	2.91	5.87	10.77	17.60	22.81	33.17
	ATG-His/pTPA	1.20	2.98	2.59	5.98	8.84	12.23	29.00	39.51	47.06	61.61	82.73
	ATG-His/pTPA	1.10	2.96	2.87	5.83	8.88	16.05	36.54	43.57	52.33	63.73	73.26
	pLMG17/pTPA	3.27	6.60	10.56	16.83	27.46	41.78	54.89	66.68	63.11	76.38	95.83
	pLMG17/pTPA	2.75	6.94	10.67	17.36	28.37	36.44	54.40	59.97	64.00	73.23	83.32
Glucose (g/L)	GTG/pTPA	10.90	10.08	10.14	11.13	9.61	8.36	5.59	4.45	4.10	3.34	2.16
	GTG/pTPA	11.03	10.61	10.64	11.44	8.91	7.75	5.32	4.17	3.73	3.80	2.08
	GTG-R52H/pTPA	11.01	10.54	10.70	11.11	10.06	9.18	6.62	5.69	4.58	2.77	0.01
	GTG-R52H/pTPA	10.44	10.70	9.75	11.88	10.31	9.15	7.05	6.00	4.92	2.73	0.01
	GTG-R52H-His/pTPA	11.27	10.52	9.95	10.68	11.31	10.45	8.92	8.19	7.78	6.00	3.31
	GTG-R52H-His/pTPA	12.15	10.27	8.47	11.40	10.49	9.48	9.32	7.66	6.68	5.17	1.46
	ATG-His/pTPA	11.55	10.66	11.23	12.26	10.68	8.36	6.33	5.68	4.11	3.18	1.70
	ATG-His/pTPA	11.22	10.20	10.32	11.00	10.16	8.53	6.45	5.05	3.90	3.12	1.49
	pLMG17/pTPA	12.24	10.46	10.86	9.64	7.51	6.30	4.74	4.43	3.90	4.04	2.58
	pLMG17/pTPA	11.00	10.52	10.19	8.84	8.23	5.77	4.60	4.25	3.86	3.62	2.40

Table 16. Raw growth, acetate, glucose, and RT-qPCR data from DASGIP bioreactor cultures.

| | Host Strain | 16C9 | | | | | | | | | | | | | |
| | Expression Vector | pTPA & pLMG17 (Inductive, no IPTG added) | | | | | | | | | | | | | |

OD

		Time (hr)	**0**	**4**	**8**	**12.5**	**16**	**18**	**19**	**20.5**	**24.5**	**28**	**32**	**36**	**37**	**38.5**
	JB017	GTG-R52H-His/pTPA	1.38	5.8	14.6	48.4	75	89	99.5	118	140	122	106	113	106	97.5
	JB018	GTG-R52H-His/pTPA	1.37	6.6	17.5	45	70	84.5	95	110	124	121	105	124.5	116	102
		Time (hr)	**0**	**4**	**8**	**12**	**16**	**21.5**	**24**	**25**	**27**	**31**	**35**	**39**		
	JB023	pLMG17/pTPA	1.14	5.85	35	50.5	73.2	81.5	93.5	99	103	126	123	118		
	JB024	GTG-R52H-His/pTPA	1.18	4.28	17.7	28	42.8	72.6	90	96	101	130	138	136		
	JB025	pLMG17/pTPA	1.1	5.25	35	53.2	79.2	114	116	112	109	101	91	78		
	JB026	GTG-R52H-His/pTPA	1.1	4.04	14	19.6	33.6	125	88	94.5	104	123	142	140		

Acetate (mM)

		Time (hr)	**0**	**4**	**8**	**12.5**	**16**	**18**	**19**	**20.5**	**24.5**	**28**	**32**	**36**	**37**	**38.5**
	JB017	GTG-R52H-His/pTPA	4.22	7.08	2.91	4.64	7.16	7.02	6.12	0.00	0.00	2.68	9.28	12.56	18.49	98.02
	JB018	GTG-R52H-His/pTPA	2.88	7.10	2.86	4.39	6.97	5.51	5.39	4.48	3.36	3.55	4.36	3.28	3.43	12.36
		Time (hr)	**0**	**4**	**8**	**12**	**16**	**21.5**	**24**	**25**	**27**	**31**	**35**	**39**		
	JB023	pLMG17/pTPA	5.25	27.69	122.30	196.27	90.95	45.83	9.45	6.63	7.51	14.24	24.28	48.89		
	JB024	GTG-R52H-His/pTPA	4.68	16.50	0.53	4.22	4.67	100.89	143.20	87.29	18.80	6.17	9.04	8.65		
	JB025	pLMG17/pTPA	3.80	27.03	97.62	50.66	11.80	9.83	4.72	5.93	6.85	17.21	81.10	203.55		
	JB026	GTG-R52H-His/pTPA	4.57	14.76	2.42	3.45	21.42	14.86	6.53	6.61	5.20	5.42	7.42	12.96		

Glucose (g/L)

