METABOLIC ENGINEERING FOR CITRAMALIC ACID PRODUCTION IN *ESCHERICHIA COLI*

by

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(Under the Direction of Mark A. Eiteman)

ABSTRACT

Citramalic acid (citramalate) is a five-carbon hydroxy-dicarboxylic acid that serves as precursor for the methacrylic acid synthesis from renewable resources. Methacrylic polymers are widely used in many fields such as construction, vehicles, furniture, medical material, and lighting equipment. The primary goal of this research is to improve the microbial production of citramalate by metabolic engineering and fermentation process development.

Citramalate production was investigated in numerous engineered strains of *Escherichia coli* that were overexpressing the citramalate synthase gene, *cimA*. Knockouts of *gltA*, citrate synthase, *leuC*, 3-isopropylmalate dehydratase and *ackA*, acetate kinase significantly increased citramalate accumulation compared to the control strain. A fed-batch process in a controlled fermenter using the strain MEC499/pZE12-*cimA* (MG1655 *gltA leuC ackA*) accumulated 46.5 g/L citramalate in 132 h with a yield of 0.63 g/g. This amounts to greater than 75% of the theoretical maximum yield from glucose of 0.82 g/g. This process also generated about 10 g/L acetate with a yield of 0.14 g/g. Strain MEC568/pZE12-*cimA* (MG1655 *ΔgltA ΔleuC ΔackA-pta ΔpoxB*) reduced acetate formation yield to less than 0.04 g/g from glycerol in a fed-batch process.

Identical citramalate production was achieved over 31 g/L when using either purified or crude glycerol at yields exceeding 0.50 g citramalate per g glycerol in 132 h.

Glutamate was previously supplemented in the medium for the *gltA* knockout strain, due to the essential role in intracellular acetyl CoA levels. This resulted in a correspondingly enhanced the citramalate production. Taking this into consideration, further protein engineering of *E. coli* citrate synthase (F383M variant) reduced but not eliminated the enzyme activity. Cell growth was restored with decreased citrate synthase activity using glucose as sole carbon source. In addition, citramalate production was dramatically increased by 125% compared to the control strain containing the native citrate synthase in the batch fermentation. Over 60 g/L citramalate with the yield 0.53 g citramalate per g glucose was achieved in a fed-batch process with exponential feeding strategy in 132 h using MEC626/pZE12-*cimA* (*gltA*-F383M).

INDEX WORDS: *Escherichia coli*, Citramalate synthase, Citrate synthase, Glucose, Glycerol, Acetate, Acetyl CoA, Pyruvate, Chemostat, Fed-batch

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Dedicated to my family

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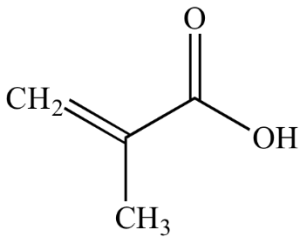
CHAPTER 1

INTRODUCTION

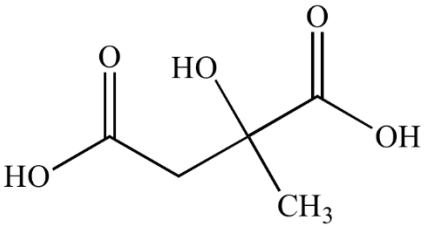
Methacrylic acid (MAA) is an important commodity chemical, which is primarily used for the synthesis of poly(methyl methacrylate) (Fig. 1-1). The global annual MAA production capacity has been almost doubled over the past 15 years, and has reached approximately 3.2 million tons per year (Choi et al., 2015). The demand for MAA is still expected to steadily grow in the future. Methacrylic polymers, which have the characteristics of good transparency and weather resistance, are widely used in many fields such as construction, vehicles, furniture, medical material, and lighting equipment.

The most common approach for MAA synthesis currently converts acetone cyanohydrin to methacrylamine sulfate using sulfuric acid followed by a hydrolysis reaction to generate MAA (Nagai, 2001). However, the main problems with this method are at the handling and transporting of volatile cyanides as well as generation of the large amounts of ammonium bisulfate waste. Many companies have investigated an alternative route for the manufacture of MAA from isobutene, isobutyric acid, and ethylene (Bauer, 2000; Nagai, 2001), although none of these pathways appear to be economically superior to the acetone cyanohydrin route. In addition, significant interest in the microbial production of MAA and acrylate from renewable resources exists, however, acrylates are extremely toxic to microorganisms such as Escherichia coli (Arya et al., 2013).

In this research, we demonstrate a biochemical-chemical approach MAA production that involves the microbial production of an MAA precursor, citramalic acid (citramalate). Citramalate can be converted directly to MAA by base-catalyzed decarboxylation and dehydration (Johnson et al., 2015). One of the goals of this research is to develop metabolically engineered strains and microbial fermentation processes to produce citramalate in high yield and final titer. In this dissertation, Chapter 3 focuses on creating platform strains and validating citramalate production in controlled fermenters. Several gene knockouts related to the pyruvate and acetyl CoA metabolism were investigated since pyruvate and acetyl CoA are the substrates of the citramalate generating reaction catalyzed by the key enzyme citramalate synthase. Chapter 4 focuses on increasing the citramalate yield on glycerol by reducing the by-product formation of acetate. Pathways regulating acetate formation and consumption were studied. Chapter 5 focuses on examining point mutations in citrate synthase to better control the metabolic production of acetyl CoA and subsequent use by the tricarboxylic acid (TCA) cycle partially. The mutations increased intracellular acetyl CoA pools and accumulated citramalate while allowing the organism to grow on glucose without any supplementation. These studies provide new insights into pathways involved in the citramalate accumulation and to how to further manipulate microbial metabolism for better production. These results also hold promise that citramalate can be produced from renewable resources and ultimately used as a source of methacrylate.



Methacrylic acid



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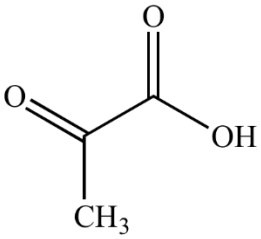
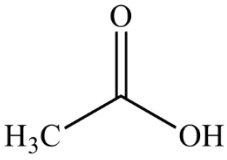


Pyruvic acid



Acetic acid

Figure 1-1. Structure of Methacrylic acid, citramalic acid, pyruvic acid and acetic acid.

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CHAPTER 2

LITERATURE REVIEW

Application of Methacrylic Acid

Methacrylic acid (methacrylate, MAA) is an important monomer that is primarily used for producing acrylic resins or polymer dispersions for paints and coatings (Nagai, 2001). Since the methacrylic polymer has good transparency and weather resistance, it is used in the synthesis of transparent thermoplastic in many fields such as building materials, furniture, vehicles, and lighting equipment. The annual market of MAA in the world has almost been doubled in the past 15 years and has reached about 3.2 million tons per year (Choi et al., 2015). The demand of MAA is still expected to grow steadily in the future.

General methods of Methacrylic Acid Production

The current most common approach for MAA synthesis can be dated back in 1930s. Rohm & Haas Co. began to manufacture a methacrylic ester (ethyl methacrylate) in 1933, and ICI improved Rohm's method and commercialized methyl methacrylate in 1937 using the acetone cyanohydrine (ACH) process. The ACH method was the only option until 1982 and is still used by most manufacturers in the world today (Nagai, 2001). The ACH method starts with the conversion of acetone and cyanide to ACH through base catalyst. The ACH is then converted to methacrylamine sulfate using sulfuric acid followed by a hydrolysis reaction to generate MAA.

There are major concerns of the ACH method that include handling and transporting toxic cyanides and dealing with the large quantities of ammonium bisulfate waste. However, until recently there were few options for replacing the ACH process. Today, other options include; (1) the direct oxidation process developed by Mitsubishi Rayon and Asahi Chemical, which consists of catalytic oxidation of isobutylene or *tert*-butanol to methacrylic acid in two steps; (2) the BASF's method, which employs ethylene, carbon monoxide, and formaldehyde as raw materials; (3) the new ACH process industrialized by Mitsubishi Gas Chemicals, which generates α-hydroxy isobutylamide as intermediate and recycles cyanide in the preparation process (Bauer, 2000; Nagai, 2001). However, none of these newer processes appears to be economically superior to the traditional ACH route.

Of significance to this dissertation is the interest in the microbial production of MAA and acrylate from renewable resources. Currently, Lucite International (a subsidiary of Mitsubishi Rayon) and Evonik have been working on the commercial production of methyl methacrylate using the processes of the decarboxylation and subsequent methylation of bio-based itaconic acid or methylation of bio-based methacrylic acid (Choi et al., 2015). However, acrylates are extremely toxic to microorganisms such as *Escherichia coli* (Todd et al., 2012; Arya et al., 2013) and therefore acrylate production in vivo presents a significant problem.

Metabolic Pathways Related to Citramalate

Citramalic acid ((R)-2-methylmalic acid, (2R)-2-hydroxy-2-methylbutanedioate, or citramalate) is a biochemical intermediate known to be involved in several aspects of bacterial metabolism. The anaerobic metabolism of glutamate via the methylaspartate pathway was reported in *Clostridium tetanomorphum* (Buckel and Barker, 1974). The linear carbon chain of

glutamate is first rearranged to the branched chain of 3-methylaspartate. Citramalate is generated through the elimination of ammonia and addition of water before being cleaved to acetate and pyruvate (Buckel and Barker, 1974). In addition, the citramalate pathway serves as the primary route of isoleucine biosynthesis in *Geobacter sulfurreducens* and *Chlorobaculum tepidum* (Risso et al., 2008; Feng et al., 2010). The key isoleucine precursor, 2-oxobutanoate, is synthesized from acetyl-CoA and pyruvate via the citramalate pathway, which accounts for 68 to 77% of the total flux (Risso et al., 2008).

He and coworkers (2000) isolated *Arthrobacter pascens* strain DMDC12 which produced D-citramalate from citraconate. The optical purity of the D-citramalate produced by this strain was 99.9%. The citraconate hydratase mediating this conversion was very stable when 0.6 M of citraconate was added as the substrate in the presence of 1 M NaCl. Under optimized conditions, 187.2 g/l of citramalate were produced from maleate and citraconate in 48 h using permeabilized resting cells, with the molar yields of 95.6%.

Howell (1999) purified and characterized the enzyme citramalate synthase (CMS, EC 2.3.1.182) from *Methanococcus jannaschii*. This enzyme is encoded by the *cimA* gene and catalyzes the specific condensation of pyruvate and acetyl CoA with the formation of citramalate. The specific activity of CMS was 2.9 μmol/min/mg protein, and the K_m for pyruvate and acetyl CoA was 0.85 mM and 0.14 mM, respectively.

Atsumi and Liao (2008) screened a CMS mutant library created via directed evolution and identified a CMS mutant (referred to as *CimA*3.7) with improved activity, with K_m values for pyruvate and acetyl CoA of 0.34 mM and 0.11 mM, respectively. Importantly, the wild type CMS is sensitive to feedback inhibition by *L*-isoleucine, while *CimA*3.7 activity was not affected by *L*-isoleucine.

CMS is a homologue of 2-isopropylmalate synthase (LeuA, EC 2.3.3.13) which mediates the first step in leucine biosynthesis. LeuA catalyzes a condensation reaction with 2-keto-isovalerate and acetyl CoA to 2-isopropylmalate, which is converted to 3-isopropylmalate by the isopropylmalate isomerase complex (LeuCD) (Calvo et al., 1962; Yang and Kessler, 1974). Like isopropylmalate, citramalate can be converted to the analogous 3-methylmalate by the LeuCD enzyme (Atsumi and Liao, 2008).

Metabolic Pathways Related to Pyruvate

Pyruvate, a key metabolite synthesized via glycolysis, occupies a crucial role in the central metabolism. During growth of *Escherichia coli* on glucose, pyruvate is generated mainly from phosphoenolpyruvate (PEP) by pyruvate kinase and from glucose u*pta*ke by the phosphotransferase system (PTS). Pyruvate is primarily converted into acetyl CoA by pyruvate dehydrogenase (PDH) aerobically or pyruvate formate lyase (PFL) anaerobically. Pyruvate can also be converted into acetate, malate and PEP by pyruvate oxidase (POX), two malic enzymes and PEP synthase (PPS), respectively. In addition to these direct pathways, the accumulation of pyruvate can be affected by many adjacent pathways such as the PEP carboxylase (Postma et al., 1993).

A large number of sugars are phosphorylated and transported into the cell via sugar specific PTSs (Postma et al., 1993; Ginsburg and Peterkofsky, 2002). In these systems, carbohydrates are phosphorylated as well as transported into the cell with the energy provided by the glycolytic intermediate PEP that is converted into pyruvate in the phosphorylation reaction. Several feedback regulation mechanisms are involved in the PTS pathway, including the membrane potential, energy-dependent efflux of PTS substrates, substrate competition for

phospho-HPr, and regulation by intracellular phosphorylated-compounds level (Postma et al., 1993).

After glucose is converted into glucose-6-phosphate and transported into the cell, a sequence of enzymes in the Embden-Meyerhof-Parnas pathway, or glycolysis, converts this 6-carbon sugar phosphate by a series of enzymes into two 3-carbon molecules of pyruvate. The final enzyme involved in glycolysis is pyruvate kinase (PYK). Pyruvate kinase (EC 2.7.1.40) catalyzes the conversion of PEP and ADP to pyruvate and ATP (Mattevi et al., 1995). This reaction is irreversible under physiological conditions.

The TCA cycle is linked to glycolysis by the conversion of pyruvate to acetyl CoA, coupled to the generation of NADH. This reaction is catalyzed by the pyruvate dehydrogenase complex (PDH). PDH comprises three enzymes: pyruvate dehydrogenase (E1, EC 1.2.4.1), dihydrolipoyl transacetylase (E2, EC 2.3.1.12), and dihydrolipoyl dehydrogenase (E3, EC 1.8.1.4) (Garrett and Grisham, 1998). These enzymes are associated noncovalently. The components E1, E2 and E3 are encoded by the gene *aceE*, *aceF* and *lpd*, respectively. First, the E1 component catalyzes the release of CO_2 from pyruvate, and the two-carbon remnant binds to thiamine pyrophosphate (TPP), forming hydroxyethyl TPP. The hydroxyethyl group is then transferred to lipoic acid by the E2 component and oxidized to form acetyl dihydrolipoamide followed by the acetyl group transferring to coenzyme A (CoA). This second step results in the production of acetyl CoA, which is the end product of pyruvate decarboxylation. Finally, lipoic acid is regenerated by the E3 component from the oxidation of dihydrolipoamide with the reduction of NAD^+ to NADH. Several coenzymes are involved in the sequential reaction, including TPP, CoA, lipoic acid, NAD^+, and FAD. The activity of the complex is inhibited by the high levels of acetyl CoA and NADH (Garrett and Grisham, 1998). In addition, PDH is highly

regulated by the intracellular energy status. PDH is activated by AMP and inhibited by GTP (Garrett and Grisham, 1998).

Under anaerobic conditions, to maintain the redox balance in *E. coli* pyruvate is metabolized to a variety of reduced organic compounds such as lactate and ethanol (Clark, 1989). The fermentative lactate dehydrogenase (LDH, EC 1.1.1.28) encoded by *ldhA* catalyzes the conversion of pyruvate to D-lactate (Tarmy and Kaplan, 1968a,b), coupled to the oxidation of NADH. LDH is activated by its substrate, pyruvate (Tarmy and Kaplan, 1968b). LDH is present in substantial basal levels under all conditions, and it is induced approximately ten-fold upon substantial acidification of the medium (Clark, 1989; Bunch et al., 1997). Under certain conditions, such as when *E. coli* has mutations which result in pyruvate accumulation, lactate is a product of aerobic conditions (Zelić et al., 2003).

Another reaction which plays an important position in the anaerobic carbon metabolism in *E. coli* is the conversion of pyruvate and CoA to formate and acetyl CoA (Guo and Himo, 2004). This nonoxidative reaction is catalyzed by pyruvate-formate lyase (PFL, EC 2.3.1.54) encoded by *pflB* (Lehtio et al., 2002). The active form of PFL is only present under anaerobic conditions due to its homodimer structure (Lehtio et al., 2002). Additionally, the expression of PFL is approximately 10-fold higher in cells grown anaerobically with glucose compared with cells grown aerobically with glucose (Sawers and Bock, 1988). PFL activity is further elevated 1.5- to 2.0-fold by the addition of pyruvate to the growth medium (Sawers and Bock, 1988).

Pyruvate oxidase (POX, EC 1.2.2.2) catalyzes the decarboxylation of pyruvate to acetate and CO_2, coupled to the reduction of flavin adenine dinucleotide (FAD) to $FADH_2$ (Gennis and Hager, 1976). Its role in metabolism is unclear. POX might serve an important role for the acetyl units synthesis under microaerobic conditions by compensating the poor function PDH and PFL

(Abdel-Hamid et al., 2001). The enzyme is encoded by the *poxB* gene and strongly activated by a variety of phospholipids which increase the maximum velocity about 20-fold and decrease the K_m for pyruvate about 10-fold (Abdel-Hamid et al., 2001).

Phosphoenolpyruvate synthase (PPS, EC 2.7.9.2), encoded by the *ppsA* gene, is a gluconeogenic enzyme which catalyzes the direct conversion of pyruvate to PEP (Patnaik et al., 1992). PPS is required when *E. coli* is grown on single three-carbon substrates such as pyruvate and lactate (Patnaik et al., 1992).

Direct fermentation processes have been used to produce pyruvate. Many recombinant microorganisms including yeast and *E. coli* can accumulate pyruvate from different carbon sources. In mineral salts medium containing glucose as the sole carbon source, an *E. coli* strain which minimized ATP yield, cell growth, and CO_2 production, converted glucose to pyruvate with a volumetric productivity of 1.2 g pyruvate/l·h and a yield of 0.75 g pyruvate/g glucose (77.9% of theoretical yield) (Causey et al., 2003). A maximum of 749 mM pyruvate was produced with excess glucose. Zelić (2004) used *E. coli ΔaceEF ΔpflB ΔpoxB Δpps ΔldhA* to obtain a yield of 0.86 g/g with a volumetric productivity of about 6 g/l·h using a repetitive fed-batch approach. Additional mutations in *atpFH* and *arcA* studied by Zhu (2008) led to a steady-state glycolytic flux of 2.38 g/g·h. In this strain, 90 g/L pyruvate with an overall productivity of 2.1 g/L·h and yield of 0.68 g pyruvate/g glucose was achieved with defined medium in a fed-batch process with an exponential feeding rate of 0.15 h^{-1}.

