Acyl-CoA quantification and the effects upon *E. coli* polyketide substrates through over-expression of native and *Ralstonia solanacearum* propionyl-CoA synthetases

A thesis

submitted by

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Abstract:

Metabolomics has become an increasingly important methodology for analyzing perturbations in biological systems along with the more established proteomics and genomics tools currently available today. The study of small molecule metabolites has been described as "the metabolic complement of functional genomics" (Villas Boas 2005), and can provide a snapshot of the complex phenotypic states of cellular systems. Metabolic studies have been mainly split into two major groups, global metabolite profiling or targeted metabolite analysis.

This study utilizes targeted metabolite analysis to allow direct quantification of small molecules of interest, which can give a snapshot of dynamic metabolic flux and help characterize genetic modifications. In particular, the short chain acyl-CoA class of metabolites, used as building blocks for the production of polyketides, was studied. The acyl-CoA levels in several engineered *Escherichia coli* strains constructed for improved heterologous polyketide production were quantified using LC-MS/MS. It was observed upon feeding propionate that the engineered *E. coli* strains had increases in both propionyl- and methylmalonyl-CoA of ~6- to 30-fold and ~3.7- to 6.8-fold respectively. The observed increases in acyl-CoA levels reflect the genetic modifications designed for improved polyketide production and correlate with the previously observed titer improvements in 6-deoxyerythronolide B (the macrolactone precursor of the potent polyketide antibiotic erythromycin) (Zhang et al. 2010, Pfeifer et al.2001).

To further improve the levels of available acyl-CoA molecules, a flexible propionyl-CoA synthetase gene from *Ralstonia solanacearum* (*prpE-RS*) was cloned and expressed in the engineered strain BAP1 (Rajashekhara et al 2004, Pfeifer et al 2000). Rajashekhara et al. demonstrated substrate flexibility of the PrpE-*RS* enzyme in vitro, which should produce an increase in propionyl-, acetyl-, and butyryl-CoA when overexpressed in *Escherichia coli*. Induction of the *prpE-RS* gene resulted in ~1.5-, 15-, and 8.5-fold increases in acetyl-, butyryl-, and propionyl-CoA, respectively, when fed with corresponding substrates. However when compared to the empty vector control, no significant increases in acyl-CoA levels were observed, indicating that the substrate flexibility observed may be a result of the native PrpE enzyme rather than the heterologously expressed PrpE-*RS* enzyme. To confirm this observation, further experiments comparing both the native and heterologous PrpE enzymes were conducted.

Additionally, the propionate transporter AtoAD was expressed with PrpE-*RS* resulting in a 1.44- and 1.34-fold increase in butyryl- and acetyl-CoA, but no significant increase in propionyl-CoA. As a result, the introduction of the flexible PrpE-*RS* and propionate transporter AtoAD did not significantly improve the acyl-CoA levels in *Escherichia coli*. To further test the availability of alternative acyl-CoA substrates observed for polyketide biosynthesis, attempts were made to quantify the production of 6-dEB, acetyl-, and butyryl-6dEB analogs (14-nor-6dEB, 15-Me-6dEB), but no significant improvements in analog production were observed.

Overall, it was observed that the native PrpE in *E. coli* demonstrates an intrinsic substrate flexibility resulting in increased acetyl-, propionyl-, and butyryl-CoA levels. This study developed a platform for acyl-CoA quantification using LC-MS/MS, helping to metabolically characterize several engineered polyketide producing *E. coli* strains. The observed improvements in acyl-CoA levels and apparent flexibility of the native PrpE enzyme provide insight into the genetic modifications that may optimize polyketide production and help direct future engineering approaches for polyketide producing *E. coli* systems.

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1. Background and Objectives

1.1. Metabolomics

Global metabolite analysis poses a challenging analytical problem, mainly due to the vast array of chemically diverse metabolites found in cellular systems and the low intracellular concentrations available upon extraction. Additionally, the rapid turnover rate and low concentrations of particular intracellular metabolite pools requires rigorous methods for extraction, quenching, and analysis to obtain a more complete and accurate snapshot of interesting dynamics. In fact, of the estimated 300 mmol l^{-1} metabolite pool of *Escherichia coli*, a large portion is composed of only a small number of metabolites, leaving many classes representing <~1% of the total pool (Bennett et al 2009, Buccholz et al 2001). Included in the <1% is the interesting metabolite family of acyl-CoAs, used in a variety of metabolic processes and serving as building blocks for valuable secondary metabolites. In particular, the acyl-CoAs serve as building blocks for the important polyketide class of natural products. Polyketide compounds exhibit a broad range of therapeutic value and serve as worthwhile targets for metabolic engineering. The analytical challenge of metabolite quantification serves as a motivating "complement to functional genomics" and provides a dynamic snapshot of the metabolic state (Villas-Boas 2005). This can provide insight into metabolic engineering modifications of industrially relevant hosts and help direct future approaches. With improvements in analytical techniques and sample extraction methods, metabolomics may serve as a necessary component of characterizing strains metabolically engineered for polyketide formation.

While global metabolite analysis utilizes methods able to cover the vast array of metabolite classes at once, targeted metabolite analysis provides an apt platform for testing hypothesis driven studies that focus on only a specific class of important metabolites, and can generate a more practical and directed means of focusing on the effects of isolated metabolic perturbations or genomic modifications. Additionally, targeted metabolite approaches help direct the analytical techniques and sample methods optimized for the particular compounds of interest. Much work is still being done to optimize and individualize extraction methods and analytical protocols for each metabolite class, but methods have been developed for a broad range of metabolites and host systems. Each developed method seeks to allow for rapid, repeatable, and accurate snapshots of metabolic flux. In comparison to the more developed proteomic and genomic realms of analytical technology, metabolomics has not defined a "standard" means of metabolite extraction or analysis (Villas-Boas et al), and a range of problems exist that require careful attention and optimization. Beyond the low levels of intracellular metabolites of interest, the extraction procedures currently in use often dilute the sample below the limit of detection. This is particularly true with the intracellular volume less than 3% of the total sample volume for most methods (Buccholz 2001), and confirms the need for selective and sensitive analytical methods, such as LC-MS/MS. The Standard Metabolic Reporting Structures group established in 2003 has outlined recommendations for standardizing the reporting of metabolic analyses and should improve future work in the field. Several attempts have been made to optimize extraction and analysis methods for particular classes, as well as the entire metabolome of select organisms (Yang 2009, Wurm 2010, Bennett 2009, Yanes 2011. One example suggests

the use of microfluidics technologies for high-throughput screening to account for the dilution of samples (Wurm 2010), while other studies focus on improving sample processing, extraction, separation, and detection for broader identification of small molecules (Zhou et al. 2011 , Wang et al. 2011).

1.2. Short chain acyl-CoAs

Using a targeted metabolite approach, several studies have sought to quantify both short and long chain acyl-CoA molecules to better understand the effects of various genetic or nutrient limited perturbations. These studies include the dynamics of varying carbon sources in bacterial culture (Yang 2009), measuring enzyme regulation (Boynton 1994), the production of biopolymeric materials (Valentin 2000), and polyketide formation and analysis (Park 2007, Se Jong 2001).