		Time (hr)	**0**	**4**	**8**	**12.5**	**16**	**18**	**19**	**20.5**	**24.5**	**28**	**32**	**36**	**37**	**38.5**
	JB017	GTG-R52H-His/pTPA	1.91	3.46	9.77	10.84	0.04	0.01	0.03	0.02	0.05	0.09	0.11	0.17	0.18	0.21
	JB018	GTG-R52H-His/pTPA	2.26	4.22	10.55	4.59	0.02	0.02	0.02	0.02	0.03	0.07	0.09	0.13	0.14	0.17
		Time (hr)	**0**	**4**	**8**	**12**	**16**	**21.5**	**24**	**25**	**27**	**31**	**35**	**39**		
	JB023	pLMG17/pTPA	2.37	2.15	0.49	0.03	0.01	0.02	0.03	0.03	0.04	0.04	0.06	0.07		
	JB024	GTG-R52H-His/pTPA	2.17	12.36	26.81	17.16	7.48	0.05	0.03	0.02	0.02	0.03	0.06	0.09		
	JB025	pLMG17/pTPA	1.90	1.66	0.04	0.00	0.03	0.05	0.05	0.05	0.07	0.09	0.11	0.09		
	JB026	GTG-R52H-His/pTPA	2.06	3.24	7.40	15.57	1.91	0.01	0.03	0.02	0.01	0.04	0.06	0.06		

QPCR

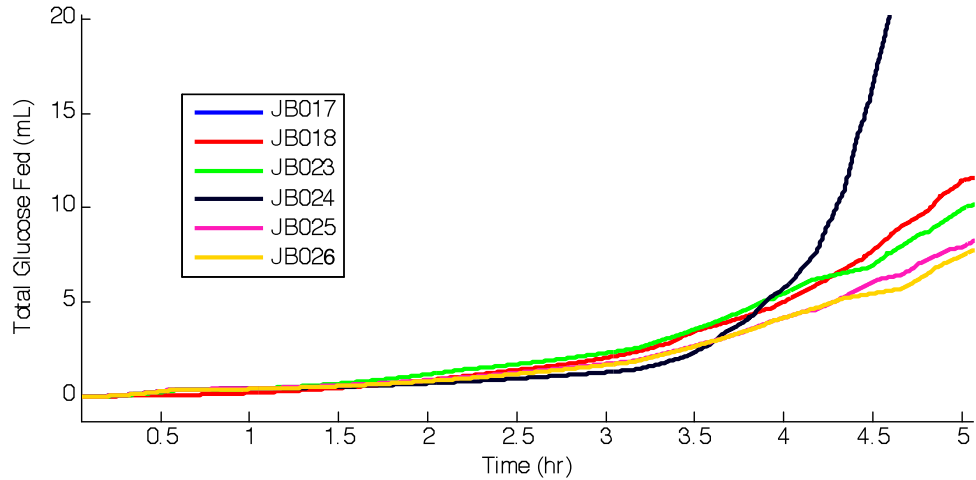|		**Time (hr)**	**0**	**4**	**8**	**12.5**	**16**	**18**	**19**	**20.5**	**24.5**	**28**	**32**	**36**	**37**	**38.5**
	JB017	GTG-R52H-His/pTPA			163	107	98.2	47.5		42.9						
	JB018	GTG-R52H-His/pTPA			160	115	68.3	39		39.2						
		Time (hr)	**0**	**4**	**8**	**12**	**16**	**21.5**	**24**	**25**	**27**	**31**	**35**	**39**		
	JB023	pLMG17/pTPA		0.294	1.12	0.963			1.23							
	JB024	GTG-R52H-His/pTPA		60	172	344	95		75.7		25.5					
	JB025	pLMG17/pTPA		0.302	0.264	1.44			1.79							
	JB026	GTG-R52H-His/pTPA		60.1	69.4	172	104		45.1		19.7					

Figure 37. Total glucose fed over the initial five hours of small scale fermentation. Note the increase in glucose fed to JB024 (overfeeding event as a result of glucose feeding algorithm). Data from JB017 was excluded due to inaccurate measurement.
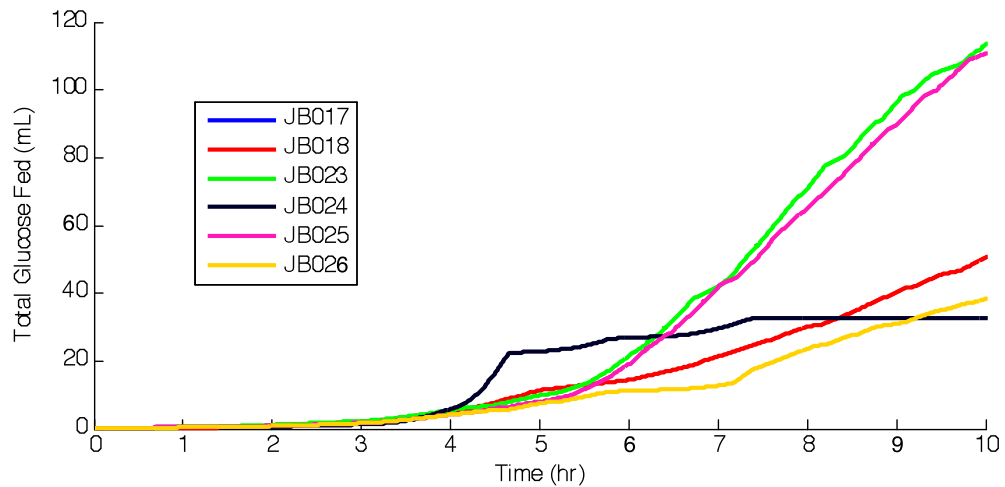

Figure 38. Total glucose fed over the initial 10 hours of small scale fermentation. Note the significant increase in glucose fed to control cultures (JB023 and JB025) after six hours.   Data from JB017 was excluded due to inaccurate measurement.