Metabolic Pathways Related to Acetyl CoA

Acetyl CoA is an important metabolite in a variety of physiological processes such as TCA cycle and synthesis of fatty acids and amino acids (Vandenberg, *et al*., 1996; Gulick et al.,

2003). In general, direct formation of acetyl CoA from pyruvate is catalyzed by PDH aerobically or PFL anaerobically (Clark, 1989; Vandenberg et al., 1996). When *E. coli* is lacking sufficient acetyl CoA regeneration, it begins to rely on the ability to scavenge for environmental acetate. The import and utilization of the external acetate is facilitated by two enzymatic pathways, acetyl CoA synthetase (ACS, EC 6.2.1.1) and the pair of enzymes phosphotransacetylase (PTA, EC 2.7.2.1) and acetate kinase (ACKA, EC 2.3.1.8).

Acetyl CoA can be generated from acetate by ACS, encoded by the *acs* gene. ACS first converts acetate and ATP to the enzyme-bound intermediate acetyladenylate (acetyl-AMP) while producing pyrophosphate. It then catalyzes the reaction of acetyl-AMP with CoA to form acetyl CoA, releasing AMP (Chou and Lipmann, 1952; Berg, 1956). Although this reaction is reversible in vitro, it is irreversible in vivo because of the presence of intracellular pyrophosphatases (PPase). This high-affinity pathway with K_m of 200 μM for acetate functions as an important way to scavenge for small amounts of environmental acetate (Brown et al., 1977; Kumari et al., 1995) as well as maintains the intracellular level of acetyl CoA, especially when microorganisms grow on acetate or due to the absence of acetyl CoA generation from pyruvate (Gulick et al., 2003; Zelić et al., 2003).

In *E. coli*, the PTA-ACKA pathway is considered as acetate catabolism (Lee et al., 1990; Matsuyama et al., 1994). PTA and ACKA are encoded by the *pta* and *ackA* gene, respectively. PTA reversibly converts acetyl CoA and inorganic phosphate to acetyl-P and CoA, while ACKA reversibly converts acetyl-P and ADP to acetate and ATP (Rose et al., 1954). Thus, the PTA-ACKA pathway couples energy metabolism with those of carbon and phosphorus (Wanner, 1993; Wanner, 1996). This pathway also can interconvert propionyl-CoA and propionate (Rose et al., 1954). Thus, it also functions in ketobutyrate metabolism (Van Dyk and LaRossa, 1987),

degradation of fatty acids with odd numbers of carbons, the assimilation of propionate, and, in *Salmonella*, growth on 1,2-propandiol as a carbon and energy source (Palacios et al., 2003). The reversible PTA-ACKA pathway also can assimilate acetate, but only in relatively large concentrations, because the enzymes of this low-affinity pathway possess K_m values for their substrates in the 7 to 10 mM range (Brown et al., 1977; Kumari et al., 1995), which is about 50 times greater than ACS.

E. coli acs mutants grow poorly on 2.5 mM acetate as the sole carbon and energy source (Kumari et al., 1995). In contrast, *E. coli* cells with deletion of all or part of the reversible PTA-ACKA pathway grow poorly on concentrations of acetate greater than 25 mM (Brown et al., 1977; Kumari et al., 1995). Since growth on low concentrations of acetate depends on the ACS pathway while growth on high concentrations requires the PTA-ACKA pathway, mutants that lack both cannot grow on acetate at any concentration (Kumari et al., 1995).

Because acetate freely permeates through the cell membrane in the undissociated form (Kihara and Macnab, 1981; Repaske and Adler, 1981; Booth, 1985; Saier and Ramseier, 1996), the u*pta*ke of acetate does not require a dedicated transport system. However, under certain circumstances acetate u*pta*ke is limited, suggesting that such a system exists (Kakuda et al., 1994). Recently, Gimenez et al. (2003) reported the existence of an acetate permease (ActP, formerly YjcG) and provided evidence for the existence of a second acetate transporter. The authors propose that these systems play critical roles when cells scavenge for micromolar concentrations of acetate.

In addition to forming acetate, the acetyl CoA flux can also be directed to the TCA cycle where the glyoxylate bypass plays an important regulation control. Citrate synthase (CS, EC 2.3.1.1) is the first enzyme of the TCA cycle, coded by the *gltA* gene (Guest, 1981). It delivers

acetyl-CoA into the cycle via binding with oxaloacetate to form citrate. Because of its key position in the TCA cycle, citrate synthase had been assumed to be an important control point for determining the metabolic rate of the cell. Its activity is inhibited by NADH (Weitzman, 1966), ATP (Jangaard et al., 1968), and α-ketoglutarate (Wright et al., 1967). The crystal structures of CS have been comprehensively studied for ligand-free and ligand-bound forms of the enzyme from various organisms, such as pig (Remington et al., 1982), chicken (Usher et al., 1995) and thermophilic archaeon (Russell et al., 1994). The *E. coli* CS composed of six identical subunits is typical of citrate synthases of gram negative microorganisms (Weitzman, 1981), and its crystal structure has been established with various point mutation to illustrate the kinetic parameter for acetyl CoA, oxaloacetate and NADH (Pereira et al., 1994). Overexpression of *gltA* decreased but not eliminated acetate production (De Maeseneire et al., 2006), while knocking out *gltA* caused a strong increase in acetate production, accompanied by an increase in formate and pyruvate excretion (Lee et al., 1994).

Two enzymes catalyze the reaction in the glyoxylate bypass, isocitrate lyase (ICL, EC 4.1.3.1) and malate synthase (MS, EC 2.3.3.9). Isocitrate is first converted to succinate and glyoxylate catalyzed by ICL: the former product returns to the TCA cycle, while the latter product binds with acetyl CoA to form malate in the condensation reaction catalyzed by MS. There are two isozymes of MS in *E. coli*: malate synthase G, encoded by the *glcB* gene, which is responsible for cell growth on glycolate (Molina et al., 1994), and malate synthase A, encoded by the *aceB* gene, which is involved in the glyoxylate bypass (Ornston and Ornston, 1969). Knocking out both *glcB* and *aceB* significantly increased the glycolic acid yield on glucose in *E. coli* (Deng et al., 2015).

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CHAPTER 3

PRODUCTION OF CITRAMALATE BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI* [1]

[1] Wu, X. and M.A. Eiteman. 2016. *Biotechnology and Bioengineering*. 113, 2670-2675.

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Abstract

Citramalic acid (citramalate) is a five carbon hydroxy-dicarboxylic acid and potential precursor for the production of methacrylic acid from renewable resources. We examined citramalate production in *Escherichia coli* expressing the citramalate synthase gene *cimA*. Although knockouts in *ldhA* coding lactate dehydrogenase and *glcB*/*aceB* coding malate synthase did not benefit citramalate accumulation, knockouts in *gltA* coding citrate synthase and *ackA* coding acetate kinase significantly increased citramalate accumulation compared to the control strain. A fed-batch process in a controlled fermenter using a glucose feed resulted in 46.5 g/L citramalate in 132 h with a yield of 0.63 g/g, over 75% of the theoretical maximum yield from glucose of 0.82 g/g.

Introduction

With growing concern about fossil-energy depletion and sustainability, innovation for "green" processes using renewable resources to produce biochemicals becomes an attractive approach for the chemical industry. Metabolic engineering and synthetic biology approaches have enabled strain modification and bioprocess optimization to generate numerous commodity chemicals such as succinate (Vemuri et al., 2002; Sanchez et al., 2005), 1,3-propanediol (Nakamura and Whited, 2003), 1,4-butanediol (Yim et al., 2011), lactic acid (Porro et al., 1999), isoprene (Lichtenthaler et al., 1997), and glutamate (Nakamura et al., 2007).

Methacrylic acid (MAA) is a commodity chemical used to form an ester polymer, polymethyl methacrylate, widely used as a transparent thermoplastic in construction, furniture, lighting, and medical technologies. The annual global market has been estimated at about 2.2 million tons (Zhang et al., 2011). The most common approach for MAA synthesis currently involves the hydrolysis of methacrylamine sulfate obtained from acetone cyanohydrin (Salkind et al., 1959; Bauer, 2000; Nagai, 2001). Sulfuric acid regeneration and hazards associated with handling and transporting volatile cyanides are major concerns for industrial MAA production. Many companies have investigated the manufacture of MAA from isobutene, isobutyric acid, and ethylene (Bauer, 2000; Nagai, 2001), though none appear to be economically superior to the acetone cyanohydrin route. Interest also exists for the microbial production of MAA and acrylate from renewable resources, which would presumably involve more renewable process and less hazards. However, acrylates are extremely toxic to microorganisms such as *Escherichia coli* (Todd et al., 2012; Arya et al., 2013), and therefore an alternative "hybrid" approach might be pursued. For example, the biochemical-chemical production of MAA could involve the microbial production of an MAA precursor, which is subsequently converted to MAA by a

purely chemical transformation. For example, both citric acid and itaconic acid, products of fungal fermentations, can be thermally decarboxylated to methacrylic acid (Carlsson et al., 1994).

Recently, citramalic acid ((R)-2-methylmalic acid, (2R)-2-hydroxy-2-methylbutanedioate, or citramalate) has been shown to be converted directly to MAA by base-catalyzed decarboxylation and dehydration (Johnson et al., 2015). Citramalate is found in bacterial metabolism, for example, anaerobic metabolism of glutamate via the methylaspartate pathway in *Clostridium tetanomorphum* (Buckel and Barker, 1974) and the isoleucine biosynthesis pathway in *Geobacter sulfurreducens* and *Chlorobaculum tepidum* (Risso *et al*., 2008; Feng *et al*., 2010). Citramalate synthase (EC 2.3.1.182) has been purified and characterized from *Methanococcus jannaschii* (Howell, 1999). This enzyme coded by the *cimA* gene catalyzes the specific condensation of pyruvate and acetyl-CoA with the formation of D-(–)-citramalate (*R*-citramalate). Using screening and directed evolution, a citramalate synthase mutant designated *CimA*3.7 was found to have improved activity and lack feedback inhibition by *L*-isoleucine (Atsumi and Liao, 2008). The particular enantiomer (i.e., *R*-citramalate, *S*-citramalate or *meso*-citramalate) does not affect the chemical conversion to MAA (Johnson et al., 2015).

In this study, we demonstrate significant citramalate accumulation by *E. coli* overexpressing citramalate synthase. Because citramalate synthase requires the availability of both pyruvate and acetyl-CoA (Fig. 3-1), the focus is on metabolic engineering strategies to block the metabolism of these two citramalate precursors.

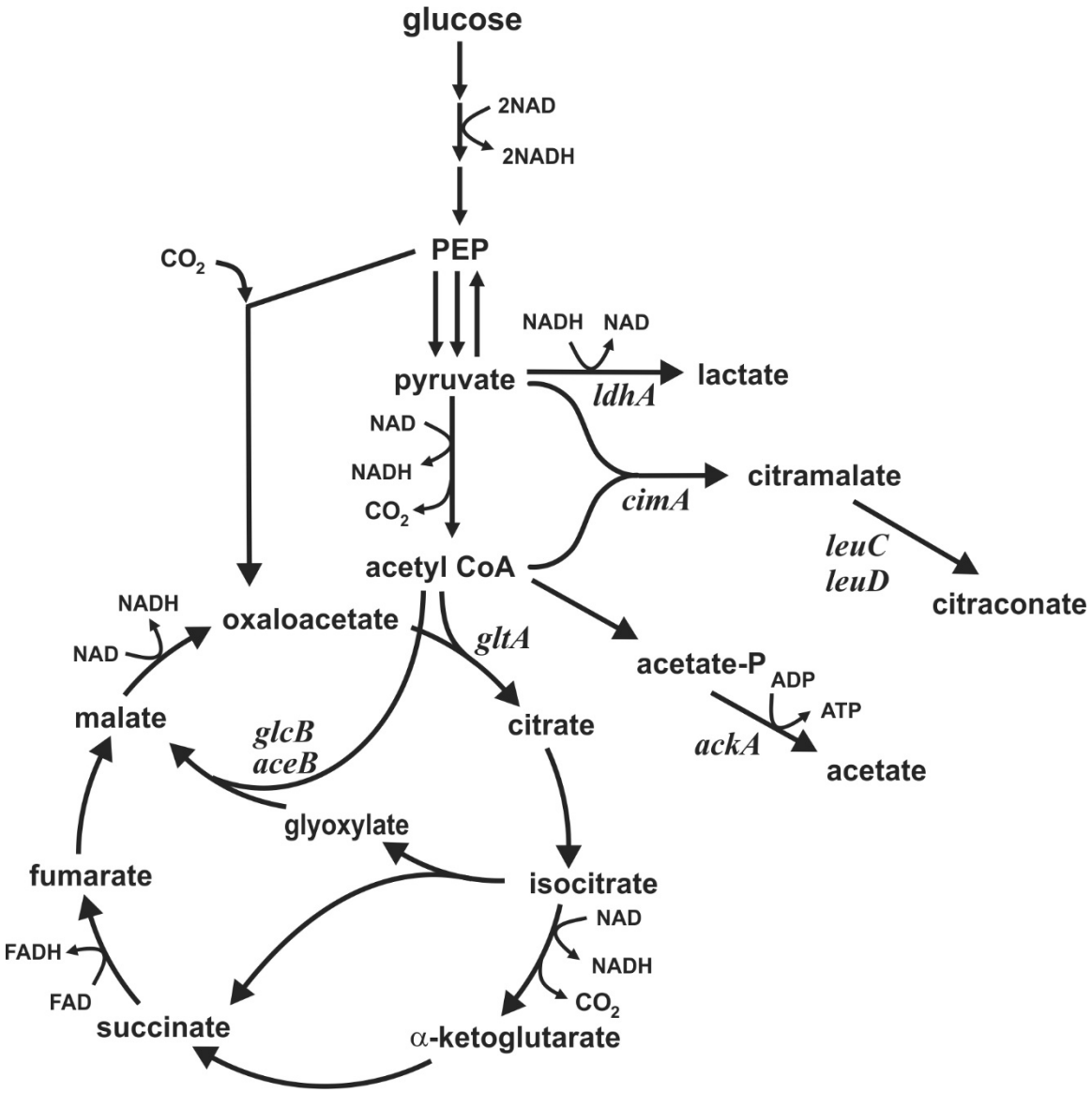

Figure 3-1. Biosynthesis of citramalate in *Escherichia coli* expressing the *cimA* gene coding citramalate synthase. Key genes and the corresponding enzymes are: *ldhA* (lactate dehydrogenase), *leuC* and *leuD* (3-isopropylmalate dehydratase), *gltA* (citrate synthase), *glcB* and *aceB* (malate synthase), *ackA* (acetate kinase).

Materials and Methods

Strain construction

Strains and plasmids used in this study are shown in Table 3-1. Gene mutations were transduced into *E. coli* MG1655 from their respective strains in the KEIO collection (Baba et al., 2006) by the P1 phage method. The knockout additional genes in a strain, the Kan antibiotic marker was removed using pCP20 (Datsenko and Wanner, 2000). In knockout strains, forward primers external to the target gene and reverse primers within the kanamycin resistance cassette were used to check for proper chromosomal integration. In cured strains, the removal of the markers was verified by PCR.

The citramalate synthase enzyme *CimA*3.7 (Atsumi and Liao, 2008) was codon optimized for expression in *E. coli* (GenScript, Piscataway, NJ, USA). The gene was PCR amplified with primers 5′-GGGAAAGGTACCATGATGGTGCGTATCTTTGACACGAC-3′ (forward) and 5′-GGGAAACTCAGATCACACCAGTTTGCCCGTCAC-3′ (reverse). To construct the plasmid pZE12-*cimA*, the 1065 bp PCR product was purified and restricted with *Kpn*I and *Xba*I, and then ligated into the regulable expression vector pZE12-luc (Lutz and Bujard, 1997) which had also been restricted with *Kpn*I and *Xba*I.

Growth medium

Defined XC medium contained (per L): 5.00 g glucose, 13.30 g KH_2PO_4, 4.00 g $(NH_4)_2HPO_4$, 8.40 mg $Na_2(EDTA)\cdot 2H_2O$, 1.20 g $MgSO_4\cdot 7H_2O$, 4.5 mg thiamine·HCl, 13 mg $Zn(CH_3COO)_2\cdot 2H_2O$, 1.5 mg $CuCl_2\cdot 2H_2O$, 15 mg $MnCl_2\cdot 4H_2O$, 2.5 mg $CoCl_2\cdot 6H_2O$, 3.0 mg H_3BO_3, 2.5 mg $Na_2MoO_4\cdot 2H_2O$, 100 mg Fe(III) citrate, and 100 mg citric acid. Unless otherwise specified, this medium was supplemented with 0.2 g/L L-leucine for the growth of all Δ*leuC* or Δ*leuD* strains, and with 2.0 g/L L-glutamate for Δ*gltA* strains because *E. coli* is unable to utilize

citrate under aerobic conditions (Koser, 1924). Additionally, 50 mg/L ampicillin and/or 100 mg/L kanamycin were added for plasmid-containing strains or strains having antibiotic resistance.

Shake flask, batch, fed-batch and chemostat process

To compare various strains for citramalate production in shake flasks, cells were first grown in 3 mL Lysogeny Broth (LB) at 37°C and 250 rpm (19 mm pitch). After 10-14 h, 0.5 mL was used to inoculate 50 mL of XC medium containing 0.2 mM IPTG in 500 mL baffled shake flasks (in triplicate). After growth at 37°C and 250 rpm (19 mm pitch) for 24 h, the cultures were analyzed for citramalate synthase activity, citramalate and intracellular acetyl-CoA concentration. To examine citramalate production under controlled conditions, cells were first grown as described above in 3 mL LB and then 50 mL XC medium. After 18 h the shake flask contents were used to inoculate the 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific Co., New Brunswick, NJ, USA) containing 1.0 L XC medium with either 20 g/L glucose (batch) or initially 25 g/L glucose and 15 g/L peptone (fed-batch). For batch and fed-batch processes, the agitation was 400 rpm and air was sparged at 1.0 L/min, which maintained the dissolved oxygen above 40% of saturation. The pH was controlled at 7.0 using 30% (w/v) NaOH, and the temperature at 30°C. Fermentations were run in duplicate. In batch processes 0.2 mM of IPTG was added initially, while in fed-batch processes 0.2 mM of IPTG was added at 9 h. For the fed-batch process, an additional 30 g glucose was added twice when the glucose decreased below 5 g/L.