The current study focuses on the short chain acyl-CoA molecules utilized as building blocks for the production of polyketide products, most specifically with engineered *Escherichia coli* hosts used for the production of 6-deoxyerythronolide B (6dEB), the macrolactone precursor to the antibiotic erythromycin. The modular biosynthesis of 6dEB requires one propionyl-CoA and six (2*S*)-methylmalonyl-CoA molecules, with additional modifications and glycosylations to produce the full erythromycin product (Figure 1). The effect of propionate was used as a supplement to the culture media as it was found previously to increase 6dEB production (Zhang BB 2009), and additionally serves as a substrate for production of both propionyl- and (2*S*)-methylmalonyl-CoA. *E. coli* does not natively produce (2*S*)-methylmalonyl-CoA and

requires genetic modifications to carboxylate propionyl-CoA to (2S)-methylmalonyl-CoA, namely with a propionyl-CoA carboxylase (PCC). Additionally, though natively a producer of propionyl-CoA, *E. coli* requires modifications to improve propionyl-CoA availability for 6dEB production (Pfeifer et al 2001), which can be accomplished through a number of routes aimed at redirecting propionate flux. Of the strains tested in this study, improvements in 6dEB production had been previously tested, but little research has been aimed at analyzing the modifications on a metabolic level. The genetic modifications analyzed include: (1) knockouts of ygfH, which catalyzes the conversion of propionyl-CoA to propionate, (2) overexpression of the native PrpE enzyme in *E. coli* and a heterologous PrpE enzyme from *Ralstonia solanacearum*, which converts propionate to propionyl-CoA with some substrate flexibility, (3) heterologous expression of a propionyl-CoA carboxylase from *Streptomyces coelicolor* which carboxylates propionyl-CoA to (2*S*)-methylmalonyl-CoA, and (4) additional hosts with the expression of the modular DEBS genes required from 6dEB biosynthesis (Table 1, Figure 3).

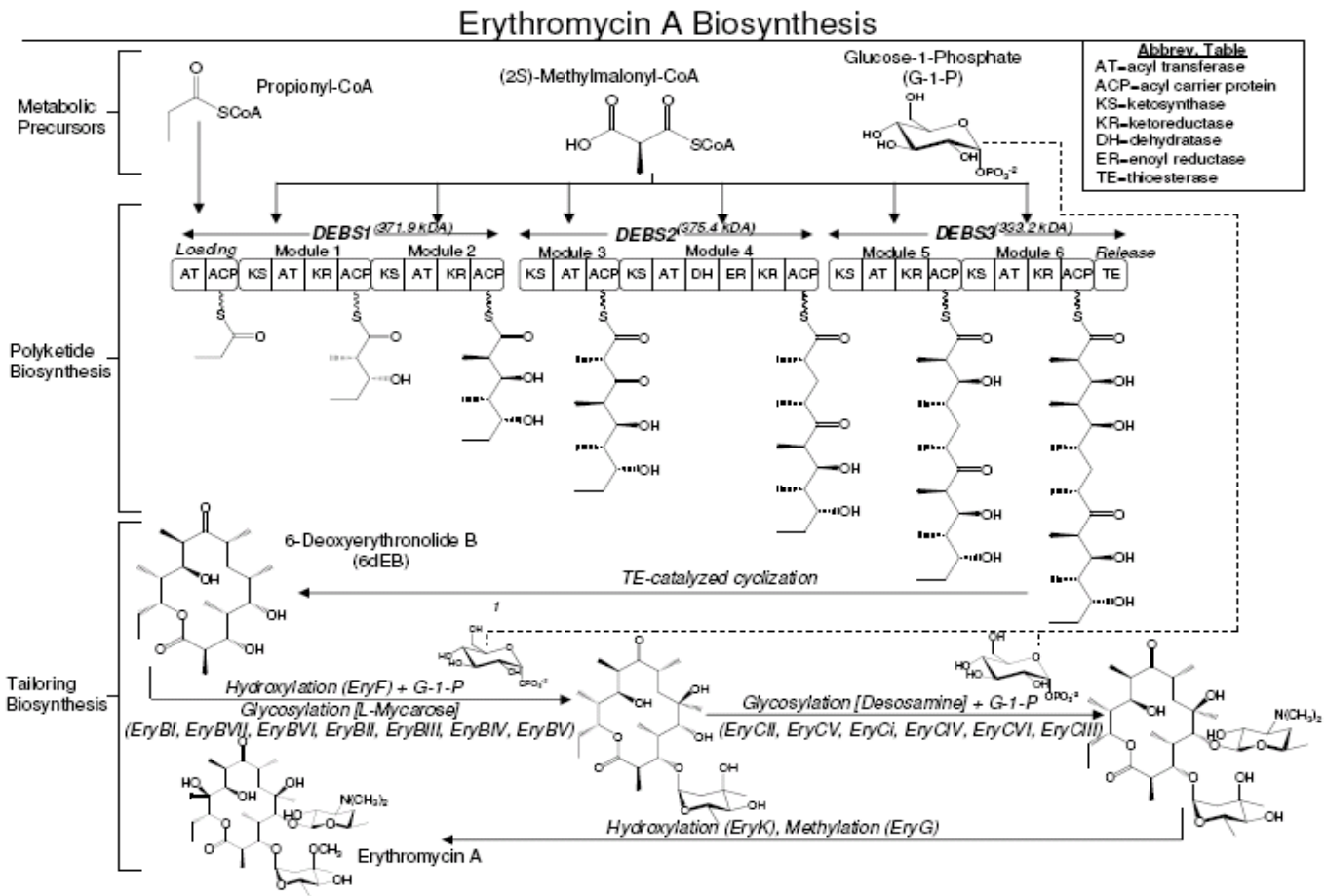


Figure 1. Biosynthesis of erythromycin A featuring the 6dEB molecule

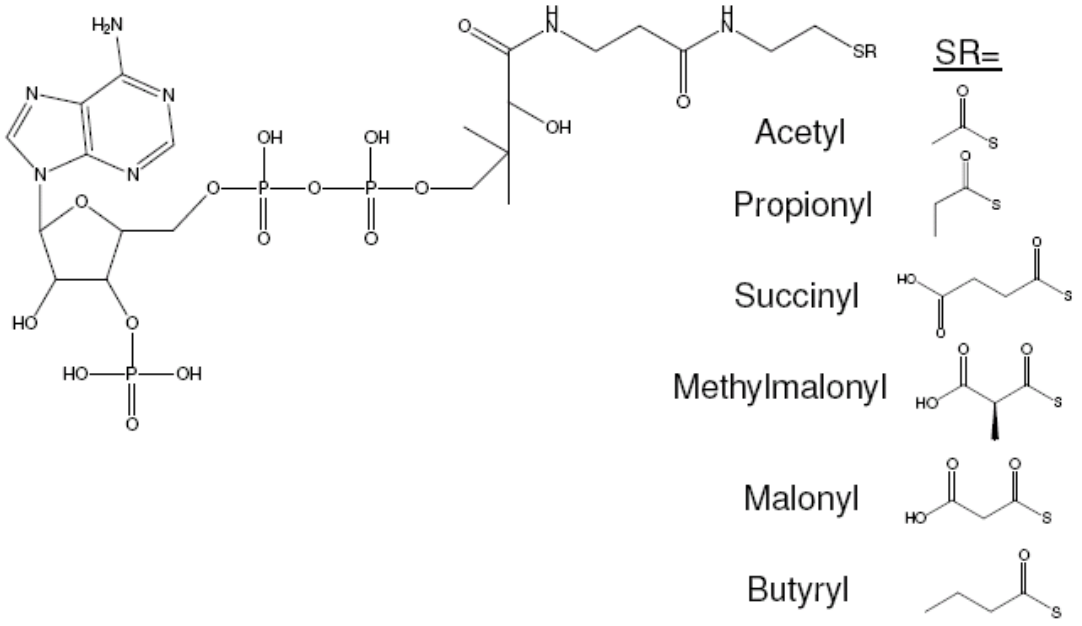


Figure 2. Family of acyl-CoA molecules analyzed in this study. All acyl-CoAs are composed of the large highly hydrophilic CoA backbone, with modifications at the acyl-thioester domain differentiating each acyl-CoA.