A continuous fermentation of 600 mL volume was operated as glutamate-limited chemostat and initiated in batch mode in a 1.0 L bioreactor (Bioflo 310, New Brunswick Scientific Co., New Brunswick, NJ, USA). The influent medium contained XC medium but with

20 g/L glucose and 0.5 g/L glutamate. A steady-state condition was assumed after five residence times at which time the oxygen and CO_2 concentrations in the effluent gas remained unchanged. For dry cell weight (DCW) measurement, three 50.0 mL samples were centrifuged (3300 × *g*, 10 min), the pellets washed by vortex mixing with 10 mL DI water and then centrifuged again. After washing three times, the cell pellets were dried at 60°C for 24 h and weighed. The pH was controlled at 7.0 using 30% (w/v) NaOH, the temperature at 30°C, an air flow rate of 0.5 L/min, and an agitation of 400 rpm to maintain the DO above 40% saturation.

Analytical methods

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, CA, USA) was used to monitor cell growth. Extracellular organic acids were analyzed by HPLC using a Refractive Index detector as previously described (Eiteman and Chastain, 1997). Glutamate concentration was measured using a glutamate assay kit (Sigma-Aldrich Co., St. Louis, MO, USA). Acetyl-CoA was analyzed by the previous method (Gao et al., 2007). Briefly, when a culture reached an OD of 1, 15 mL was centrifuged (3300 × *g*, 10 min, 4°C), the pellet washed with 3 mL Tris·HCl (pH 7.5), and centrifuged again. After washing three times, cell-free extracts were prepared with a French® pressure cell (Thermospectronic, Rochester, NY, USA) at a pressure of 14,000 psi. Cell debris was removed by centrifugation (20,000 × *g*, 15 min, 4°C), and the extract used for acetyl-CoA quantification by HPLC using a Dionex Ultimate 3000 (Thermo Scientific, Bannockburn, IL, USA) with a reverse-phase 50 × 4.6 mm C-18 column (Dionex Acclaim PolarAdvantage II, Thermo Scientific, Bannockburn, IL, USA). Samples were eluted at a flow rate of 600 µL/min using a gradient of 25 mM ammonium acetate (mobile phase A) and 25 mM ammonium acetate in 90% acetonitrile (mobile phase B). Solution B was

delivered from 0% to 80% over 5 min at a flow rate of 0.6 mL/min. Acetyl-CoA was detected and quantified by monitoring absorbance at 254 nm.

Cell-free extracts were also used to measure citramalate synthase activity by the generation of free CoA and its reaction product with 5,5′-Dithiobis(2-nitrobenzoic acid) detected at a wavelength of 412 nm (Howell et al., 1999). One Unit of activity is the amount of enzyme which generates one μmole of CoA in one minute at 37°C.

Results

Comparison of citramalate formation in various strains

Citramalate is generated from the condensation of pyruvate and acetyl-CoA mediated by the enzyme citramalate synthase coded by the *cimA* gene (Fig. 3-1). We first examined citramalate formation after 24 h in wild-type *E. coli* expressing *cimA* (MG1655/pZE12-*cimA*) using 5 g/L glucose as sole carbon source (Fig. 3-2). Because acetyl-CoA is a precursor to citramalate, we also examined its accumulation using a medium containing 5 g/L glucose and 1 g/L acetate. About 1 g/L citramalate accumulated when glucose was the sole carbon source, and 0.81 g/L accumulated in the presence additionally of acetate (Fig. 3-2). Interestingly, the intracellular acetyl-CoA concentration was also slightly lower in the acetate-containing medium (38.7 μg/g versus 36.3 μg/g).

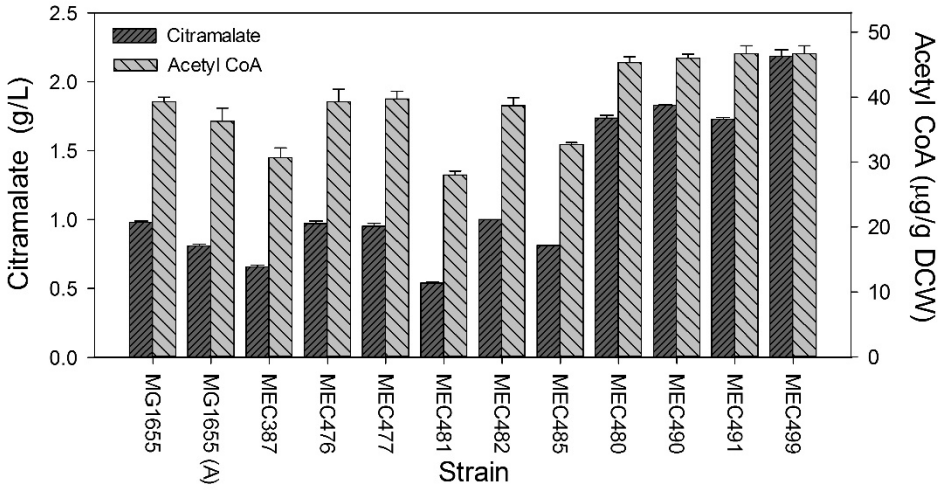

Figure 3-2. Comparison of citramalate production and intracellular acetyl-CoA concentrat ion in shake flasks using various knockout strains of *E. coli* expressing the *cimA* gene. The defined medium contained 5 g/L glucose, and in triplicate experiments measurements were made at 24 h. The (A) indicates the addition of 1 g/L acetate. The *leuC* or *leuD* strains additionally contained 0.2 g/L leucine, while *gltA* strains contained 1 g/L glutamate. See Table 3-1 for information on strains.

Because citramalate synthase requires pyruvate as a substrate, we next examined the effect of a knockout in the *ldhA* gene coding lactate dehydrogenase. Although lactate does not normally accumulate during aerobic growth of *E. coli*, this gene is transcribed during aerobic growth, especially at low pH (Bunch et al., 1997). Surprisingly, MEC387/pZE12-*cimA* accumulated only 0.66 g/L citramalate, 34% less than observed in MG1655/pZE12-*cimA*. Similarly, MEC387/pZE12-*cimA* resulted in an intracellular acetyl-CoA concentration of 30.7 μg/g, about 20% less than MG1655 expressing citramalate synthase (Fig. 3-2).

Citramalate could potentially be metabolized in *E. coli* by 3-isopropylmalate dehydratase coded by the *leuC* (large subunit) and *leuD* (small subunit) genes (Fultz et al., 1979; Fultz and Kemper, 1981). Those two subunits are both required for the activity of isopropylmalate isomerase, which catalyzes the second step in leucine biosynthesis in *E. coli* (Yang and Kessler, 1974). With the deletion of either *leuC* or *leuD*, *E. coli* did not grow in XC medium containing glucose as the sole carbon source. Growth was restored by the addition of leucine into the medium (data not shown), and therefore 0.2 g/L leucine was used for studies involving strains with either of these gene knockouts. Both MEC476/pZE12-*cimA* and MEC477/pZE12-*cimA* accumulated citramalate to about 1.0 g/L, unchanged from the citramalate concentration generated by MG1655/pZE12-*cimA* (Fig. 3-2). We also observed no difference in intracellular acetyl-CoA concentration, suggesting that citramalate degradation by 3-isopropylmalate dehydratase is not significant in shake flask cultures. Of course, this potential degradation pathway might become relevant with additional knockouts or under extended fermentation conditions.

We next studied knockouts in enzymes which are involved in the metabolism of acetyl-CoA. Acetyl-CoA enters the glyoxylate shunt via malate synthase coded by the *glcB* and *aceB*

genes (Ornston and Ornston, 1969; Molina et al., 1994) and the tricarboxylic acid cycle via citrate synthase coded by the *gltA* gene (Eikmanns et al., 1994). We therefore constructed MEC480 (MG1655 *gltA*), MEC481 (MG1655 *aceB*), MEC482 (MG1655 *glcB*) and MEC485 (MG1655 *aceB glcB*). Compared to MG1655/pZE12-*cimA*, MEC481/pZE12-*cimA* showed about 50% lower citramalate accumulation, while MEC482/pZE12-*cimA* resulted in a statistically identical citramalate concentration. The strain having knockouts in both malate synthase genes, MEC485/pZE12-*cimA*, resulted in an intermediate concentration of citramalate. Not surprisingly, MEC480 was unable to grow on XC medium with glucose as sole carbon source, though growth was restored when the medium additionally contained 1 g/L glutamate. This *gltA* strain accumulated 1.74 g/L citramalate, and also 13% greater intracellular acetyl-CoA (45.3 μg/g) than MG1655/pZE12-*cimA*. Since MG1655/pZE12-*cimA* grown in XC medium supplemented with 1 g/L glutamate also yielded about 1 g/L citramalate (data not shown), we attribute the 74% increase in citramalate formation in MEC480/pZE12-*cimA* to the *gltA* knockout, and not to the presence of glutamate.

The effects of *leuC* and *leuD* gene knockouts on citramalate production in the *gltA* strain were also investigated. The final citramalate attained by MEC490 (MG1655 *gltA leuC*) expressing citramalate synthase was 1.83 g/L while 1.73 g/L was obtained by MEC491/pZE12-*cimA*. Acetyl-CoA levels were similar in MEC490/pZE12-*cimA* and MEC491/pZE12-*cimA*, just slightly higher than MEC480/pZE12-*cimA* (Fig. 3-2).

Knocking out *gltA* grown in the presence of 1 g/L glutamate, resulted in a significant increase in acetate production compared to the other strains, and similar to previous results which also reported an increase in pyruvate accumulation (Lee et al., 1994). In this study, MEC490/pZE12-*cimA* generated 0.26 g/L acetate in 24 h, while MG1655/pZE12-*cimA* and other

strains accumulated negligible acetate (0.03 g/L). To reduce acetate formation in the *gltA* knockout and potentially further increase acetyl CoA availability and citramalate yield, we constructed MEC499 (MG1655 *gltA leuC ackA*) having additionally a knockout in the *ackA* gene encoding acetate kinase (Lee et al., 1990; Matsuyama et al., 1994). A deletion of the *ackA* gene reduces acetate formation and correspondingly increases acetyl-CoA accumulation (Diaz-Ricci et al., 1991). Compared to MEC490/pZE12-*cimA*, MEC499/pZE12-*cimA* generated only 0.06 g/L acetate in 24 h. Moreover, MEC499/pZE12-*cimA* achieved the highest citramalate concentration of 2.19 g/L, over twice the final concentration as MG1655/pZE12-*cimA*. The intracellular acetyl-CoA concentration of 47.0 μg/g was about 20% higher than observed in MG1655/pZE12-*cimA*.

Steady-state fermentation

Since MEC499/pZE12-*cimA* showed greatest citramalate and intracellular acetyl-CoA concentrations, we selected this strain for a chemostat experiment using glutamate-limited conditions. We reasoned that a glutamate-limited process would allow the highest yield of citramalate from (excess) glucose. Using a dilution rate of about 0.06 h^{-1}, the yield of citramalate on glucose was 0.77 g/g compared to a maximum theoretical yield of 0.82 g/g, and acetate was not detected in the effluent.

Citramalate production in controlled fermenters

We next compared citramalate production by MG1655, MEC490, or MEC499 expressing citramalate synthase in duplicate under controlled batch conditions using a defined medium composed of 20 g/L glucose. The media additionally contained 2 g/L glutamate (for the *gltA* knockout) and 1 g/L leucine (*leuC/leuD*). MG1655/pZE12-*cimA* reached an OD of 8.3 in only 18 h, and in 30 h accumulated 4.9 g/L citramalate with no detectable acetate (Fig. 3-3). During

exponential growth, the citramalate synthase activity was 21 IU/g DCW, and the intracellular acetyl-CoA concentration was 39 μg/g DCW. MEC490/pZE12-*cimA* reached an OD of 6.3 in 36 h, and accumulated 6.9 g/L citramalate and 7.5 g/L acetate in 60 h (Fig. 3-4). During exponential growth, the citramalate synthase activity was 20 IU/g, and the intracellular acetyl-CoA concentration was 46 μg/g. MEC499/pZE12-*cimA* reached an OD of 5.1 in 84 h and accumulated 14.8 g/L citramalate and 1.9 g/L acetate in 100 h (Fig. 3-5). During exponential growth, the citramalate synthase activity was 19 IU/g, and the intracellular acetyl-CoA concentration was 46 μg/g. During the growth of these strains, the potential byproducts of succinate, lactate, ethanol and pyruvate were not detected. Also, the results show that citramalate synthase expression was fortunately not affected by the *E. coli* strain genotype. The *gltA ackA* knockouts were critical to attaining a high concentration of citramalate, resulting in an increase citramalate yield on glucose threefold from 0.25 g/g (MG1655/pZE12-*cimA*) to 0.75 g/g (MEC499/pZE12-*cimA*).

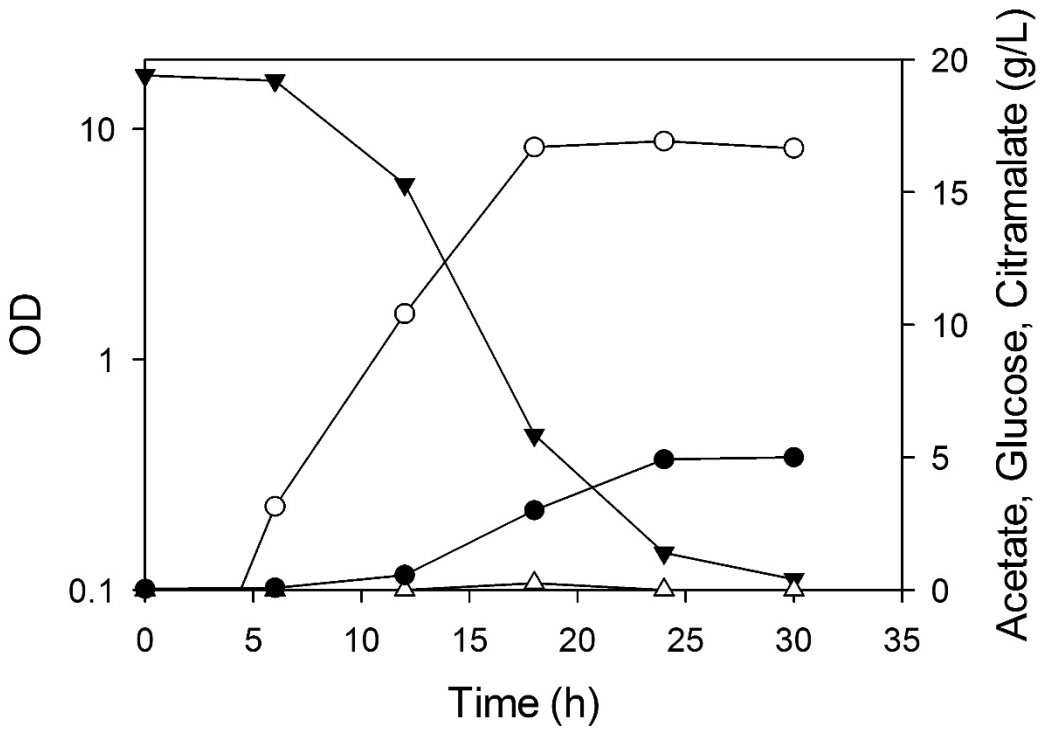

Figure 3-3. Citramalate production in a batch fermentation using MG1655/pZE12-*cimA*: OD (○), citramalate (●), glucose (▼), and acetate (△).

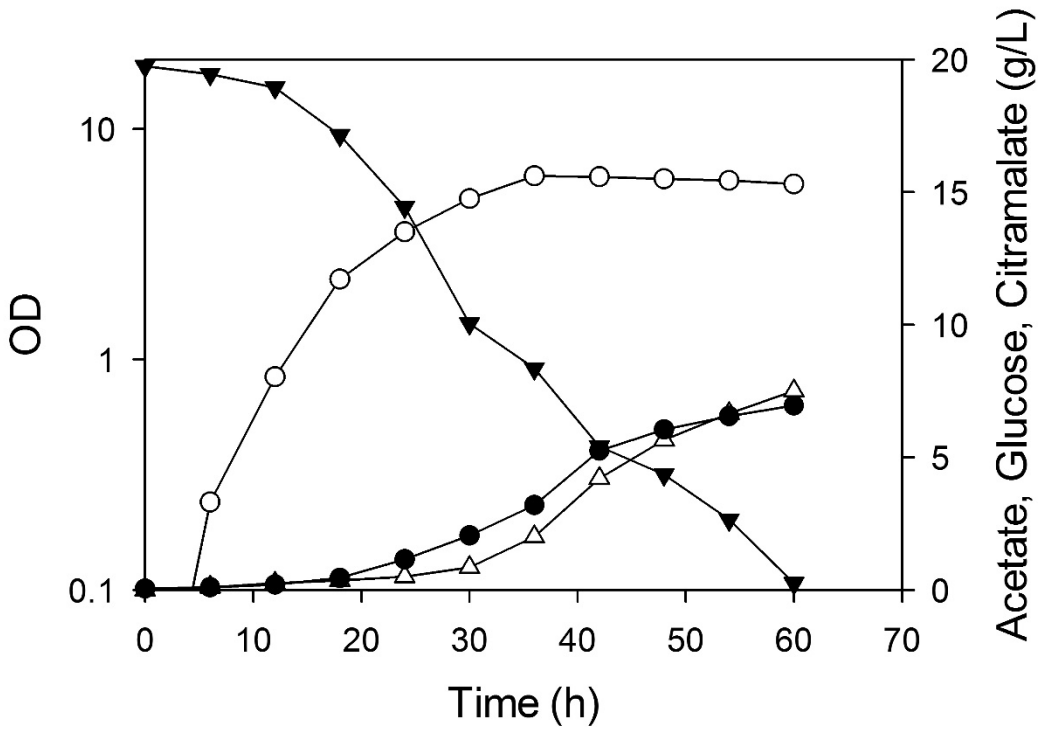

Figure 3-4. Citramalate production in a batch fermentation using MEC490/pZE12-*cimA*: OD (○), citramalate (●), glucose (▼), and acetate (△).