1.3 Acyl-CoA detection

To analyze similar short-chain acyl-CoA molecules, liquid chromatography and tandem mass spectrometry has been the most successful analytical tool, as it is readily accessible for analyzing an array of diverse chemical classes, does not require derivatization, and provides high levels of selectivity and sensitivity. While NMR has additionally become a popular method for metabolomic studies, it suffers from low sensitivity, and is not as suitable for the detection of low concentrations of acyl-CoAs, while GC-MS often requires cumbersome derivatization procedures to analyze non-

volatile metabolites. Mass spectrometry has become the dominant means of metabolite analysis particularly with the advent of soft ionization techniques including APCI and ESI. Both techniques allow the detection of polar, ionic, and neutral metabolites extracted with volatile solvents from complex mixtures. The sensitivity and selectivity provided by a paired chromatography and tandem mass spectrometry technique further improves the analysis of complex extraction mixtures, particularly with very low sample concentrations. Although not as mature a technique as gas chromatography paired MS, the compatibility of liquid chromatography with developed extraction procedures has provided success for several previous studies (Villas-Boas 2005) and continues to be the analytical method of choice. Paired LC-MS/MS is particularly powerful for short chain acyl-CoA analysis as the class has the same hydrophilic CoA core which makes reverse phase separation a challenge (Figure 2), and an orthogonal analytical selectivity is often desired. The key to C18 reverse phase separation lies in the small acyl portion of the molecule. But the orthogonal selectivity of mass spectrometry allows quantification without an optimized separation of each acyl-CoA. This study ensured separation of all acyl-CoAs of interest (Figure 4), as both succinyl- and methylmalonyl-CoA have the same parent and product ion pairs (m/z) in positive ion mode.

1.4 Project Objectives

A broad range of hosts were tested to determine the acyl-CoA profile of five acyl-CoA molecules including acetyl-, propionyl-, butyryl-, methylmalonyl-, and malonyl-CoA. Testing and comparing the acyl-CoA profiles of previously engineered host strains served as a means of comparing both the genetic modifications present in each host and to better

estimate and understand, on a metabolic level, the optimal host for polyketide production (Figure 3).

To help improve and expand the acyl-CoA substrate pools, and help alleviate a potential substrate bottleneck in polyketide biosynthesis, a heterologous propionyl-CoA synthetase was expressed in the engineered strain BAP1. Propionyl-CoA synthetase enzymes (PrpE) belong to the acyl-CoA synthetase family that catalyze the formation of acyl-CoA molecules from acid substrates. Propionyl-CoA synthetases convert propionate to its -CoA thioester derivative, with varying enzymatic efficiencies and, more interestingly, substrate flexibilities. Rajashekhara et al. characterized the atypical kinetics of PrpE enzymes from both *Ralstonia solancearum* and *Salmonella choleraesuis* indicating substrate flexibility of each enzyme. Both PrpEs were able to catalyze acetyl-, propionyl-, butyryl-, and acrylyl-CoA molecules in vitro, with the greatest specificity for propionate. Pfeifer et al discovered that elevated levels of propionyl-CoA and (2*S*)-methylmalonyl-CoA may improve natural product production in *E. coli* and over-expression of the native *prpE* gene and a propionyl-CoA carboxylase gene from *Streptomyces coelicolor* was demonstrated to improve production of 6dEB. This study attempts to quantify the effects on the acyl-CoA substrate pool of several genetic modifications as described in Table 1, and additionally to test the heterologous expression of the PrpE enzyme from *Ralstonia solanacearum* to diversify and increase the acyl-CoA substrate pool even further, allowing a metabolic view of improvements for 6dEB and 6dEB analog production in the industrial host, *E. coli*.

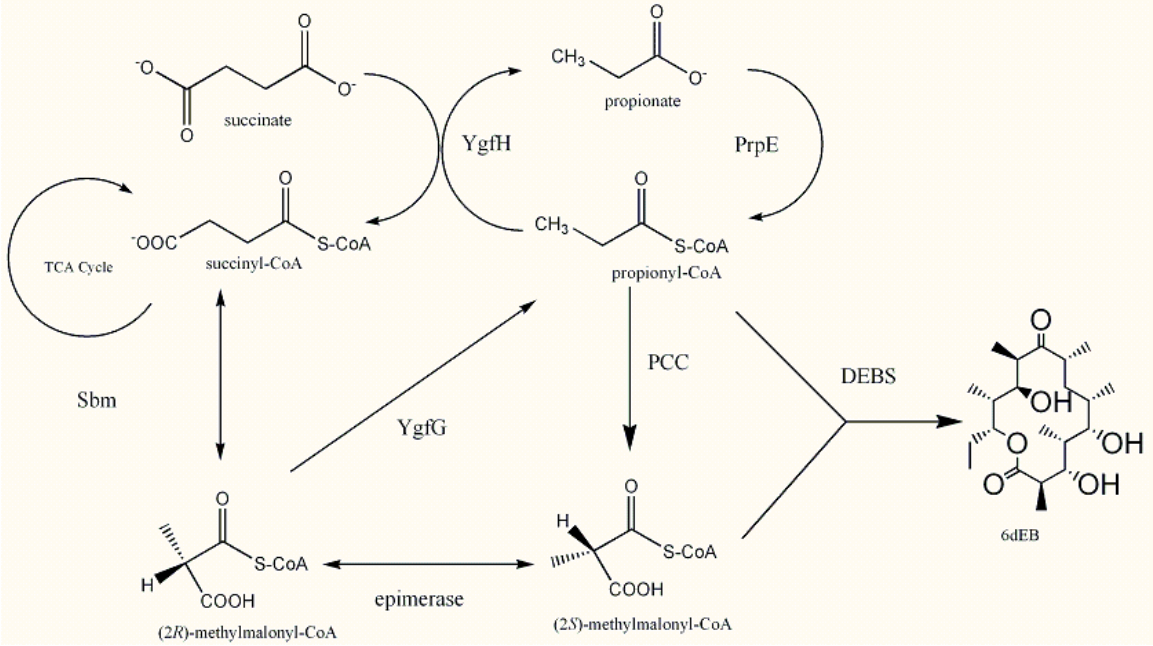


Figure 3. Summary of reactions and enzymes involved in the pathway to 6dEB biosynthesis.

2. Materials and Methods

2.1 Bacterial Strains and Plasmids

The *Escherichia coli* strains used in this study contained several genetic modifications designed to improve the production of 6dEB by increasing the intracellular level of available acyl-CoA substrates. A summary of the strains used in this study and previous literature in which the production of 6dEB was analyzed are listed in Table 1. Additionally, Figure 3 summarizes a simplified metabolic pathway highlighting the enzymes over-expressed or knocked out.