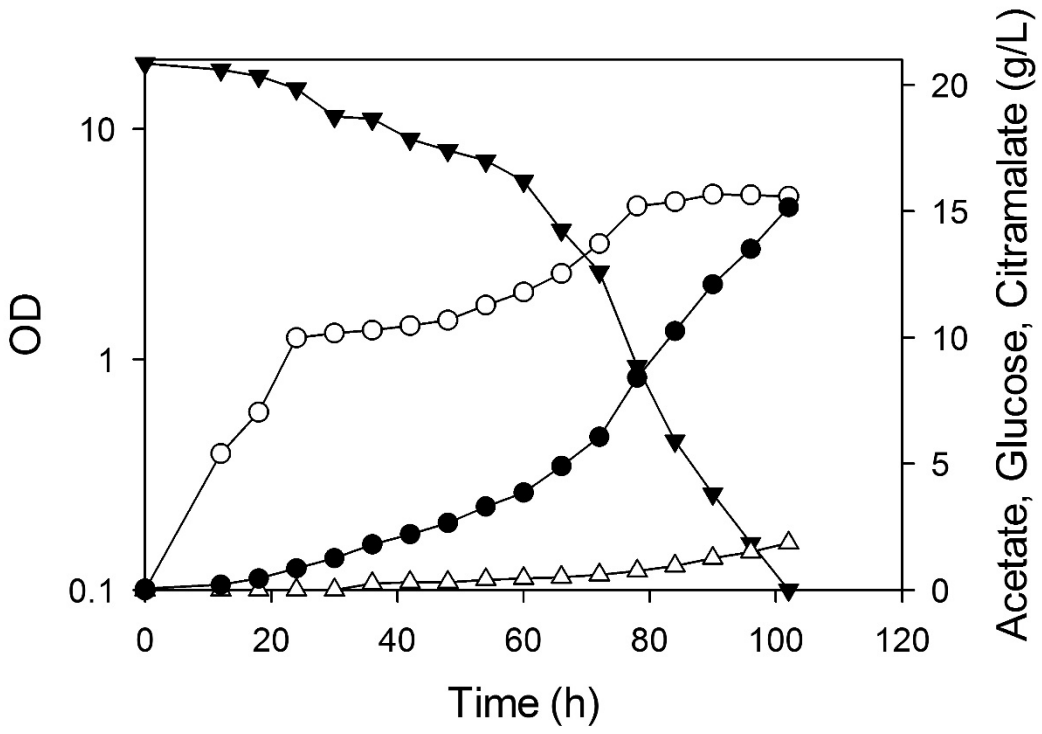

Figure 3-5. Citramalate production in a batch fermentation using MEC499/pZE12-*cimA*: OD (○), citramalate (●), glucose (▼), and acetate (△).

Although the *gltA* and *ackA* gene deletions in MEC499 expressing citramalate synthase significantly increased citramalate yield, the *ackA* knockout also reduced growth rate by over 50% in the defined medium. A high level of intracellular acetyl-CoA might lead to the accumulation of NADH, which would affect glucose u*pta*ke by inhibiting the glyceraldehyde phosphate dehydrogenase (D'Alessio and Josse, 1971) and consequently decrease the rate of PEP synthesis. To overcome these potential growth deficiencies, we completed additional duplicate experiments in the fed-batch mode using a medium containing 15 g/L peptone as a complete source of amino acids (replacing leucine and glutamate addition). Additionally, approximately 30 g glucose was twice added to the fermenter when ~~time~~ the glucose concentration decreased below 5 g/L. For these fed-batch processes the OD reached 9.0 within 21 h at which time the citramalate concentration was 12 g/L (Fig. 3-6). After 132 h, the final citramalate concentration reached an average of 46.5 g/L with a yield of 0.63 g/g glucose. Surprisingly, despite the *ackA* knockout, 10 g/L acetate was formed as byproduct.

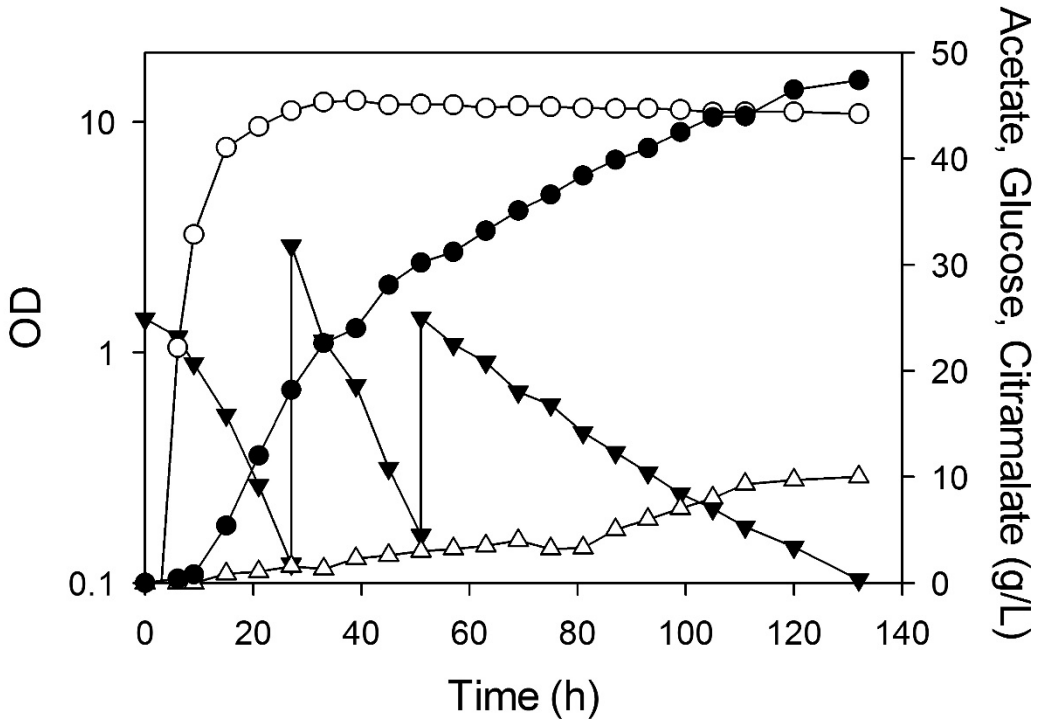

Figure 3-6. Citramalate production in a fed-batch fermentation using MEC499/pZE12-*cimA*: OD (○), citramalate (●), glucose (▼), and acetate (△). Approximately 30 g of glucose was added at 27 h and 51 h.

Discussion

This study demonstrates that citramalate, a chemical precursor to the commodity chemical MAA, will accumulate by *E. coli* expressing citramalate synthase, an enzyme which forms the 5-carbon dicarboxylic acid directly from pyruvate and acetyl CoA as co-substrates. The key knockout necessary to facilitate acetyl CoA accumulation and hence optimal citramalate formation is the *gltA* gene coding citrate synthase. Since a *gltA* deletion prevents growth by the elimination of entry into the TCA cycle, necessary for generating precursors for biosynthesis (Neidhardt and Curtiss, 1996), glutamate was supplemented into the medium as a precursor of α-ketoglutarate. With this medium supplement cell growth was partially recovered in the *gltA* mutant.

Acetate was observed in several processes, particularly in the higher density cultures in the controlled bioreactor. The *ackA* deletion significantly reduced but not eliminate acetate production. Generally, acetyl CoA synthetase (coded by *acs*) is considered to be an acetate assimilation pathway (Lin et al., 2006), and is not initially suspected in the reverse formation of acetate from acetyl CoA. Phosphotransacetylase (or phosphate acetyltransferase) coded by *pta* converts acetyl CoA to acetyl-phosphate. Acetyl-phosphate can itself be used as a phosphate donor in the process of gene regulation and protein-dependent transportation systems (Hong et al., 1979; Wanner and Wilmes-Riesenberg, 1992). Since acetyl-phosphate can therefore result in acetic acid formation even in the absence of acetate kinase (*ackA*), an additional knockout target for improved citramalate formation and reduced acetate formation would be the *pta* gene.

Pyruvate oxidase (*poxB*) also might play a significant role in the aerobic growth of *E. coli* and in acetate formation (Abdel-Hamid et al., 2001). Typically, the Pta-AckA pathway operates during the growth phase, while the PoxB pathway functions during stationary phase (Dittrich et

al., 2005). Moreover, PoxB would bypass acetyl CoA formation altogether. The prospect for PoxB involvement is supported by observations during the fed-batch process, over 80% of the acetate was formed after 30 h when cell growth had ceased, while pyruvate did not accumulate.

Acetate might also result from alternative anabolic pathways or from central carbon metabolism. Several metabolic reactions including acetylornithine deacetylase (ArgE), acetoacetyl-CoA transferases (AtoA and AtoD), cysteine synthases (CysM and CysK), UDP-3-O-acyl-Nacetylglucosamine deacetylase (LpxC), and N-acetylglucosamine-6-phosphate deacetylase (NagA) also generate acetate and could be more significant in a triple knockout strain. An interesting result is that the shake flask studies with MEC499 (MG1655 *gltA leuC ackA*) showed insignificant acetate formation, whereas the controlled, prolonged fed-batch process resulted in about 10 g/L acetate. Clearly, results in shake flasks are weak predictors of results in controlled processes.

In the fed-batch process, *E. coli* MEC499/pZE12-*cimA* produced nearly 50 g/L at a yield reaching over 75% of the theoretical maximum. This result suggests a hybrid biochemical-chemical route could provide a cost-effective approach to producing MAA using renewable resources. Further studies are underway to increase the performance of citramalate production by reducing acetate accumulation further and increasing the specific citramalate productivity under aerobic conditions.

Acknowledgments

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Table 3-1. Strains used in this study.

Strain	Genotype	Notes
MG1655	*E. coli* F- λ- *ilvG rfb*-50 *rph*-1	Wild type
MEC387	MG1655 Δ*ldhA*744::(FRT)	This study
MEC476	MG1655 Δ*leuC*778::(FRT)	This study
MEC477	MG1655 Δ*leuD*778::(FRT)	This study
MEC480	MG1655 Δ*gltA*770::(FRT)	This study
MEC481	MG1655 Δ*aceB*781::(FRT)	This study
MEC482	MG1655 Δ*glcB*749::Kan	This study
MEC485	MG1655 Δ*aceB*781::(FRT) Δ*glcB*749::Kan	This study
MEC490	MG1655 Δ*gltA*770::(FRT) Δ*leuC*778::Kan	This study
MEC491	MG1655 Δ*gltA*770::(FRT) Δ*leuD*778::Kan	This study
MEC499	MG1655 Δ*gltA*770::(FRT) Δ*leuC*778::(FRT) Δ*ackA*778::Kan	This study

CHAPTER 4

CITRAMALIC ACID IS PRODUCED FROM GLYCEROL IN METABOLICALLY ENGINEERED *ESCHERICHIA COLI*

[1] Wu, X. and M.A. Eiteman. Submitted to *Journal of Industrial Microbiology & Biotechnology*, 07/09/17

Abstract

The microbial product citramalic acid (citramalate) serves as a five-carbon precursor for the chemical synthesis of methacrylic acid. We compared citramalate and acetate accumulation from glycerol in shake flasks using numerous *Escherichia coli* mutant strains overexpressing the citramalate synthase gene *cimA*. These studies revealed that *gltA* coding citrate synthase, *leuC* coding 3-isopropylmalate dehydratase, and acetate pathway genes (in particular *poxB* coding pyruvate oxidase) play important roles in elevating citramalate formation and minimizing acetate formation. Controlled batch experiments at the 1.0 L scale confirmed that deletions in all three acetate-production genes (*poxB*, as well as *ackA* coding acetate kinase and *pta* coding phosphotransacetylase) were necessary to minimize acetate formation during citramalate formation to less than 1 g/L from 30 g/L glycerol. Fed-batch processes using MEC568/pZE12-*cimA* (*gltA leuC ackA pta ackA*) generated over 31 g/L citramalate and less than 2 g/L acetate from either purified or crude glycerol at yields exceeding 0.50 g citramalate / g glycerol in 132 h. These results hold promise for the viable formation of citramalate from unrefined glycerol.

Introduction

The commercial manufacture and use of biodiesel has been rapidly emerging in Europe and US during the last two decades. As an alternative to petrochemical fuels, biodiesel is superior in its health and environmental impact, including low sulfur content, lower emission of harmful off-gases and a better lifecycle of CO_2 (Bournay et al., 2005). One key challenge in the development and adoption of biodiesel is the low value by-product glycerol, which is generated at about 10% mass ratio from the esterification or transesterification of vegetable oil and animal fats (Ma and Hanna, 1999). Fortunately, many microorganisms can naturally utilize glycerol as the sole carbon and energy source, and glycerol is a potential substitute for traditional carbohydrates such as sucrose or starch in industrial fermentation processes (Behr et al., 2008). Glycerol has been evaluated as a raw material for the production of many microbial products, including hydrogen (Sabourin-Provost and Hallenbeck, 2009), 1,3-propanediol (Chatzifragkou et al., 2011), 2,3-butanediol (Yang et al., 2015) and succinic acid (Gao et al., 2016).

Methacrylic acid (MAA) is a commodity chemical with an estimated annual global market of about 2.2 million tons, and it is used primarily for the synthesis of poly(methyl methacrylate) (Zhang et al., 2011). This polyester is widely used as a transparent thermoplastic in construction, furniture, medical material, and display technologies. The most common route for MAA synthesis converts acetone cyanohydrin to methacrylamine sulfate using sulfuric acid (Salkind et al., 1959; Bauer, 2000; Nagai, 2001). Sulfuric acid regeneration and hazards associated with volatile cyanides are concerns for industrial MAA production, and companies have sought other routes from isobutene, isobutyric acid, and ethylene (Bauer, 2000; Nagai, 2001). Although direct microbial production of MAA and

acrylate with its reduced hazards has been proposed, acrylates are extremely toxic to microorganisms such as *Escherichia coli* (Todd et al., 2012; Arya et al., 2013).

Recently, we reported a microbial approach to produce citramalic acid (citramalate, (R)-2-methylmalic acid, (2R)-2-hydroxy-2-methylbutanedioate) from renewable carbohydrates. Citramalate can be directly converted to MAA by base-catalyzed decarboxylation and dehydration (Johnson et al., 2015). In a fed-batch fermentation, 46.5 g/L citramalate was formed with a yield of 0.63 g/g from glucose using an engineered *Escherichia coli* overexpressing the *cimA* gene coding citramalate synthase (Wu and Eiteman, 2016). Despite the deletion of citrate synthase (coded by *gltA*) and acetate kinase (*ackA*), about 10 g/L acetate were still formed as an undesirable by-product from glucose. The maximum theoretical yield of citramalate from glycerol in *E. coli* is 0.80 g/g (Fig. 4-1), and the stoichiometric equation for the biochemical conversion is:

$$2 \text{ glycerol} + 5 \text{ NAD} + 2 \text{ Pi} + 2 \text{ ADP} \rightarrow \text{citramalate} + 5 \text{ NADH} + 2 \text{ ATP} + CO_2$$

The goals of this study was to examine citramalate formation from glycerol by *Escherichia coli*. In addition to studying whether 5-carbon citramalate can be generated directly from both purified and crude 3-carbon glycerol at high yield, we examined strategies to reduce the formation of acetate as a by-product (see Fig. 4-1).

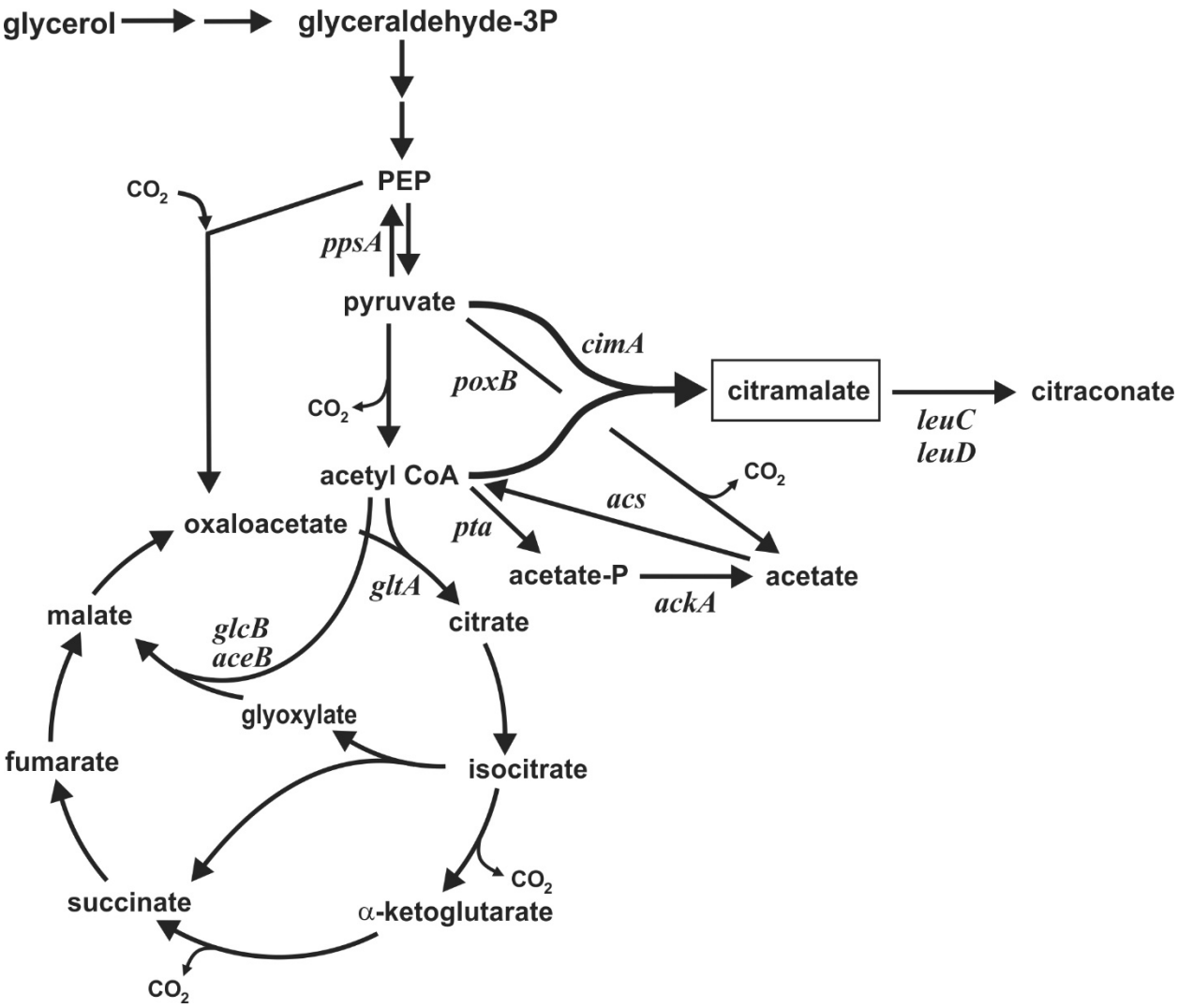

Figure 4-1. Biosynthesis of citramalate in *Escherichia coli* expressing the *cimA* gene coding citramalate synthase. Key genes (and coded enzymes) are: *leuC* and *leuD* (3-isopropylmalate dehydratase), *gltA* (citrate synthase), *glcB* and *aceB* (malate synthase), *pta* (phosphotransacetylase), *ackA* (acetate kinase), *poxB* (pyruvate oxidase), *ppsA* (phosphoenolpyruvate synthetase).