For this study, plasmid pJexpress411-*prpE*-RS was constructed by DNA 2.0 (Menlo Park, CA) based upon the NCBI reference sequence number NP_519639.1, corresponding to the propionyl-CoA synthetase from *Ralstonia solanacearum* (PrpE-*RS*). The *prpE-RS* gene from pJexpress411-*prpE*-RS was subcloned using *NdeI* and *XhoI* into the MCS2 of pACYCDuet-1 (Novagen) to be selectable/compatible with plasmids pBP130 and pBP144 required for 6dEB production (Pfeifer 2001). All transformations, SDS-PAGE analysis, and other standard molecular biology techniques were carried out as described by Sambrook *et al*. (1989).

Strain	***Source***	***Modification***
BAP1	(Pfeifer 2001)	BL21(DE3), Δ*prpRBCD*: $T7_{prom}$-*sfp*-$T7_{prom}$-*prpE*
BAB2	(Boghigian 2011)	BAP1; Δ*sbm-ygfDGH*::FRT
TB3	(Zhang et al 2009)	BAP1; Δ*ygfH*::*kan*
YW22	(Boghigian 2011)	JM109(DE3); *araA*::$T7_{prom}$-*dxs*-$T7_{term}$-$T7_{prom}$-*idi*-$T7_{term}$-$T7_{prom}$-*ispDF*-$T7_{term}$
BL21(DE3)	Novagen	F^- *ompT hsdSB* (r_B^-, m_B^-) *gal dcm* λ(DE3)
Plasmid		
pJexpress-*prpE-RS*	(DNA 2.0)	$T7_{prom}$-*prpE-RS*-$T7_{term}$; pJexpress411 [Kan-resistant]
pJWA1	(this study)	$T7_{prom}$-*prpE-RS*-$T7_{term}$; pACYCDuet-1 [Cm-resistant]
pACYCDuet-1	(Novagen)	[Cm-resistant]
pAtoAD	(Boghigian, Park)	AtoA, AtoD in MCS1 and MCS2 of pACYCDuet-1 respectively [Cm-resistant]
pBP130	(Pfeifer 2001)	*bla ; T7$_{prom}$-eryA2-eryA3*-$T7_{term}$
pBP144	(Pfeifer 2001)	*kan:* $T7_{prom}$-*pccB-accA1*-$T7_{prom}$-*eryA1*-$T7_{term}$

Table 1. Strains and plasmids used in the study.

2.2 Culture Conditions

Strains were made electrocompetent and transformed with pJexpress411-*prpE-RS*, pJWA1, pACYCDuet-1, pBP130 and pBP144, or a combination of each; resulting transformants were stored as glycerol stocks at -80°C. Analytical experiments began with 3 ml cultures inoculated from glycerol stocks and grown overnight at 37°C and 250 rev min^{-1} in LB medium with appropriate antibiotics (included at 34 [chloramphenicol], 50 [kanamycin], and 100 [carbenicillin] μg ml^{-1}). The overnight cultures were used to inoculate 15 ml LB medium in baffled Erlenmeyer flasks supplemented with 20 mmol l^{-1} sodium acetate, sodium propionate, or sodium butyrate, and induced with 100 μmol l^{-1} IPTG as indicated. Cultures were inoculated to an OD_{600nm} = 0.1 in triplicate. The production cultures were allowed to incubate at 22°C and 250 rev min^{-1} for 24 hours before extraction and subsequent LC-MS/MS analysis. Cell density was measured spectrophotometrically at 600 nm prior to extraction.

For 6dEB production, an additional 2 mmol l^{-1} sodium propionate was used to supplement sodium acetate and sodium butyrate feeds to supply the necessary propionyl-CoA and (2*S*)-methylmalonyl-CoA precursors. Cultures were inoculated to an OD_{600nm} = 0.1 in triplicate. The production cultures were allowed to incubate at 22°C and 250 rev min^{-1} for 72 hours before extraction and subsequent LC-MS/MS analysis. Cell density was measured spectrophotometrically at 600 nm prior to extraction.

2.3 Extraction Procedure

A sample (3 ml) of the final culture was centrifuged for 10 min at 3000 rev min^{-1} and 4°C. Pellets were resuspended in 1 ml ddH_2O and centrifuged for 1 min at 10,000 rev min^{-1} and 4°C. The resulting cell pellet was subjected to a cell lysis and extraction protocol adapted from Bennett et al (2009). Pellets were rapidly quenched and extracted to a final volume of 130 µl with 45:45:10 acetonitrile:methanol:H_2O + 0.1% glacial acetic acid at -20°C and spiked with 10 µmol l^{-1} glutaryl-CoA as an internal standard. The resuspended culture was incubated on ice with intermittent vortexing for 15 min. An equal molar volume of ammonium hydroxide was added post-incubation to neutralize the acetic acid and each extract was centrifuged for 3 min at 13,000 rev min^{-1} and 4°C, transferred to a new 1.5 ml eppendorf tube, and centrifuged for 5 min at 13,000 rev min^{-1} and 4°C. Clarified medium (10 µl) was injected for HPLC-MS/MS analysis.

2.4 LC-MS/MS Development and Quantification

2.4.1 MS/MS detection and compound optimization

MS and MS/MS conditions (declustering potential (DP), entrance potential (EP), collision energy (CE), and product ions) were optimized in positive ion mode (5.5 kV) for each acyl-CoA by direct injection of 100 µmol l^{-1} standards diluted in 50:50 H_2O:MeOH + 5 mmol l^{-1} ammonium formate to an Applied Biosystems 3200 Q-Trap triple quadrupole at a flow rate of 5 µl min^{-1}. A mixture of methanol, water, and

ammonium formate was used to aid in desolvation of the solvent mixture and ionization of the compounds of interest. The mass spectrometer conditions were nebulizer gas pressure of 12 psi, auxiliary gas pressure of 5 psi, source temperature of 100°C, and curtain gas pressure of 10 psi. The optimal parent/product ion pairs in Table 3 were used for further study and determined through the Analyst software compound optimization program summing the intensities at varying collision energies. Dry nitrogen was used as desolvation gas. Optimal parent/product pairs were found from the loss of 507 Da from the positive ion precursor as previously described (Gao et al 2007, Dalluge et al 2002, Gan-Schreier 2005, Park et al 2007)

Acyl-CoA	***Parent ion***	***Product ion***
Acetyl-CoA	810	303
Butyryl-CoA	838	331
Malonyl-CoA	854	347
Methylmalonyl-CoA	868	361
Propionyl-CoA	824	317
Succinyl-CoA	868	361
Glutaryl-CoA	882	375

Table 3. Parent and product ion pairs used in LC-MS/MS analysis for acyl-CoAs in positive ion mode $[M - H]^+$.

2.4.2 Chromatographic separation and optimization

Chromatographic elution was optimized on a GL Sciences Inertsil ODS-3 C18 analytical column (4.6 x 150 mm, 3 μm; Torrance, CA). The extracted cultures and standards were eluted at a flow rate of 400 μl min^{-1} with a gradient of 10 to 22.5% B for 30 min, to 100% B in 5 min, and re-equilibrated and washed at 10% B for 10 minutes at 500 μl min^{-1} (Table 4). Buffer A was composed of 20 mmol l^{-1} ammonium acetate in water (pH 7.4) and Buffer B was composed of 20 mmol l^{-1} ammonium acetate in HPLC grade methanol. Gas and temperature conditions were further optimized for LC-MS/MS by analyzing high and low level conditions of source temperature, nebuilizer gas, and

auxiliary gas for a total of 11 experimental combinations (Appendix). The sum of signal intensity, measured for acetyl-, butyryl-, propionyl-, glutaryl-, malonyl-, and methylmalonyl-CoA was used to determine the most optimal method. The optimized parameters to obtain maximum signal intensity were determined to be curtain gas pressure of 30 psi, nebulizer gas pressure of 50 psi, auxiliary gas pressure of 30 psi, and source temperature of 350°C. Acyl-CoAs were eluted in the following order: malonyl-CoA (21.5 min), succinyl-CoA (26 min), methylmalonyl-CoA (28 min), glutaryl-CoA (31 min), acetyl-CoA (39 min), propionyl-CoA (40.5 min), and butyryl-CoA (41.5 min) (Figure 4).