Materials and Methods

Strain construction

Strains used in this study are shown in Table 4-1. Gene mutations were transduced into *E. coli* MG1655 from their respective strains in the KEIO collection (Baba et al., 2006) by the P1 phage method. The Δ*pta* knockout was constructed using the λ Red recombination (Datsenko and Wanner, 2000). The sequence of the *ackA-pta* operon was confirmed revealing that Δ*ackA-pta* was knocked out (Eurofins Scientific, Louisville, KY, USA).To knockout multiple genes in single strain, the Kan antibiotic marker was removed using pCP20 (Datsenko and Wanner, 2000). In knockout strains, forward primers external to the target gene and reverse primers within the kanamycin resistance cassette were used to check for proper chromosomal integration. In cured strains, the removal of the markers was verified by PCR. Plasmid pZE12-*cimA* was transformed into all strains for citramalate production (Wu and Eiteman, 2016).

Growth medium

XP medium contained (per L): 3.00 g glycerol, 1.00 g/L peptone, 1.44 g KH_2PO_4, 2.11 g K_2HPO_4, 2.00 g K_2SO_4, 3.50 g NH_4Cl, 20.00 mg $Na_2(EDTA)\cdot 2H_2O$, 0.15 g $MgSO_4\cdot 7H_2O$, 20 mg thiamine·HCl, 0.25 mg $ZnSO_4$, 0.125 mg $CuCl_2\cdot 2H_2O$, 1.25 mg $MnSO_4\cdot H_2O$, 0.875 mg $CoCl_2\cdot 6H_2O$, 0.06 mg H_3BO_3, 0.25 mg $Na_2MoO_4\cdot 2H_2O$, 5.50 mg $FeSO_4\cdot 7H_2O$, and 20 mg citric acid. For the growth of strains having *leuC* or *leuD* knockouts, the medium was supplemented with 0.20 g/L L-leucine. For the growth of strains having *gltA* knockouts, the medium was supplemented with 1.00 g/L L-glutamate. *E. coli* is unable to utilize citrate under aerobic conditions (Koser, 1924). Additionally, 50 mg/L ampicillin and/or 100 mg/L kanamycin were added for plasmid-containing strains or

strains having antibiotic resistance. The crude glycerol from biodiesel process was generously provided by a local biodiesel producer (Down To Earth Energy, LLC, Monroe, GA, USA) and contained 58.6% w/w glycerol and 0.3% w/w methanol.

Shake flask, batch and fed-batch processes

To compare various strains for citramalate production in shake flasks, cells were first grown in 3 mL Lysogeny Broth (LB) at 37°C and 250 rpm (19 mm pitch). After 10-14 h, 0.5 mL was used to inoculate 50 mL of XP medium containing 0.2 mM IPTG in 500 mL baffled shake flasks (in triplicate). After growth at 37°C and 250 rpm (19 mm pitch) for 24 h, these shake flask cultures were analyzed for citramalate synthase activity, citramalate and intracellular acetyl-CoA concentration.

To examine citramalate production under controlled bioreactor conditions, cells were first grown as described above in 3 mL LB and then 50 mL XP medium. After 18 h the shake flask contents were used to inoculate a 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific Co., New Brunswick, NJ, USA) containing 1.0 L XP medium modified to contain 30 g/L glycerol, 5 g/L peptone, 3 g/L glutamate and 1 g/L leucine (but otherwise as described above) and 0.2 mM IPTG initially. For duplicate batch and fed-batch processes, the agitation was 400 rpm, and air was sparged at 1.0 L/min, which maintained the dissolved oxygen above 40% of saturation. The pH was controlled at 7.0 using 20% (w/v) NaOH, and the temperature was controlled at 37°C. For the fed-batch process, an additional 30 g glycerol and 5 g peptone dissolved in 60 mL were added when the glycerol concentration decreased below 5 g/L.

Analytical methods

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, CA, USA) was used to monitor cell growth. Extracellular organic acids were analyzed by HPLC using a Refractive Index detector as previously described (Eiteman and Chastain, 1997). Glutamate concentration was measured using a glutamate assay kit (Sigma-Aldrich Co., St. Louis, MO, USA). Acetyl-CoA was analyzed by the previously established method (Gao et al., 2007).

Cell-free extracts were also used to measure citramalate synthase activity by the generation of free CoA and its reaction product with 5,5′-dithiobis(2-nitrobenzoic acid) detected at a wavelength of 412 nm (Howell et al., 1999). One Unit of activity is the amount of enzyme that generates one μmole of CoA in one minute at 37°C.

Results and Discussion

Comparison of citramalate and acetate formation by various strains

In *E. coli* overexpressing citramalate synthase coded by the *cimA* gene, citramalate accumulates as the reaction product of the condensation of pyruvate and acetyl-CoA. In wild-type *E. coli* expressing citramalate synthase (MG1655/pZE12-*cimA*) just over 1 g/L citramalate formed from 3 g/L glycerol, resulting in a citramalate yield of 0.36 g/g (Fig. 4-2). This wild-type strain expressing citramalate synthase generated substantial acetate in shake flasks, resulting in a yield of 0.033 g acetate/g glycerol (Fig. 4-2). Since acetyl CoA and pyruvate are involved in numerous enzyme reactions, we compared citramalate formation from glycerol using several strains having knockouts in genes associated with these metabolites.

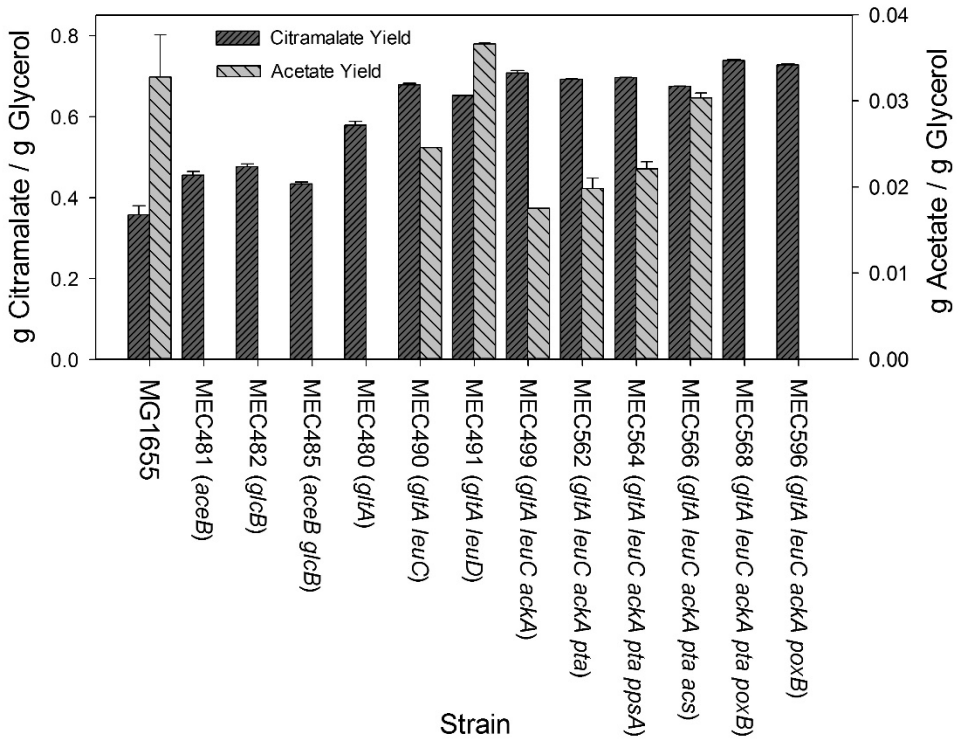

Figure 4-2. Comparison of citramalate yield and acetate yield from 3 g/L glycerol in triplicate shake flasks using various knockout strains of *E. coli* expressing the *cimA* gene. The *leuC* or *leuD* strains additionally contained 0.2 g/L leucine, while *gltA* strains contained 1 g/L glutamate.

Acetyl-CoA is converted to malate via malate synthase coded in *E. coli* by the *glcB* and *aceB* genes (Ornston and Ornston, 1969; Molina et al., 1994). We therefore constructed MEC481 (MG1655 *aceB*), MEC482 (MG1655 *glcB*) and MEC485 (MG1655 *aceB glcB*). Compared to MG1655/pZE12-*cimA*, MEC481/pZE12-*cimA* and MEC482/pZE12-*cimA* showed about 28% and 35% higher citramalate accumulation, respectively (Fig. 4-2). The strain having knockouts in both malate synthase genes, MEC485/pZE12-*cimA*, resulted in only 22% greater citramalate compared to the wild-type. Acetyl CoA is also converted to citrate via citrate synthase coded by the *gltA* gene (Eikmanns et al., 1994), and we therefore examined citramalate production in MEC480 (MG1655 *gltA*) expressing citramalate synthase. MEC480/pZE12-*cimA* grew poorly on XP medium, but growth was restored when the medium additionally contained 1 g/L glutamate. MEC480/pZE12-*cimA* grown with supplemented glutamate accumulated 0.58 g citramalate/g glycerol, 63% more than MG1655/pZE12-*cimA*. Since MG1655/pZE12-*cimA* grown in XP medium supplemented with 1 g/L glutamate also generated the same yield of citramalate as the same strain without added glutamate (data not shown), we attribute the 63% increase in citramalate formation in MEC480/pZE12-*cimA* to the *gltA* knockout and not to the presence of glutamate. Therefore, media for strains having the *gltA* knockout were henceforth supplemented with 1 g/L glutamate. These strains having knockouts of enzymes associated with the glyoxylate shunt or the TCA cycle (i.e., *aceB*, *glcB*, *gltA*) accumulated no detectable acetate.

Citramalate could be potentially metabolized in *E. coli* by 3-isopropylmalate dehydratase coded by the *leuC* (large subunit) and *leuD* (small subunit) genes (Fultz et al.,

1979; Fultz and Kemper, 1981). The two subunits are both required for the activity of isopropylmalate isomerase, an enzyme which is necessary for leucine biosynthesis in *E. coli* (Yang and Kessler, 1974), and each of these individual deletions were examined by comparing MEC490 (MG1655 *gltA leuC*) and MEC491 (MG1655 *gltA leuD*). With the deletion of either *leuC* or *leuD*, *E. coli* did not grow in XP medium containing glycerol as the sole carbon source, despite the presence of peptone in the medium. Growth was restored by the addition of 0.2 g/L leucine, and MEC490/pZE12-*cimA* accumulated 0.68 g citramalate/g glycerol, 13% greater than MEC480/pZE12-*cimA*, while MEC491/pZE12-*cimA* accumulated 0.65 g citramalate/g glycerol (Fig. 4-2). MEC490/pZE12-*cimA* and MEC491/pZE12-*cimA* both accumulated similar acetate as MG1655/pZE12-*cimA*.

Although *leuC* led to more citramalate in the *gltA* strain, this additional knockout also led to acetate formation from glycerol. To reduce acetate formation in the *E. coli gltA leuC* expressing citramalate synthase, we examined several pathways related to the acetate and pyruvate metabolism. Four enzymes exist in *E. coli* related to acetate and acetyl-CoA. Acetate kinase coded by *ackA* and phosphotransacetylase coded by *pta* (Lee et al., 1990; Matsuyama et al., 1994) are typically considered the primary routes for the conversion of acetyl-CoA to acetyl-phosphate (acetyl-P) and to acetate. Acetyl-P can form acetate via other routes, also, since it can serve as a phosphate donor in gene regulation and protein-dependent transport systems (Hong et al., 1979; Wanner and Wilmes-Riesenberg, 1992). On the other hand, acetyl-CoA synthetase coded by *acs* functions as an anabolic route and scavenges acetate to acetyl-CoA (Lin et al., 2006). Finally, pyruvate oxidase coded by *poxB* can play a role in aerobic growth of *E. coli* and in acetate formation from pyruvate (Abdel-Hamid et al., 2001). We also examined phosphoenolpyruvate synthetase coded by *ppsA*,

which could affect the intracellular pyruvate and acetyl CoA pools (Niersbach et al., 1992). We constructed several strains having these knockouts, expressed citramalate synthase and determined the citramalate and acetate formation in shake flasks (Fig. 4-2).

The additional deletion in the *ackA* gene or the combination of *ackA* and *pta* genes increased citramalate yield slightly to 0.71 g/g and 0.69 g/g, respectively. However, both MEC499/pZE12-*cimA* and MEC562/pZE12-*cimA* still formed acetate with yields of about 0.018 g/g – 0.020 g/g (Fig. 4-2). Compared to *E. coli gltA leuC ackA pta* expressing citramalate synthase, an additional *ppsA* deletion did not affect citramalate or acetate formation significantly, while an additional *acs* knockout actually elevated acetate yield to 0.030 g/g. Inexplicably, one previous investigation of an *acs* deletion strain resulted in lower specific acetate formation from glucose (Contiero et al., 2000), while in another study overexpression of *acs* significantly reduced acetate formation (Lin et al., 2006). In our study using strains with additional gene deletions, the increase in acetate formation when *acs* is deleted (in the *ackA pta* background) suggests that some acetate is formed via pyruvate oxidase, and that acetyl CoA synthase provides the cells with a means to metabolize that acetate partially. In support of this conclusion, the *poxB* knockout (in the *ackA pta* background) eliminated acetate formation in the shake flask culture, and increased citramalate yield from glycerol to 0.74 g/g. To determine whether *poxB* or the combination of *pta poxB* was important to eliminate acetate formation, we also examined MEC596/pZE12-*cimA*, which generated 0.73 g citramalate/g glycerol and no detectable acetate. These results conclusively show that pyruvate oxidase is a key enzyme in the accumulation of acetate during citramalate production in *E. coli*. The deletion of *poxB* has

similarly reduced acetate in an *ackA pta* strain during the aerobic production of succinate by *E. coli* (Lin et al., 2005).

Acetyl CoA is an important substrate for citramalate synthase, and we measured intracellular acetyl CoA concentration in all triplicate shake flask experiments. These results were used to determine whether any correlation exists between intracellular acetyl CoA and citramalate yield in the 13 different strains (Fig. 4-3). The results show that increased citramalate yield correlates strongly with increased acetyl CoA concentration.

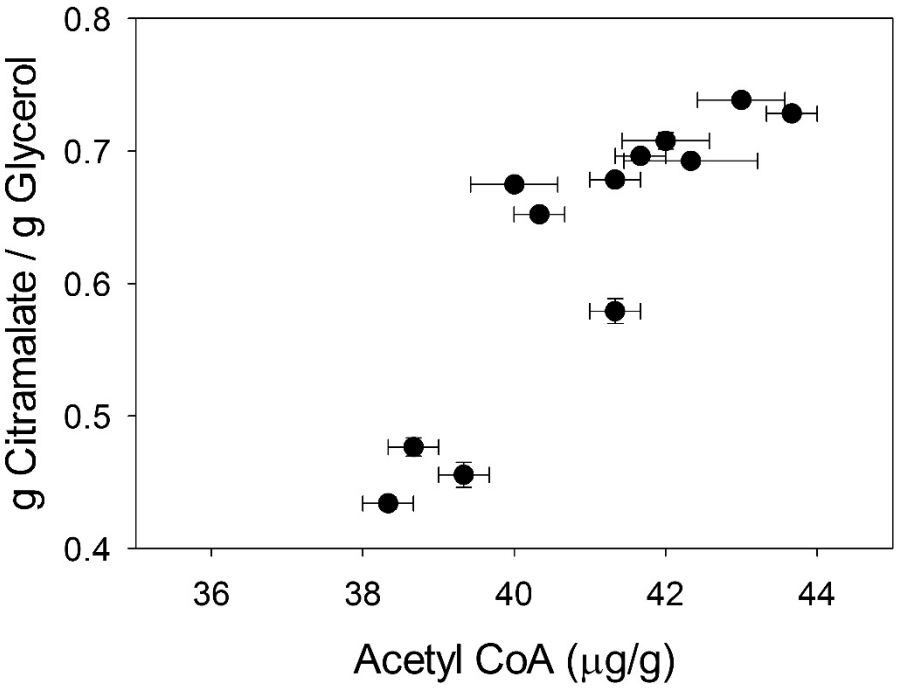

Figure 4-3. Relationship between citramalate yield and intracellular acetyl CoA concentration in shake flasks using various knockout strains of *E. coli* expressing the *cimA* gene (shown in Fig. 4-2).

Controlled batch citramalate production from glycerol

In order to determine whether shake flask results were transferable to larger scale, we next examined citramalate production at the 1.0 liter scale in controlled bioreactors. In duplicate, we compared six strains expressing citramalate synthase: MG1655, MEC490, MEC499, MEC562, MEC568, or MEC596. To accommodate greater cell growth, the medium contained 30 g/L glycerol and 5 g/L peptone, as well as 3 g/L glutamate and 1 g/L leucine (for strains with *gltA leuC* knockouts). The results of these batch processes are shown in Table 4-2.

MG1655/pZE12-*cimA* reached an OD of over 20 in 24 h and accumulated 4.3 g/L citramalate (yield of 0.143 g/g) and 0.05 g/L acetate in 30 h (yield of 0.002 g/g). All other strains examined had the *gltA* and *leuC* knockouts which significantly slowed growth despite the presence of glutamate and leucine, and they generally reached an OD of 10 in 24 – 30 h. The *gltA leuC* knockouts alone (MEC490/pZE12-*cimA*) resulted in only 5.2 g/L citramalate (yield of 0.175 g/g) and 11.4 g/L acetate (yield of 0.380 g/g). In comparison the addition of an *ackA* deletion increased citramalate and diminished acetate formation. Nevertheless, the *ackA* deletion was insufficient to prevent acetate formation. The addition of either a *pta* or a *poxB* deletion to the *gltA leuC ackA* strain further decreased acetate formation, with the *poxB* resulting in a better reduction of this by-product. The lowest accumulation of acetate was observed under controlled batch conditions using the strain with all three acetate pathway knockouts (*gltA leuC ackA pta poxB*), and MEC568/pZE12-*cimA* also led to the greatest citramalate production (about 17.5 g/L). Typically, the phosphotransacetylase and acetate kinase operate during cell growth, while pyruvate oxidase appears to become important during the stationary phase (Dittrich et al., 2005).