Acyl-CoAs were detected in the multiple reaction monitoring (MRM) mode for quantification with the m/z parent > m/z daughter (pairs listed in Table 2) and previously optimized. A dwell time of 75 ms was used for each MRM transition for the 45 min elution. LC-ESI-MS/MS chromatograms were analyzed using Analyst software v.1.5.1, and manually integrated after 2x Gaussian smoothing to standardize and aid in accurate peak integration. Acyl-CoA areas were normalized to the glutaryl-CoA internal standard area, and concentrations were determined from an intra-run generated calibration curve of 0.5, 0.75, 1, 2.5, 5, 7.5, and 10 μmol l^{-1} standard acyl-CoA concentrations with 10 μmol l^{-1} used for glutaryl-CoA as internal standard. All comparative samples were run during the same elution batch with the same intra-run calibration curve. Concentration of a given acyl-CoA was calculated by correlating the acyl-CoA area/area internal standard to intra-run calibration curves. The total nanomoles of a given acyl-CoA was further normalized to mg DCW by first calculating total acyl-CoA amount (using the extraction volume of

130 µl) and then using the previously determined correlation (data not shown) of 1 OD_{600nm} = 0.52 gDCW l^{-1}.

$$\frac{\text{nmol}}{\text{mg}\cdot\text{DCW}} = \left(\frac{\text{Area}\cdot\text{acyl}-\text{CoA}}{\text{Area}\cdot\text{glutaryl}-\text{CoA}}\right)\cdot a\cdot\left(\frac{\text{umol}}{\text{L}}\right)\cdot 130\text{ul}\cdot\left(\frac{10^3\text{nmol}}{\text{umol}}\right)\cdot\left(\frac{1\text{L}}{10^6\text{ul}}\right)$$

Where "a" is the coefficient from the linear fit calibration curve for each individual acyl-CoA, relating the normalized peak area of acyl-CoA to µmol l^{-1}. The resulting nanomoles of acyl-CoA was then normalized to mg DCW by the above listed correlation.

Time	***% Buffer B***	***Flow (ul/min)***
0	10	400
30	22.5	400
35	100	400
35.01	10	500
45	10	500
45.01	10	400

Table 2. LC-MS/MS elution method. Buffer A: 20 mmol ammonium acetate, Buffer B: 20 mmol ammonium acetate in methanol.

2.4.3 Limit of Detection/Quantification

The limit of detection was determined by injecting 10 µl standard mixtures including acetyl-, butyryl-, glutaryl-, propionyl-, methylmalonyl-, succinyl-, and malonyl-CoA in 50:50 methanol:H_20 with 5 mmol l^{-1} ammonium formate. Seven concentrations of standard acyl-CoAs were run to generate calibration curves at 0.050, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 µmol l^{-1} concentrations. The standards were eluted and detected as described above. The signal-to-noise ratio was determined by dividing the maximum intensity for each acyl-CoA at the determined concentration by the max intensity measured by a blank 50:50 methanol:H_2O with 5 mmol l^{-1} ammonium formate. The limit of detection (LOD) was determined as the concentration with a signal to noise ratio = 3, while the limit of

quantification (LOQ) was determined as a signal to noise ratio = 5. The calculated LOD and LOQ's are listed in Table 3.

Acyl-CoA	*LOD (μmol l^{-1})*	*LOQ (μmol l^{-1})*
Acetyl	0.200	0.333
Butyryl	0.092	0.154
Glutaryl	0.281	0.468
Propionyl	0.078	0.130
MM	0.017	0.029
Succinyl	0.698	1.164
Malonyl	0.758	1.263

Table 4. Limit of detection and limit of quantification of acyl-CoAs using developed method.

2.5 6-deoxyerythronolide B Quantification

TB3/pACYCDuet-1/pBP130/pBP144 and TB3/pJWA1/pBP130/pBP44 were inoculated from glycerol stocks and cultured overnight at 37°C and 250 rev min^{-1}. Overnight cultures were used to inoculate 15 ml LB medium in Erlenmeyer flasks to an OD_{600nm} of 0.1. The resulting cultures were supplemented with 20 mmol l^{-1} sodium acetate or sodium butyrate, 2 mmol l^{-1} sodium propionate, and 100 μmol l^{-1} IPTG and grown for 72 hours at 22°C and 250 rev min^{-1} before a 500 μl sample was extracted with an equal volume of ethyl acetate and vortexed for 2 min. Extracted cultures were centrifuged for 1 min at 10,000 rev min^{-1}, and the organic layer was transferred to a new 1.5 ml microfuge tube and air dried overnight. The dry product was re-suspended in 50 μl of methanol with 2.5 μg l^{-1} roxithromycin as an internal standard. Cultures were infused at 15 μl min^{-1} on a Finnigan LTQ Mass Spec and the product masses in Table 3 were used to quantify 6dEB, 14-nor-6dEB, 15-methyl-6dEB, and roxithromycin. Signal intensities in positive ion mode were summed for both the sodium adduct and protonated product and normalized to the signal intensities of the roxithromycin internal standard. A

calibration curve was generated by spiking 0.5, 1, 1.5, 2, and 2.5 µg 6dEB standards each into 500 µl LB medium and extracting as described above. Normalized signal intensities were correlated to the intra-run calibration curve and used to determine the mg/l of 6dEB.

Product	***[M+H]$^+$***	***[M+Na$^+$]$^+$***
Roxithromycin	837	859
6-dEB	387	409
14-nor-6dEB	373	395
15-methyl-6dEB	401	423

Table 5. 6dEB and 6dEB analog m/z ratios used for quantification. Values from sodium adduct formation as $[M+Na]^+$.

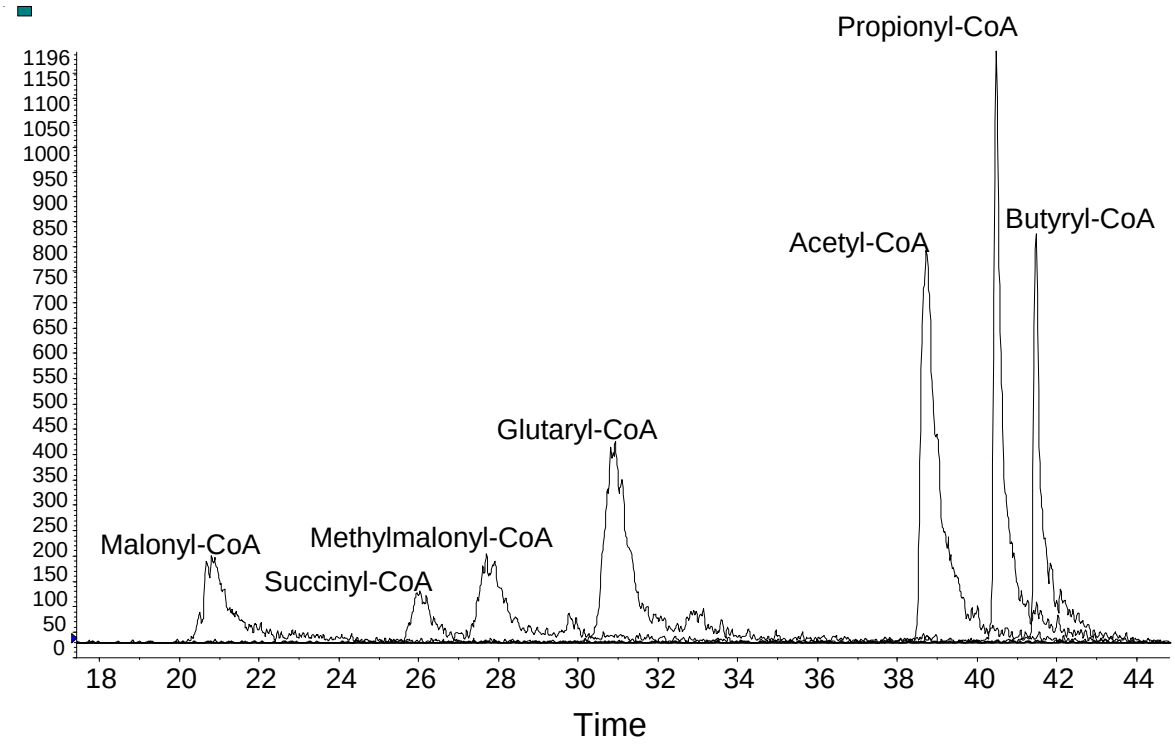


Figure 4. Representative chromatogram for optimized elution method of standard acyl-CoA molecules. Acyl-CoAs infused at 100 µmol l^{-1} in 50:50 methanol:water + 5 mmol l^{-1} ammonium formate. Optimized elution conditions are described above.

3. Results

3.1 LOD and LC-MS/MS conditions optimization

The conditions for LC-MS/MS detection were optimized by testing 11 combinations of source temperature, nebulizer gas, auxiliary gas, and flow rate. The signal intensity of each acyl-CoA at the 11 conditions (Appendix) tested was summed and the combination yielding the greatest signal for all acyl-CoAs was used for future studies. The optimal method had a total signal approximately 2 times that of the second most optimal condition and approximately 100x greater signal than the blank.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by fitting linear curves to the signal-to-noise ratio (S/N) vs. concentration for each acyl-CoA. The signal-to-noise ratios were determined by dividing the maximum signal intensity at each concentration and dividing by a blank injected sample containing only sample buffer. The linear curves were used to determine the LOD at a signal-to-noise ratio of 3 and an LOQ at a signal-to-noise ratio of 5. The LOD and LOQ values are listed in Table 6. All linear curves showed an R^2 value > 0.75.

3.2 Acyl-CoA Profiling of Engineered *Escherichia coli* strains

After establishing an intracellular LC-MS/MS method (Figure 4), five strains with varying genetic modifications (Table 1) were tested to profile the level of five acyl-CoA molecules of interest under different induction and feed conditions. Four conditions were tested: (1) 20 mmol l^{-1} propionate and 100 μmol l^{-1} IPTG, (2) propionate only, (3) IPTG only, (4) no propionate or IPTG. Upon feeding with propionate and IPTG, it was

observed that BAB2, BAP1, and TB3 showed fold increases between ~6-30 for propionyl-CoA, ~3.7-6.8 for methylmalonyl-CoA, and ~4-6.8 for butyryl-CoA with respect to the un-engineered controls YW22 (a K12 strain of *E. coli*) and BL21(DE3) (the parent strain of BAB2, BAP1, and TB3). Acetyl-CoA levels were comparatively lower for BAB2, BAP1, and TB3 strains, while malonyl-CoA showed no significant increases. No significant increases in propionyl- or methylmalonyl-CoA were observed with only propionate or only IPTG conditions or in the case of no propionate and IPTG (Figure 5).

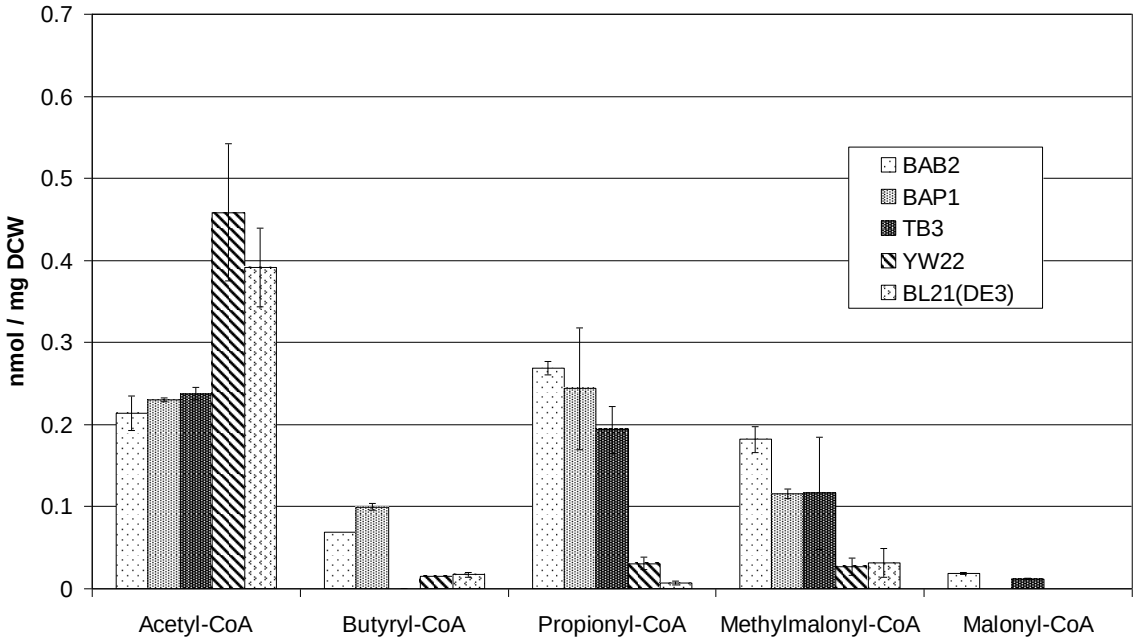
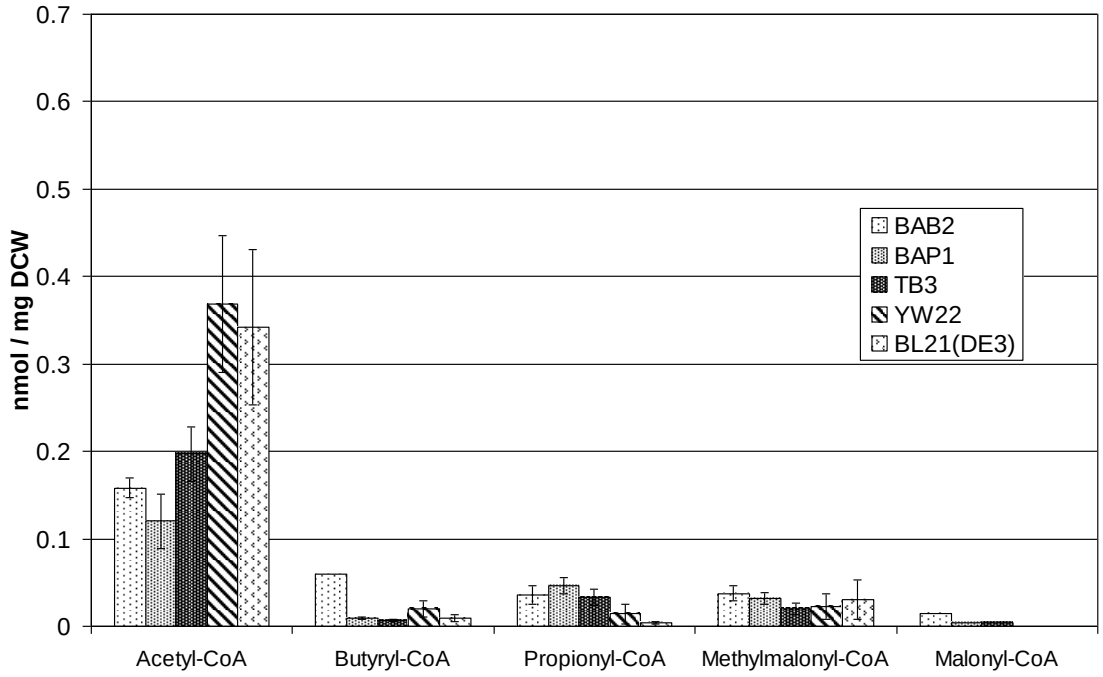
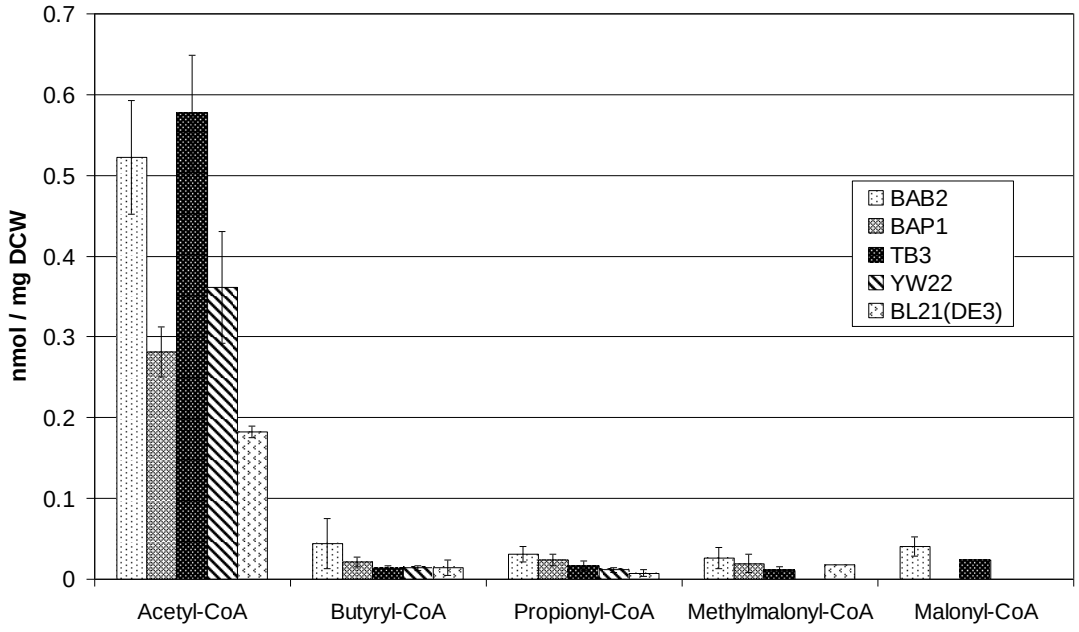