Pyruvate oxidase moreover bypasses acetyl-CoA formation altogether. The controlled batch experiments contrast with previous shake flask results and demonstrate that shake flask results are weak predictors of larger scale processes. In particular, MEC490, MEC499, MEC596 and MEC568 showed insignificant acetate formation in shake flasks, whereas in the controlled and prolonged batch processes acetate accumulation was observed for all these strains.

During the growth of these strains, succinate, lactate, ethanol and pyruvate were not detected, and citramalate synthase activity was not affected by the *E. coli* strain genotype (data not shown). The combination of *gltA leuC ackA-pta* and *poxB* knockouts were important to achieve a high yield of citramalate and minimal acetate, and therefore MEC568 was used for further studies.

Fed-batch production of citramalate

The final concentration of a fermentation product can often be maximized by continuous feeding of the carbon source. We therefore next completed duplicate experiments using a fed-batch process in which 30 g glycerol and 5 g peptone were added to the fermenter once when the glycerol concentration decreased below 5 g/L. MEC568/pZE12-*cimA* was selected for this study because this strain achieved the greatest citramalate yield in batch processes (Table 4-2). Like the batch process described above, for these fed-batch processes the OD reached 10.0 within 36 h at which time the citramalate concentration was 12.5 g/L (Fig. 4-4). After 132 h, the final citramalate concentration reached an average of 31.4 g/L, corresponding to a yield of 0.52 citramalate g/g glycerol. In addition, only 1.8 g/L acetate was formed as byproduct.

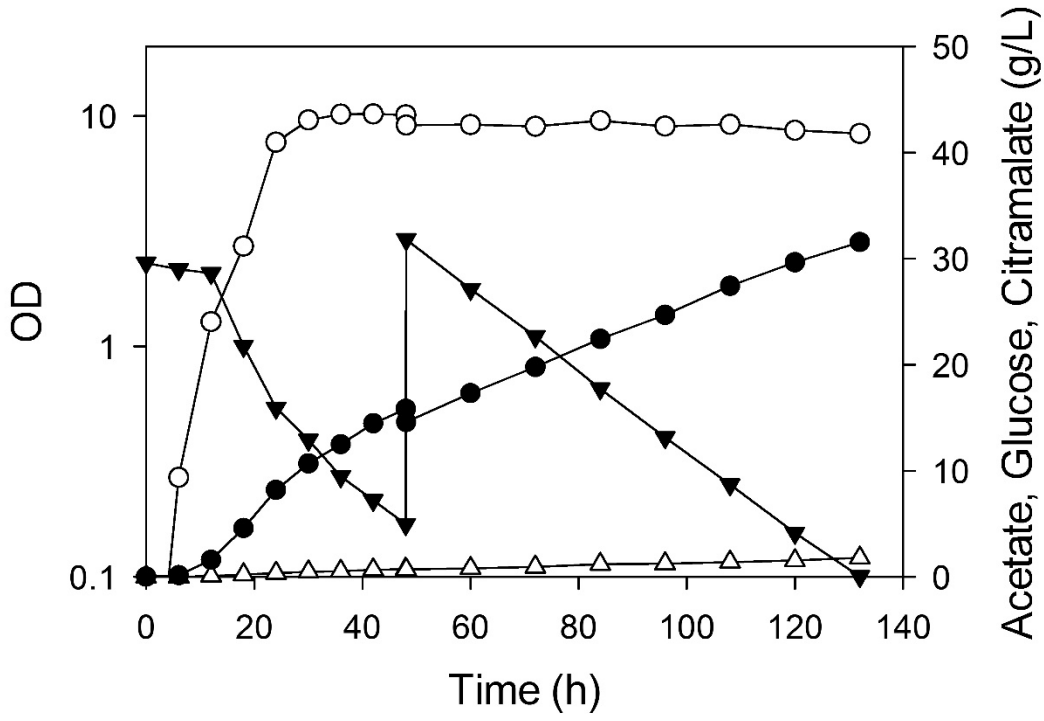

Figure 4-4. Citramalate production using pure glycerol in a 1.0 L fed-batch fermentation with MEC568/pZE12-*cimA*: OD (○), citramalate (●), glycerol (▼), and acetate (△). Approximately 30 g purified glycerol and 5 g peptone in 60 mL was added at 48 h.

Citramalate production using crude glycerol

The rapid growth of the biodiesel industry has resulted in surplus availability of crude glycerol production, which has a purity of 60%–80% based on the type of oil used as feedstock (Ayoub and Abdullah, 2012). Crude glycerol also often contains 10%–15% methanol, 1.5%–2.5% ash, and 3.0%–5.0% soap as impurities (Ayoub and Abdullah, 2012). To determine if *E. coli* could be used to generate citramalate from crude glycerol, we next examined the fed-batch process using unrefined glycerol obtained directly from a local biodiesel manufacturer in place of purified glycerol. In this fed-batch process, about 31 g/L citramalate (0.51 g/g yield) and 1.9 g/L acetate were obtained using MEC568/pZE12-*cimA* (Fig. 4-5). This result is virtually identical to the fed-batch process using purified glycerol, and demonstrates that refining glycerol is not necessary for citramalate production by *E. coli*. Interestingly, the final OD was 22% greater when crude glycerol was used (10.3 vs. 8.4), possibly because of the presence of other unidentified carbon sources in the crude material. Crude glycerol has been used in other studies of biological conversions to value-added chemical. For example, ethanol formation was similar for purified and unrefined glycerol by a *Klebsiella pneumoniae* mutant (Oh et al., 2011), and the same 1,3-propanediol concentration was achieved using purified or crude glycerol in a fed-batch fermentation, although the productivity was lower using crude glycerol (Hiremath et al., 2011).

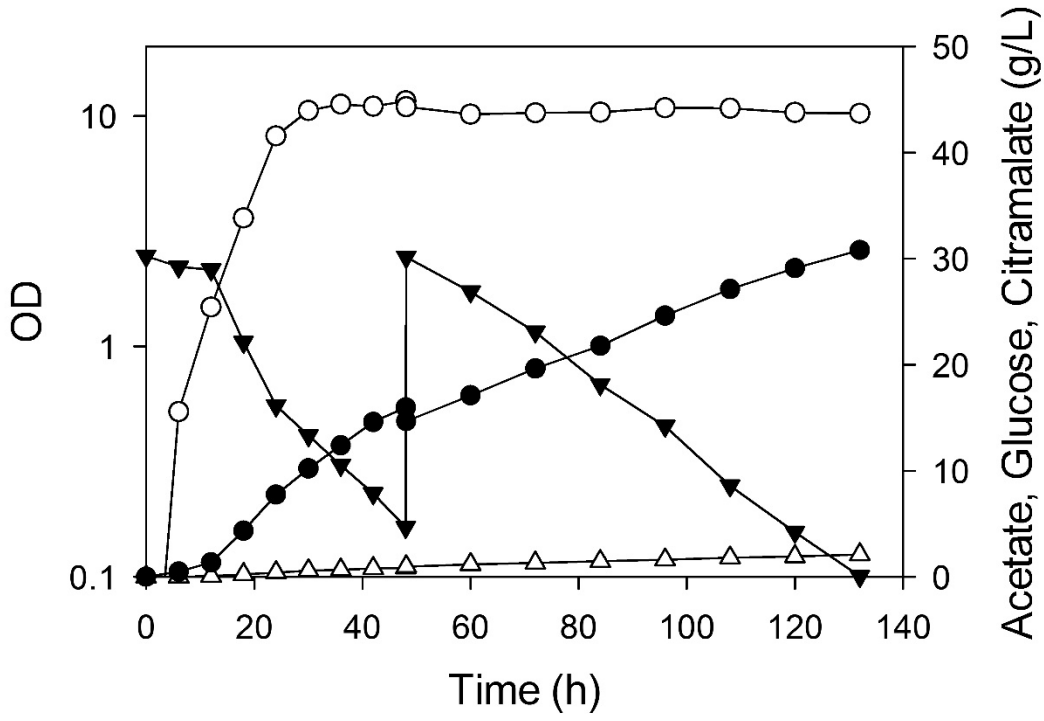

Figure 4-5. Citramalate production using crude glycerol in a 1.0 L fed-batch fermentation with MEC568/pZE12-*cimA*: OD (○), citramalate (●), glycerol (▼), and acetate (△). Approximately 30 g crude glycerol and 5 g peptone was added at 48 h.

Conclusions

Gene knockouts and fermentation optimization improve citramalate production from glycerol and also reduce acetate accumulation. Near elimination of acetate formation necessitates deletions in genes for both pathways associated with acetate formation: *ackA* coding acetate kinase, *pta* coding phosphotransacetylase, and *poxB* coding pyruvate oxidase. Fed-batch fermentation demonstrated that identical citramalate over 30 g/L can be generated from pure or crude glycerol at yield greater than 0.50 g citramalate/g glycerol. This result holds promise that crude glycerol could be used as for citramalate production and ultimately as a source of methacrylate.

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Table 4-1. Strains used in this study.

Strain	Genotype	Notes
MG1655	*E. coli* F- λ- *ilvG rfb*-50 *rph*-1	Wild type
MEC480	MG1655 Δ*gltA*770::Kan	Wu and Eiteman, 2016
MEC481	MG1655 Δ*aceB*781::Kan	Wu and Eiteman, 2016
MEC482	MG1655 Δ*glcB*749::Kan	Wu and Eiteman, 2016
MEC485	MG1655 Δ*aceB*781::(FRT) Δ*glcB*749::Kan	Wu and Eiteman, 2016
MEC490	MG1655 Δ*gltA*770::(FRT) Δ*leuC*779::Kan	Wu and Eiteman, 2016
MEC491	MG1655 Δ*gltA*770::(FRT) Δ*leuD*778::Kan	Wu and Eiteman, 2016
MEC498	MG1655 Δ*gltA*770::(FRT) Δ*leuC*779::(FRT)	Wu and Eiteman, 2016
MEC499	MEC498 Δ*ackA*778::Kan	Wu and Eiteman, 2016
MEC562	MEC498 Δ*ackA*778-*pta*-779::Kan	This study
MEC564	MEC498 Δ*ackA*778-*pta*-779::(FRT) Δ*pps*-776::Kan	This study
MEC566	MEC498 Δ*ackA*778-*pta*-779::(FRT) Δ*acs*-763::Kan	This study
MEC568	MEC498 Δ*ackA*778-*pta*-779::(FRT) Δ*poxB*772::Kan	This study

MEC596	MEC498 Δ*ackA*778::(FRT) Δ*poxB*772::Kan	This study

Table 4-2. Summary of citramalate and acetate formation from 30 g/L glucose in controlled batch bioreactor using various *E. coli* strains.

Strain	Key gene deletions	Time (h)	Citramalate Yield (g/g)	Acetate Yield (g/g)
MG1655/pZE12-*cimA*	N/A	30	0.143	0.002
MEC490/pZE12-*cimA*	*gltA leuC*	48	0.175	0.380
MEC499/pZE12-*cimA*	*gltA leuC ackA*	60	0.476	0.181
MEC562/pZE12-*cimA*	*gltA leuC ackA-pta*	60	0.485	0.143
MEC596/pZE12-*cimA*	*gltA leuC ackA poxB*	66	0.560	0.086
MEC568/pZE12-*cimA*	*gltA leuC ackA-pta poxB*	60	0.585	0.032

CHAPTER 5

ENGINEERING CITRATE SYNTHASE IMPROVES CITRAMALIC ACID PRODUCTION IN ESCHERICHIA COLI

[1] Wu, X. and M.A. Eiteman. To be submitted to *Metabolic Engineering*.

Abstract

The microbial product citramalic acid (citramalate) serves as a five-carbon precursor for the chemical synthesis of methacrylic acid, and accumulates in *Escherichia coli* expressing the *cimA* gene. A knockout in the *gltA* gene coding citrate synthase increases citramalate accumulation. However, a *gltA* knockout strain requires a secondary carbon source such as glutamate to supply the tricarboxylic acid cycle. In this study citrate synthase was engineered with rational point mutations to decrease enzyme activity. Citrate synthase mutant strains expressing were compared for cell growth, enzyme activity and citramalate production in shake flasks and controlled fermenters. These studies revealed that *E. coli* expressing citrate synthase F383M grew in the absence of glutamate, but showed 125% greater citramalate production compared to the control strain containing the native citrate synthase. An exponential feeding strategy was employed using MEC626/pZE12-*cimA* (*gltA*-F383M), which generated over 60 g/L citramalate with the yield 0.53 g citramalate/g glucose in 132 h. These results demonstrated protein engineering aimed at decreasing enzyme activity is a powerful tool to improve the microbial production of traditional commodity chemicals.

Introduction

Metabolic engineering of microbes has been widely applied in microbial production of fuels, chemicals, pharmaceuticals, and materials (Bommareddy et al., 2014; Chen et al., 2015; Lee and Kim, 2015). Most of the genetic toolboxes currently used in metabolic engineering are based on altering metabolic flux through the modification of gene expression. For example, pathway flux can be modulated by altering the promoter strength (Alper et al., 2005), ribosome binding sites (Salis et al., 2009), codon usage (He et al., 2014), mRNA secondary structure (Liang et al., 2011), and deleting a gene for a competing pathway altogether. In the context of enzyme kinetics, each one of these approaches affects the quantity of active enzyme (i.e., V_{MAX}), but typically does not alter the intrinsic enzyme-substrate affinity (K_M). Thus, these strategies would not address protein-level limitations such as feedback inhibition or substrate specificity. In contrast, enzyme engineeringis an approach to effect both enzyme activity and selectivity to further optimize the production pathway (reviewed by Otte and Hauer, 2015). One strategy is to evolve key enzymes in a biosynthesis pathway to achieve higher activity or specificity, and thus to obtain higher yields of the target molecule (Yoshikuni et al., 2008; Leonard et al., 2010; Machado et al., 2012). Protein engineering is rarely used as a strategy to reduce flux through a competing pathway (e.g., increase K_M of an existing enzyme), or more generally towards the goal of altering enzyme properties to affect pathway flux.

Acetyl CoA is a key central metabolite at the junction of glycolysis and the tricarboxylic acid (TCA) cycle. Of the 11 precursors in central metabolism withdrawn for the synthesis of *Escherichia coli* biomass, more acetyl CoA on a molar basis is consumed for biomass than any other precursor (Zhao et al., 2004). Acetyl CoA is also the starting material for a surprisingly diverse suite of biochemical products of commercial interest, including butanol (Anfelt et al.,

2015), poly(hydroxyalkanoate)s (Centeno-Leija et al., 2014), polyketides (Choi and Da Silva, 2014) and isoprenoids (Lv et al., 2016). Most studies concerned with elevating acetyl CoA availability have focused on eliminating acetate formation, for example, by knocking genes coding for phosphotransacetylase, acetate kinase and pyruvate oxidase (Dittrich et al., 2005). These enzymes mediate the conversion of acetyl CoA or pyruvate to acetate, and their deletion reduces the formation of this by-product without preventing growth. However, ^{13}C-labelling experiments using wild-type *E. coli* at steady-state demonstrate that over 62% of the acetyl CoA generated is directed to the TCA cycle, over 22% is directed to biomass generation, while actually less than 16% is directed to the by-product acetate (Zhao et al., 2004). These results suggest that a reduction of flux toward acetate might minimally impact the acetyl CoA pool, since the other two acetyl CoA sinks might readily absorb this fairly small metabolic perturbation. Because entry into the TCA cycle actually constitutes the principal use of acetyl CoA, reducing this flux mediated by citrate synthase should have a more profound effect on the availability of acetyl CoA for any metabolically engineered pathways leading from acetyl CoA.

With an estimated annual global market of about 3.2 million tons, the commodity chemical methacrylic acid (MAA) is primarily used for the synthesis of poly(methyl methacrylate) (Choi et al., 2015). This polyester finds application as a transparent thermoplastic in construction, furniture, medical materials, and display technologies. The most common current production route for MAA synthesis converts acetone cyanohydrin to methacrylamine sulfate using sulfuric acid (Salkind et al., 1959; Bauer, 2000; Nagai, 2001). Sulfuric acid regeneration and volatile cyanides are concerns for industrial MAA production, and companies have sought other routes from isobutene, isobutyric acid, and ethylene (Bauer, 2000; Nagai, 2001). Although direct microbial production of MAA and acrylate has been proposed, acrylates

are extremely toxic to microorganisms such as *E. coli* (Todd et al., 2012; Arya et al., 2013). An alternative is a hybrid approach, whereby a microbially-derived biochemical is converted to MAA in a chemical step. For example, citramalic acid, or citramalate, can be directly converted to MAA by base-catalyzed decarboxylation and dehydration (Johnson et al., 2015). Recently, we demonstrated the microbial formation of nearly 50 g/L citramalate from renewable carbohydrates using *E. coli* (Wu and Eiteman, 2016). The key enzyme citramalate synthase coded by the *cimA* gene uses pyruvate and acetyl CoA as substrates (Fig. 5-1). An elevated concentration of intracellular acetyl CoA correlated with high citramalate formation and were associated with a deletion in *gltA* coding citrate synthase (Wu and Eiteman, 2016). Because α-ketoglutarate (i.e., 2-oxoglutarate) is a precursor for several amino acids, an active citrate synthase is required for growth on glucose as the sole carbon source (Lakshmi and Helling, 1976). Thus, *E. coli gltA* strains growing on glucose must be supplemented with an intermediate of the TCA cycle such as glutamate.