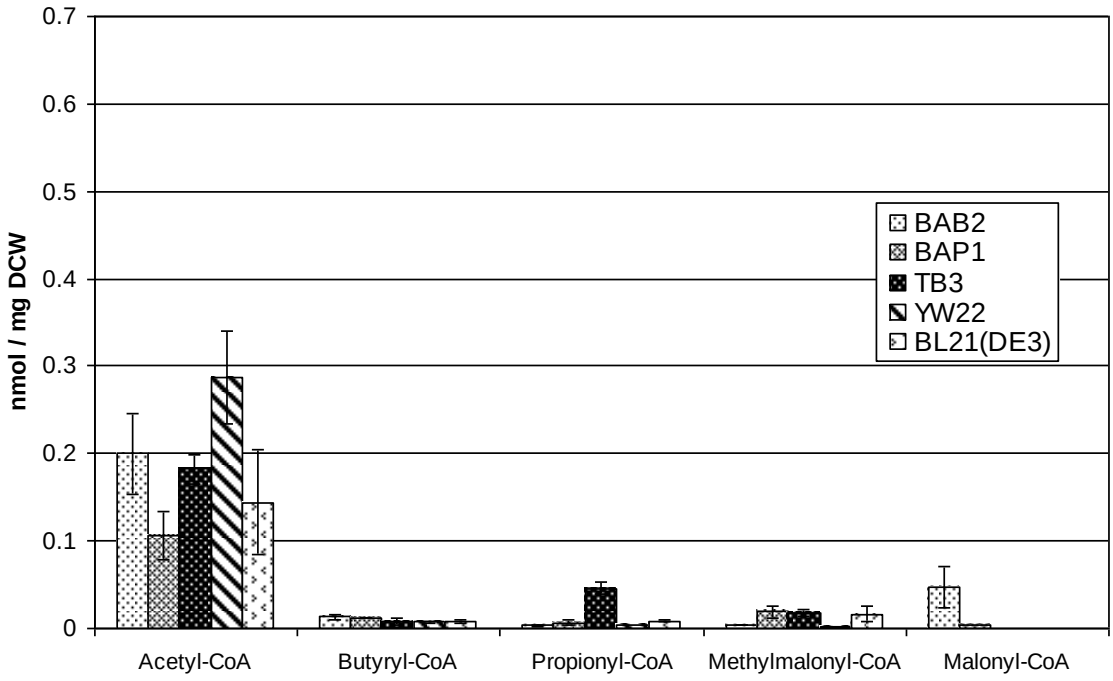
Figure 5. Acyl-CoA strain profiling of engineered *E. coli* strains for 6-deoxyerythronlide B production, with **a.)** 100 μmol l^{-1} IPTG and 20 mmol l^{-1} sodium propionate cultured for 24 hours, and analyzed via LC-MS/MS.



b.) 20 mmol l^{-1} sodium propionate only



c.) 100 µmol l^{-1} IPTG only

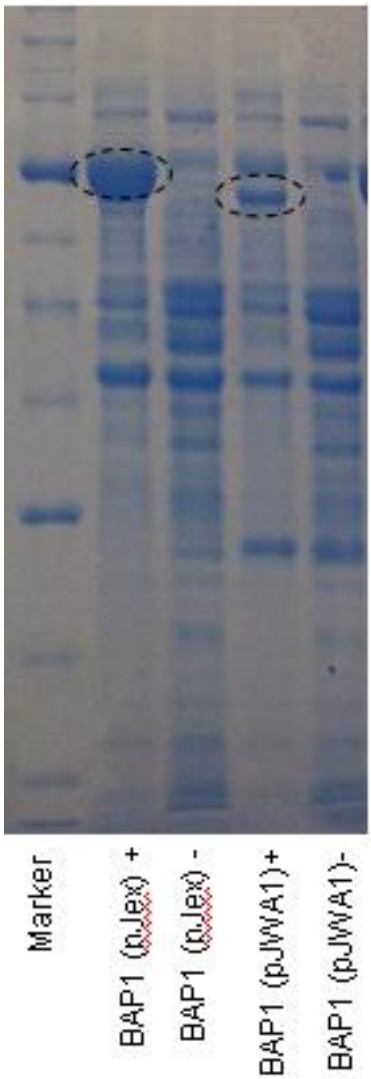


d.) No IPTG or sodium propionate

3.3 PrpE-*RS* and Native PrpE analysis in *Escherichia coli* BAP1 and BL21(DE3)

SDS-PAGE was first used to confirm production of PrpE-RS from pJexpress411-*prpE-RS* and pJWA1 (Figure 6). BAP1/pJWA1 showed fold increases of 1.6, 15.0, and 8.7 of acetyl-, butyryl-, and propionyl-CoA, respectively, when fed with the corresponding substrates (sodium acetate, butyrate, or propionate) at 20 mmol $l^{-1}$and induced with 100 μmol l^{-1} IPTG when compared to the uninduced control (Figure 7). Each triplicate run had student t-test p values < 0.05. Significant increases in the remaining acyl-CoA molecules were either not observed or not detected.