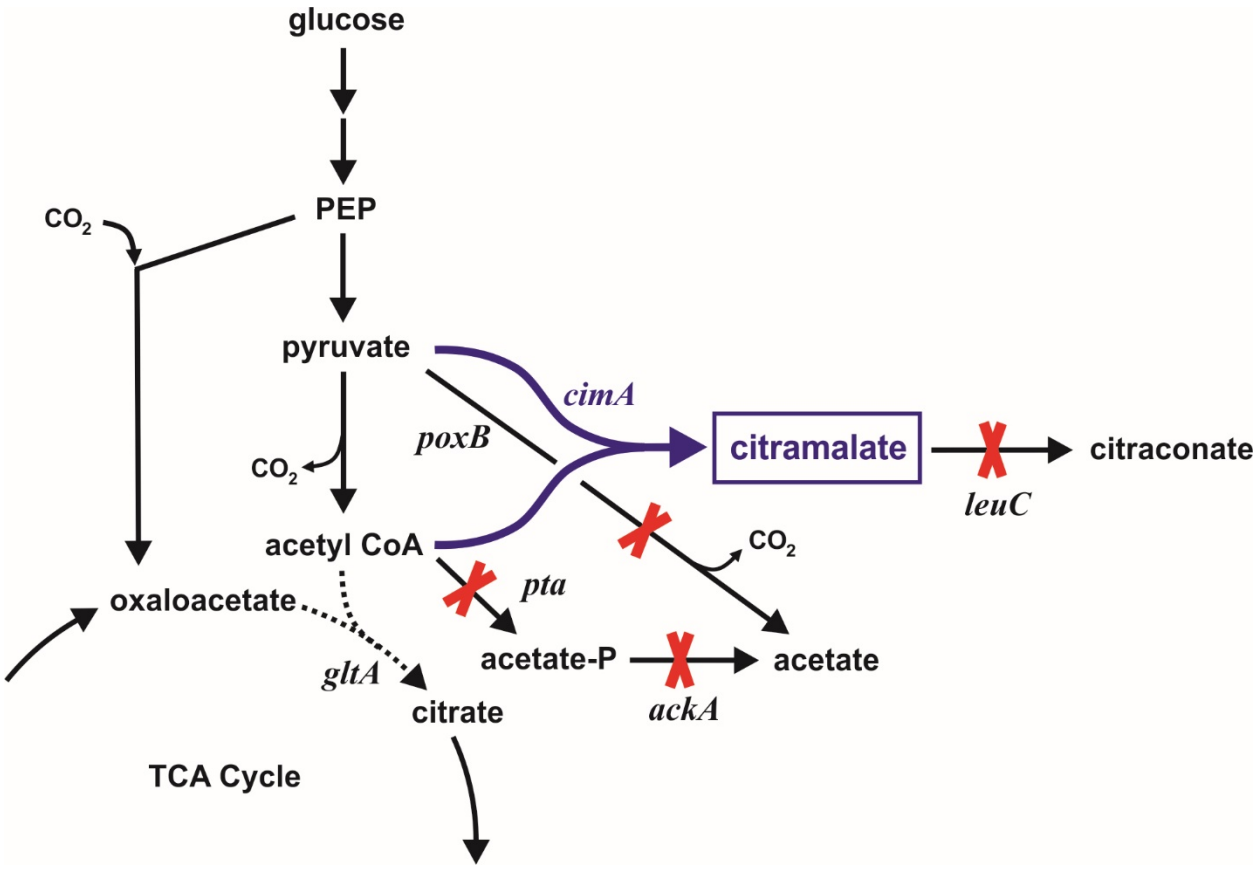

Figure 5-1. Biosynthesis of citramalate in *Escherichia coli* expressing the *cimA* gene coding citramalate synthase. Key genes which were knocked out (and coded enzymes) are: *leuC* (3-isopropylmalate dehydratase), *pta* (phosphotransacetylase), *ackA* (acetate kinase), and *poxB* (pyruvate oxidase). Point mutations were introduced into citrate synthase coded by the *gltA* gene (indicated by dotted curve) which reduced the activity of this enzyme.

Citrate synthase has been comprehensively studied, and crystal structures are established for ligand-free and ligand-bound forms of the enzyme from various organisms (Remington et al., 1982; Russell et al., 1994; Usher et al., 1995). Although the cofactor NADH does not play a direct role in the conversion, NADH is a strong and very specific allosteric inhibitor of citrate synthase, binding at a location remote from the active site (Weitzman, 1966; Weitzman and Danson, 1976). The elevated NADH concentration which normally exists at high growth rates or under anaerobic conditions at least partly explains the reduced flux through the TCA cycle under those conditions. Numerous studies on citrate synthase have explored the structure of the active sites and the NADH allosteric binding pocket (for example, Pereira et al., 1994; Stokell et al., 2003). The typical technique employed is to express and purify citrate synthases having point mutations at locations hypothesized to play a role in substrate and inhibitor binding, and then determine the effect of those mutations on kinetic parameters. Research has therefore demonstrated that H264, D362 and F383 are associated with the acetyl CoA binding pocket (Pereira et al., 1994). The effect of mutations in these or other residues in citrate synthase on *E. coli* growth and product formation has not been considered.

The goal of our study is to examine point mutations in citrate synthase to block partially the metabolic flux into the TCA cycle. We hypothesize that a reduction in citrate synthase activity would increase the intracellular pool of acetyl CoA and production of citramalate as an example product from acetyl CoA while allowing growth on glucose without supplementation of the TCA cycle intermediate glutamate.

Materials and Methods

Strain construction

Strains used in this study are shown in Table 1. The Kan antibiotic marker was first removed from MEC568 (Parimi et al., 2017) to construct MEC569 using pCP20 (Datsenko and Wanner, 2000). Then, site-directed mutagenesis of *gltA* was performed using overlap extension PCR (OE-PCR) (Braman, 2010). The *gltA* variant sequences, including the native and the mutant sequences, were amplified using the primers listed in Table 2. For the kanamycin resistance cassette insertion, PCR products were amplified with the primers Kan-For and Kan-Rev, using pKD4 as a template (Datsenko and Wanner, 2000). The OE-PCR products, which were amplified with the primers *gltA*-Up-For and *gltA*-Down-Rev, contained 500 nt of sequence identical to the target locus upstream, the variant *gltA* gene, the kanamycin resistance cassette flanked by FRT (Flp recognition target) and 500 nt of sequence identical to the target locus downstream. The entire native *gltA* gene was replaced with different variants by transforming OE-PCR products into cells expressing λ Red recombinase proteins encoded on pKD46 (Datsenko and Wanner, 2000). The native *gltA* gene was reconstructed on-site with the adjacent kanamycin resistance cassette insertion downstream. Gene replacement was selected for using kanamycin plates and then verified by PCR with the primers *gltA*-Verif-For and *gltA*-Verif-Rev. Furthermore, the sequence of the *gltA* operon was confirmed for each mutant (Eurofins Scientific, Louisville, KY, USA). The kanamycin cassette remained in the final mutant strains during the fermentation experiments. The plasmid pZE12-*cimA* containing a codon-optimized citramalate synthase was transformed into strains to examine citramalate production (Wu and Eiteman, 2016).

Growth medium

XC medium contained (per L): 5.00 g glucose, 0.20 g/L L-leucine, 1.44 g KH_2PO_4, 2.11 g K_2HPO_4, 2.00 g K_2SO_4, 3.50 g NH_4Cl, 20.00 mg $Na_2(EDTA)\cdot 2H_2O$, 0.15 g $MgSO_4\cdot 7H_2O$, 20 mg thiamine·HCl, 0.25 mg $ZnSO_4$, 0.125 mg $CuCl_2\cdot 2H_2O$, 1.25 mg $MnSO_4\cdot H_2O$, 0.875 mg $CoCl_2\cdot 6H_2O$, 0.06 mg H_3BO_3, 0.25 mg $Na_2MoO_4\cdot 2H_2O$, 5.50 mg $FeSO_4\cdot 7H_2O$, and 20 mg citric acid. The medium was supplemented with 0.2 mM IPTG initially for strains which contained the pZE12-*cimA* plasmid. Additionally, 50 mg/L ampicillin and/or 100 mg/L kanamycin were added as appropriate for plasmids/strains having antibiotic resistance.

Shake flask, batch and fed-batch processes

For measuring specific growth rate of strains (i.e., not containing the pZE12-*cimA* plasmid), cells were first grown in 3 mL Lysogeny Broth (LB) at 37°C and 250 rpm (19 mm pitch). After 12-16 h, 0.5 mL was used to inoculate triplicate 50 mL of XC medium in 500 mL baffled shake flasks growing at 37°C and 250 rpm. The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, CA, USA) was used to monitor cell growth of samples measured every 45 min. These shake flask cultures were harvested and analyzed for citrate synthase activity when the OD reached about 1. For other studies using strains harboring the pZE12-*cimA* plasmid, analogous procedures were performed except that the cultures were harvested at 24 h and analyzed for citramalate synthase activity, citramalate and intracellular acetyl CoA concentration.

For the comparison of strains under controlled bioreactor conditions, cells were grown as described above first in 3 mL LB and then in shake flasks with 50 mL XC medium. After 18 h the shake flask contents were used to inoculate a 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific Co., New Brunswick, NJ, USA) containing 1.0 L XC medium modified to contain 30 g/L glucose, 0.5 g/L leucine and 0.2 mM IPTG initially. The agitation was 400 rpm, and air was

sparged at 1.0 L/min, which maintained the dissolved oxygen above 40% of saturation. The pH was controlled at 7.0 using 30% (w/v) KOH, and the temperature was controlled at 37°C. For fed-batch processes, the medium was modified to contain 1.5 g/L NH_4Cl, 30 g/L glucose, 0.5 g/L leucine and 0.2 mM IPTG initially. Also, a solution of 300 g/L NH_4Cl was fed exponentially to maintain the cell growth rate of 0.15 h^{-1} after OD reached about 3. A 50 mL volume containing 30 g glucose and 0.5 g leucine was added four times when the glucose concentration decreased below 5 g/L. The batch and fed-batch processes were completed in duplicate.

Analytical methods

Extracellular organic acids were analyzed by HPLC using a Refractive Index detector (Eiteman and Chastain, 1997). Intracellular acetyl CoA was analyzed by the previously established method (Gao et al., 2007). For dry cell weight (DCW) measurement, three 25.0 mL samples were centrifuged (3300 × *g*, 10 min), the pellets washed by vortex mixing with 10 mL DI water and then centrifuged again. After washing three times, the cell pellets were dried at 60°C for 24 h and weighed.

Cell-free extracts were prepared using French press and used to measure citramalate synthase activity (Howell et al., 1999) and citrate synthase activity (Srere et al., 1963) separately. In both assays, free CoA was generated and then reacted with 5,5′-dithiobis(2-nitrobenzoic acid), which product can be detected at a wavelength of 412 nm. One Unit of activity is the amount of enzyme that generates one μmole of CoA in one minute at 37°C.

Results

Strain construction

Citramalate synthase (coded by the *cimA* gene) catalyzes the conversion of pyruvate and acetyl CoA to citramalate. A knockout in the *gltA* coding for citrate synthase elevated the concentration of intracellular acetyl CoA and enhanced the citramalate production in *E. coli* (Wu and Eiteman, 2016). However, L-glutamate was a required nutrient for cell growth in the *gltA* strain. In order to prevent a glutamate requirement but maintain an elevated intracellular concentration of acetyl CoA, several single-residue point mutations were introduced into the chromosomal citrate synthase of *E. coli leuC ackA-pta poxB*. Specifically, five different single point mutations associated with the acetyl CoA binding site on citrate synthase were compared (Table 1), resulting in the following strains (sequence change): MEC613 (native *gltA* reintroduced), MEC624 (F383L), MEC626 (F383M), MEC649 (F383I), MEC654 (F383V), and MEC648 (D362E).

Growth and enzyme activity in shake flasks

Using these six strains (a native *gltA*-containing strain and five with point mutations), we examined the specific growth rate using 5 g/L glucose as the sole carbon source (Fig. 5-2). Each strain differed in the citrate synthase sequence only, and all contained knockouts in the *leuC ackA-pta poxB* knockouts. The strain with the native citrate synthase (MEC613) and each strain containing a point mutation in citrate synthase was able to grow without a glutamate supplement. MEC613 attained a maximum specific growth rate of 0.42 h^{-1}. Among the point-mutation strains, MEC626 (F383M) achieved the highest growth rate of 0.31 h^{-1}, while the other four strains attained growth rates in the range of 0.13 – 0.19 h^{-1}. Citrate synthase activity measured during the exponential phase correlated closely with growth rate: MEC613 showed a citrate synthase

activity over 30 IU/g DCW, while MEC626 displayed the next greatest citrate synthase activity of 17 IU/g DCW. The citrate synthase activities were 8-10 IU/g DCW among the other four point-mutation strains. These results conclusively show that the growth rate of strains is reduced by introducing point mutations into citrate synthase.

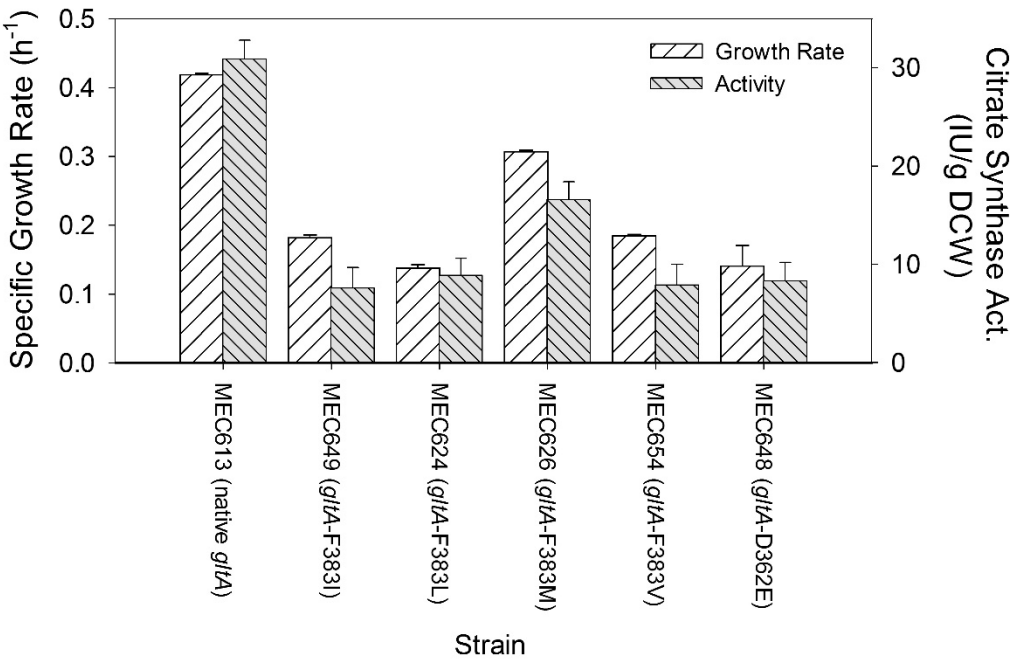

Figure 5-2. Comparison of specific growth rate and citrate synthase activity using various strains of *E. coli leuC ackA-pta poxB* containing point mutations in citrate synthase coded by the *gltA* gene. MEC613 contains the wild-type *gltA* gene. All results represent triplicate shake flask experiments.

Citramalate formation in shake flasks

We next investigated 24 h citramalate production in defined medium containing 5 g/L glucose using the *E. coli gltA*-mutant strains overexpressing citramalate synthase coded by the *cimA* gene (Fig. 5-3). MEC613/pZE12-*cimA* with native citrate synthase generated 1.72 g/L citramalate with a yield of 0.47 g/g glucose. Four of the point-mutation strains MEC624, MEC648, MEC649 or MEC654 harboring the plasmid pZE12-*cimA* each accumulated 30% – 70% less citramalate concentration than MEC613/pZE12-*cimA*. Despite the lower citramalate titer, the citramalate yields for these four mutant strains were 0.40 g/g or greater. The lower citramalate concentration is attributed to the lower growth rate (Fig. 5-2) and incomplete utilization of glucose over 24 h. For MEC626/pZE12-*cimA*, 1.81 g/L citramalate accumulated with a yield of 0.57 g/g. Despite the citrate synthase mutation (F383M) and exhibiting slower growth, these cells generated more citramalate than the strain containing the native citrate synthase in shake flasks. Citramalate synthase activity was also measured in all shake flasks studied and was not affected by citrate synthase mutation (data not shown).

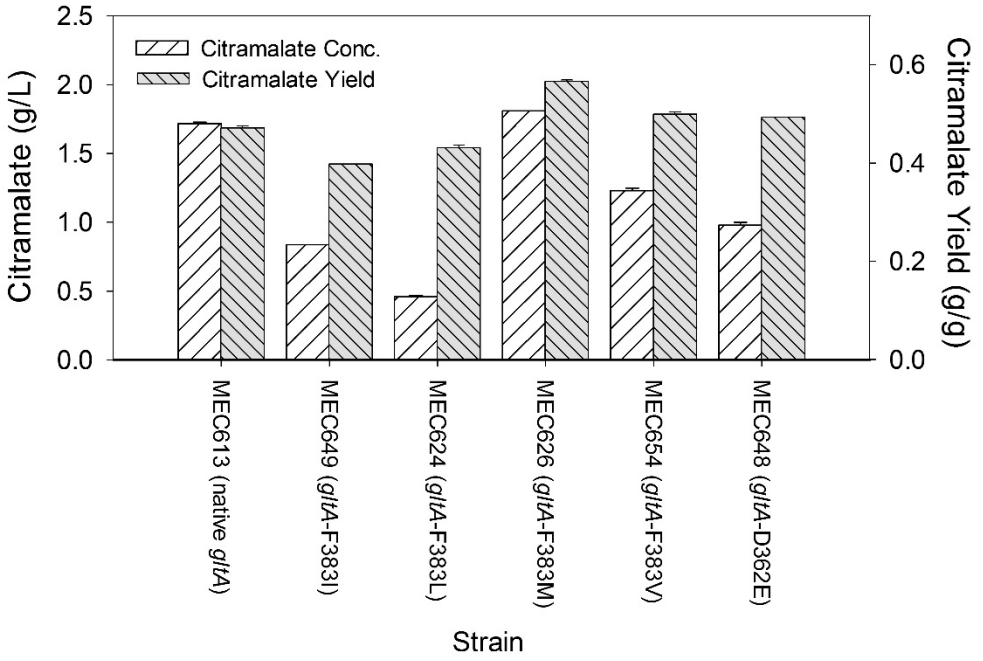

Figure 5-3. Comparison of citramalate concentration and citramalate yield from 5 g/L glucose in various strains of *E. coli leuC ackA-pta poxB* containing point mutations in citrate synthase coded by the *gltA* gene. MEC613 contains the wild-type *gltA* gene. All strains contained the pZE12-*cimA* plasmid expressing citramalate synthase and were induced initially with 0.2 mM IPTG.

Citramalate formation in controlled batch conditions

We next compared citramalate production by MEC613/pZE12-*cimA* and MEC626/pZE12-*cimA* under controlled batch conditions using defined medium with 30 g/L glucose as the sole carbon source. MEC613/pZE12-*cimA* reached an OD of 8.5 in only 18 h, and by 30 h had accumulated 4.9 g/L citramalate with no detectable acetate (Fig. 5-4). In contrast, MEC626/pZE12-*cimA* reached an OD of 7.8 in 30 h, and accumulated 11 g/L citramalate and 0.75 g/L acetate in 48 h (Fig. 5-5). During the growth of these strains, the potential by-products succinate, lactate, ethanol and pyruvate were not detected. The F383M mutation in citrate synthase more than doubled the citramalate yield on glucose from 0.16 g/g (MEC613/pZE12-*cimA*) to 0.37 g/g (MEC626/pZE12-*cimA*). Moreover, because the activity of citrate synthase was reduced but not eliminated, a TCA cycle intermediate such as glutamate was not a required component of the medium for either strain.

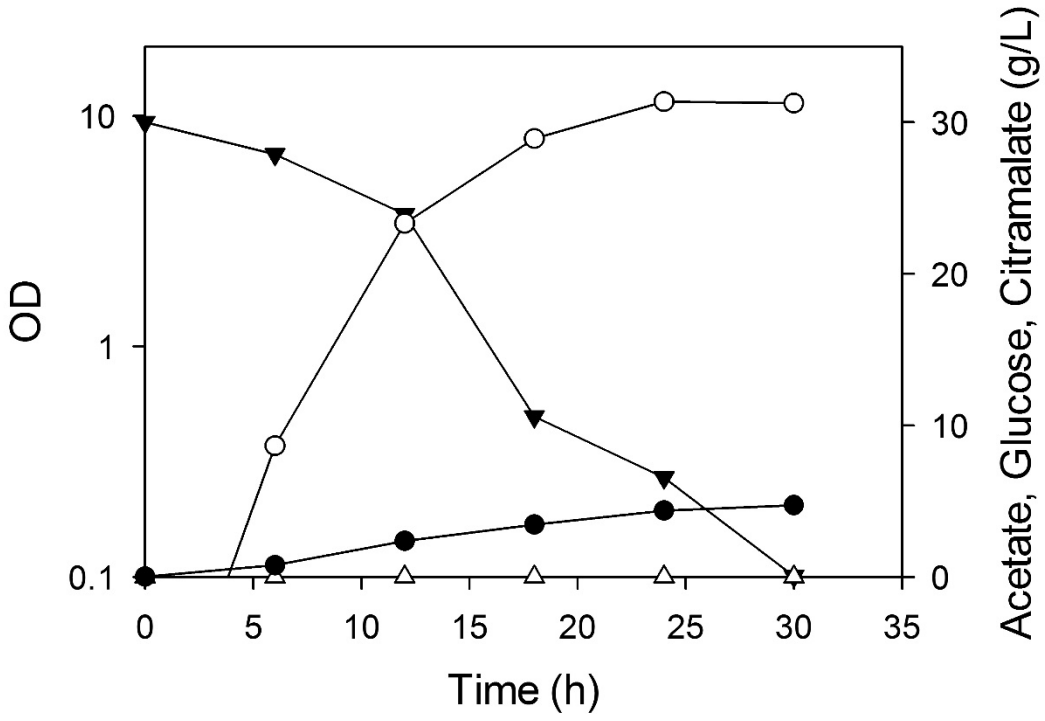

Figure 5-4. Citramalate production using 30 g/L in a 1.0 L batch fermentation with MEC613/pZE12-*cimA*: OD (○), citramalate (●), glucose (▼), and acetate (△). MEC613 contains the wild-type *gltA* gene.

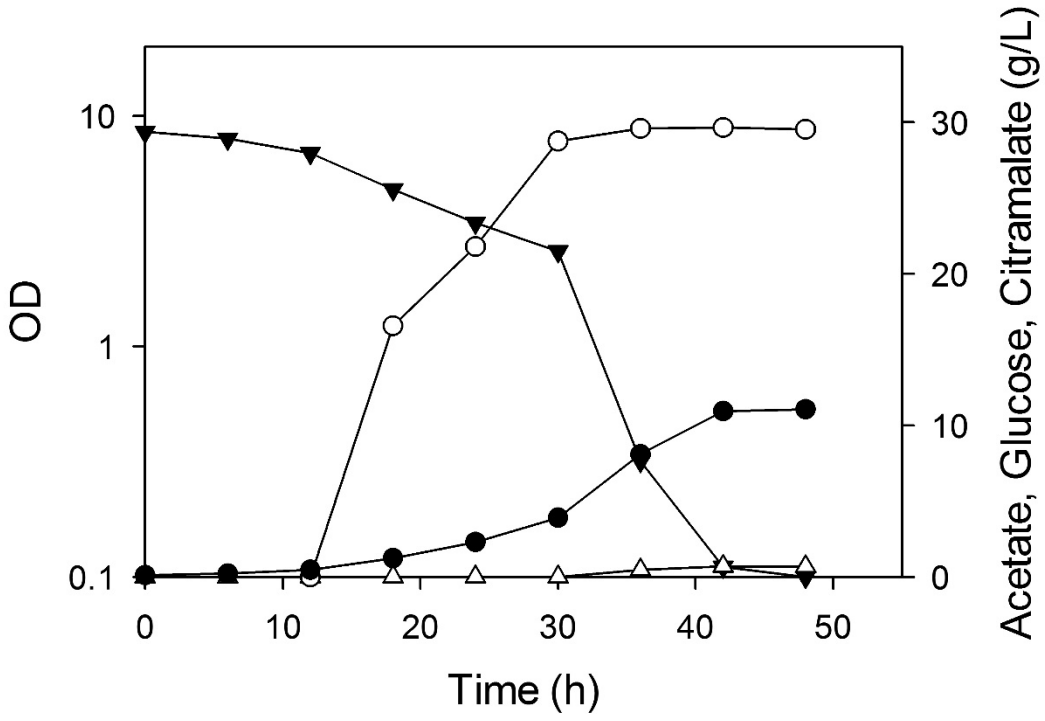

Figure 5-5. Citramalate production using 30 g/L in a 1.0 L batch fermentation with MEC626/pZE12-*cimA*: OD (○), citramalate (●), glucose (▼), and acetate (△). MEC626 contains the F383M point mutation in the *gltA* gene.

Exponential fed-batch fermentation

To increase the citramalate titer and yield, we designed a fed-batch experiment with an exponential feed strategy using nitrogen (NH_4Cl) as the growth limiting resource. Additionally, approximately 30 g glucose was added four times to the fermenter when the glucose concentration decreased below 5 g/L. For these fed-batch processes the OD reached 32 within 42 h at which time the citramalate concentration was 13.5 g/L (Fig. 5-6). After 132 h, the citramalate concentration reached an average of 60 g/L with a yield of 0.53 g/g glucose. Despite the *ackA-pta poxB* gene deletions and the aerobic conditions, 3.1 g/L acetate and 1.3 g/L lactate were formed as by-products (latter not shown).

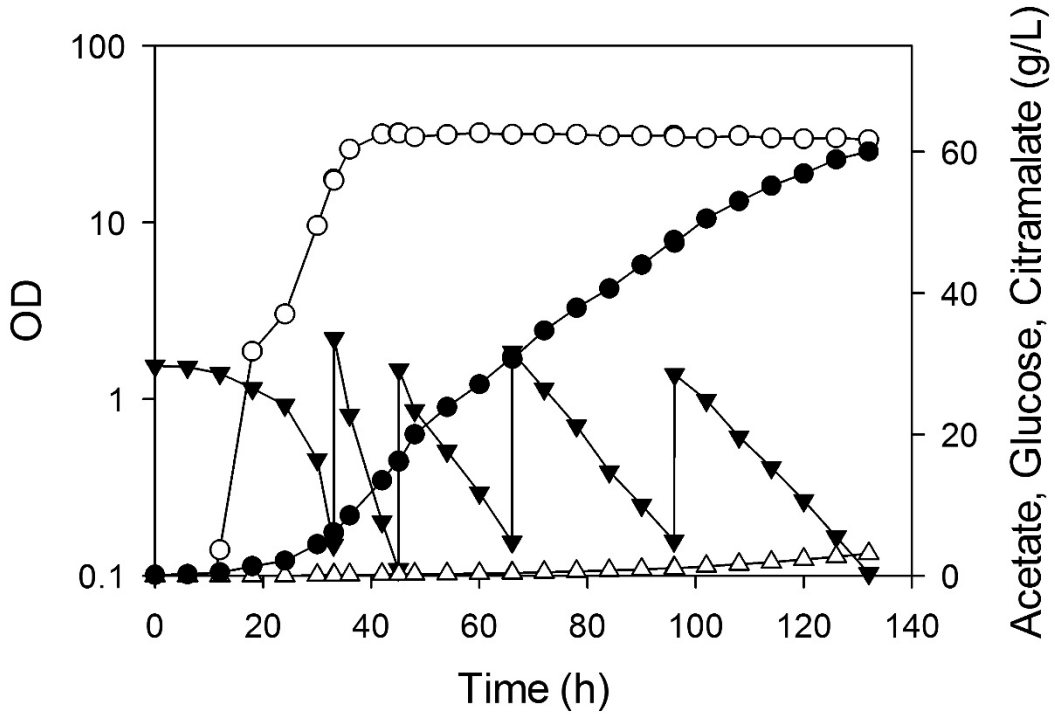

Figure 5-6. Citramalate production using 30 g/L in a 1.0 L fed-batch fermentation with MEC626/pZE12-*cimA*: OD (○), citramalate (●), glucose (▼), and acetate (△). Approximately 30 g glucose was added four times to the fermenter when the glucose concentration decreased below 5 g/L. MEC626 contains the F383M point mutation in the *gltA* gene.

Discussion

This study demonstrates that citramalate, a chemical precursor to the commodity chemical MAA, will accumulate from glucose as sole carbon source by *E. coli* overexpressing citramalate synthase coded by the *cimA* gene. In order to reduce the acetyl CoA flux towards the TCA cycle as well as maintain cell growth without the addition of glutamate, protein engineering of *E. coli* citrate synthase was investigated. Specifically, several point mutations were examined to residues in the acetyl CoA binding pocket, with the ultimate goal of decreasing the conversion of acetyl CoA into citrate. All citrate synthases studied did not completely eliminate acetyl CoA entering the TCA cycle, and hence allowed cell growth in the defined medium containing glucose as sole carbon source.

All the 5 citrate synthase mutations we investigated showed lower enzyme activities, and the cells carrying those mutations grew significantly slower than the control strain having the reintroduced native citrate synthase. The lower acetyl CoA flux entering the TCA cycle caused by the mutations resulted in slower generation of metabolites, energy and CO_2. Similar results have been reported by Quandt et al. (2005). In their study, several single point citrate synthase mutations were isolated during the Lenski long-term evolution experiment. Both A162V and A124T substitution increased the K_m value for acetyl CoA and reduced citrate synthase activity, and therefore significantly decreased the cell growth rate.

Since citrate synthase plays a pivotal role in the central metabolism, manipulating its overall activity has been studied previously in several different ways. For example, native citrate synthase overexpressed in *Corynebacterium glutamicum* drives more carbon towards succinate biosynthesis, increasing the succinate yield and decreasing the pyruvate yield (Zhu et al., 2013). Alternatively, in L-lysine production by *C. glutamicum*, the citrate synthase activity was

dampened by switching *gltA* promoter (van Ooyen et al., 2012) or engineering lysine riboswitches (Zhou and Zeng, 2015). Recently, Heo et al. (2017) demonstrated that controlling citrate synthase expression by CRISPR/Cas9 genome editing effectively enhanced the *n*-butanol production in *E. coli*. However, these studies each involved modulation of the quantity of active enzyme present, not the enzyme's properties such as substrate affinity. In our study, the protein engineering of citrate synthase revealed as a powerful tool for altering kinetic parameters of acetyl CoA, and therefore redistributing carbon flux. Citramalate production also benefitted as cells grew on glucose as sole carbon source.

In conclusion, the engineering of citrate synthase *E. coli* is very effective at modulating the flux of carbon into the TCA cycle. This diminished flux allows greater formation of compounds derived from acetyl CoA such as citramalate. This strategy also avoids requiring a TCA cycle intermediate such as glutamate as a medium supplement. Further metabolic engineering could similarly lead to a strain capable of growth without leucine. Improvements in reducing equivalents and cofactor balance and developing techniques to maintain plasmid or gene integration into the genome will all likely refine and enhance upon the citramalate production in *E. coli*.

Acknowledgments

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Table 1. Strains used in this study.

Strain	Genotype	Ref
MEC568	MG1655 Δ*leuC*778::(FRT) Δ(*ackA*778-*pta*-779)::(FRT) Δ*poxB*772::Kan Δ*gltA*770::(FRT)	Parimi et al., 2017
MEC569	MG1655 Δ*leuC*778::(FRT) Δ(*ackA*778-*pta*-779)::(FRT) Δ*poxB*772::(FRT) Δ*gltA*770::(FRT)	This study
MEC613	MG1655 Δ*leuC*778::(FRT) Δ(*ackA*778-*pta*-779)::(FRT) Δ*poxB*772::(FRT) Δ*gltA*770::*gltA*-Kan	This study
MEC624	MG1655 Δ*leuC*778::(FRT) Δ(*ackA*778-*pta*-779)::(FRT) Δ*poxB*772::(FRT) Δ*gltA*770::*gltA*-F383L-Kan	This study
MEC626	MG1655 Δ*leuC*778::(FRT) Δ(*ackA*778-*pta*-779)::(FRT) Δ*poxB*772::(FRT) Δ*gltA*770::*gltA*-F383M-Kan	This study
MEC648	MG1655 Δ*leuC*778::(FRT) Δ(*ackA*778-*pta*-779)::(FRT) Δ*poxB*772::(FRT) Δ*gltA*770::*gltA*-D362E-Kan	This study
MEC649	MG1655 Δ*leuC*778::(FRT) Δ(*ackA*778-*pta*-779)::(FRT) Δ*poxB*772::(FRT) Δ*gltA*770::*gltA*-F383I-Kan	This study
MEC654	MG1655 Δ*leuC*778::(FRT) Δ(*ackA*778-*pta*-779)::(FRT) Δ*poxB*772::(FRT) Δ*gltA*770::*gltA*-F383V-Kan	This study

Table 2. Primers used in this study.

Primer	Sequence
gltA-Up-For	5′-TCATGCAAAACACTGCTTCCAGATG-3′
gltA-D362E	5′-AGAGTAGAATTCGACGTTCGGGTACAG-3′
	5′-TGTACCCGAACGTCGAATTCTACTCTG-3′
gltA-F383I	5′-CGTGCCATTGCAATAATGACGGTGAAC-3′
	5′-GTTCACCGTCATTATTGCAATGGCACG-3′
gltA-F383L	5′-GCCATTGCCAGAATGACGGTGAACATG-3′
	5′-CCGTCATTCTGGCAATGGCACGTACC-3′
gltA-F383M	5′-GTGCCATTGCCATAATGACGGTGAACATG-3′
	5′-CGTCATTATGGCAATGGCACGTAC-3′
gltA-F383V	5′-GTGCCATTGCCACAATGACGGTGAAC-3′
	5′-CGTCATTGTGGCAATGGCACGTAC-3′
gltA-Bot-R	5′-GAAGCAGCTCCAGCCTACACCAACTTAGCAATCAACCATTAACGC-3′
Kan-For	5′-GCGTTAATGGTTGATTGCTAAGTTGGTGTAGGCTGGAGCTGCTTC-3′
Kan-Rev	5′-CATATGAACGGCGGGTTAAAATATTTAATGGGAATTAGCCATGGTCCATATG-3′
gltA-Down-For	5′-CATATGGACCATGGCTAATTCCCATTAAATATTTTAACCCGCCGTTCATATG-3′
gltA-Down-Rev	5′-GTTGTCGTGACTTGTCCAAGATCTATG-3′
gltA-Verif-For	5′-ACTACGGGCACAGAGGTTAACTTTC-3′
gltA-Verif-Rev	5′-CTGCCTCGTCCTGCAGTTCATTC-3′

CHAPTER 6

CONCLUSION

The fundamental goal of this research was to understand the microbial metabolism and develop fermentation process to maximize the citramalate production. The intracellular concentration of acetyl CoA was demonstrated to be a critical aspect for accumulating citramalate, though it is tightly regulated in wild-type *E. coli*. Therefore, enzymes catalyzing the catabolism of acetyl CoA, especially citrate synthase coded by *gltA*, were deleted to achieve high product yield and final titer, which was demonstrated in Chapter 2. The elevated intracellular acetyl CoA pools led to high by-product formation as acetate. By knocking out *ackA-pta* and *poxB*, the citramalate production was further improved by minimizing the acetate formation as illustrated in Chapter 3. One undesirable effect of deleting *gltA* is the supplement of glutamate in the medium to support the growth of the engineered *E. coli* strains. Protein engineering of the *E. coli* citrate synthase (F383M) recovered the growth as well as facilitated citramalate production without the addition of glutamate, which was elucidated in Chapter 5. These results revealed promise that citramalate could be converted from renewable resouces such as crude glycerol and ultimately be used for the methacrylate synthesis.

Although high citramalate concentrations and yields have been achieved, further improvement could be made. Citramalate synthase can be screened for a higher conversion rate, considering that this enzyme was isolated from thermophilic archaea. As illustrated in the protein engineering of citrate synthase, a similar protein modification of 3-isopropylmalate dehydratase might release the demanding of leucine addition in the medium for cell growth.

When citramalate accumulates to high titers in the bioreactor, significant amount of base solution, such as NaOH or KOH, is added to adjust the pH. Due to the limited tolerance of *E. coli* to osmotic stress, further study of seeking alternative host strains with high salt tolerance or low pH might benefit the overall citramalate production as well as improve the downstream purification process.

In this study, citramalate synthase was overexpressed and the *cimA* gene was carried on the plasmid. The stability of plasmid replication and the addition of antibiotics would compromise this approach at large scale in the industrial. This issue can also be addressed by seeking an alternative host strain or expression system.