Figure 6. SDS-PAGE of BAP1 expressing both high copy-number pJexpress411-T7-prpE-*RS* and low copy number pJWA1 (cloned into pACYCDuet-1 MCS2)



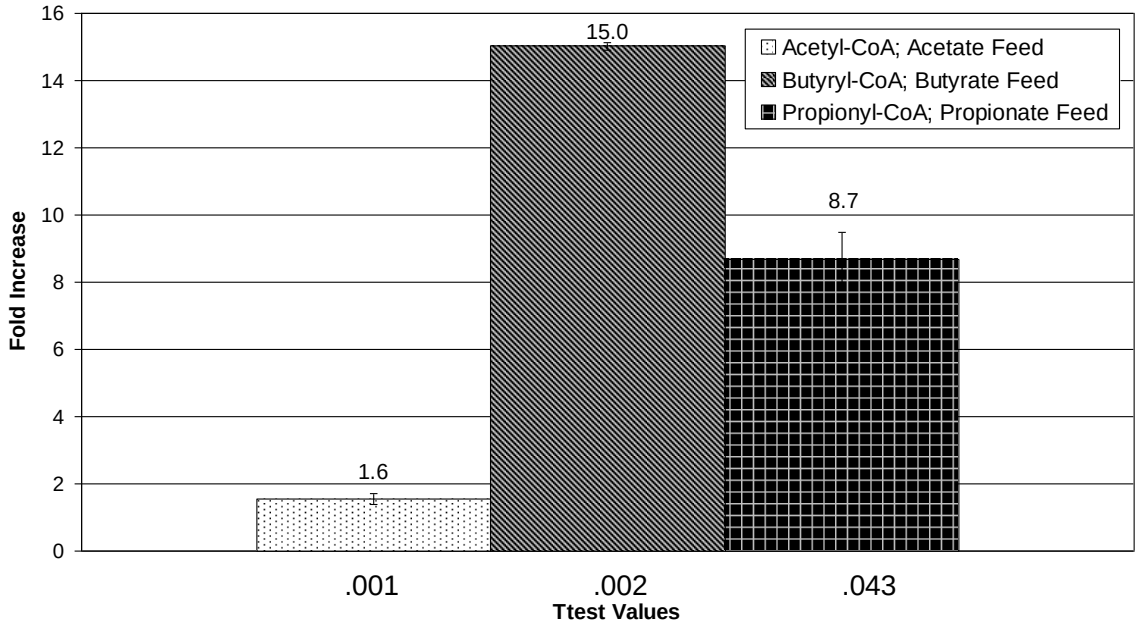


Figure 7. Comparison of BAP1/pJWA1 fold increase in acyl-CoAs of induced:uninduced cultures extracted at 24 hours post-induction from LB media supplemented with 20 mmol l^{-1} sodium propionate, acetate, or butyrate and 100 μmol l^{-1} IPTG if indicated. Acyl-CoA concentrations normalized to mg DCW.

When compared to the BAP1/pACYCDuet-1 empty vector control, BAP1/pJWA1 had fold increases of 2.1, 1.2, and 1.3 for acetyl-, butyryl, and propionyl-CoA, respectively, when the corresponding substrates were fed as described above (Figure 8). Butyryl- and propionyl-CoA fold increases were statistically significant but not reflective of the fold increases observed in Figure 7.

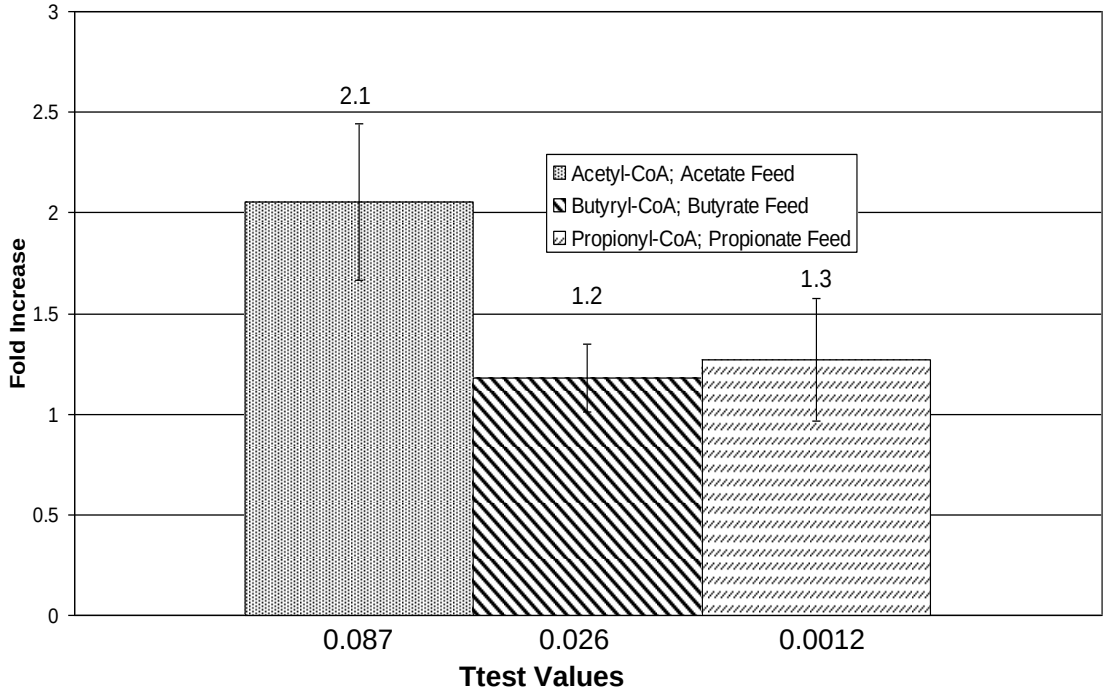


Figure 8. Fold increases in acyl-CoAs comparing BAP1/pJWA1:BAP1/pACYCDuet-1. Cultures extracted 24 hours post-induction, from LB media supplemented with 20 mmol l^{-1} sodium propionate, acetate, or butyrate and 100 μmol l^{-1} IPTG. Acyl-CoA concentrations normalized to mg DCW.

To further probe the fold increases originally observed, a third control was tested where pJWA1 and pACYC were transformed into BL21(DE3) and compared to BAP1 with no additional plasmids. In this comparison, BAP1 showed fold increases in butyryl-CoA of 7.76 and 6.01, and 13.7 and 2.2 for propionyl-CoA with respect to BL21(DE3)/pJWA1 and BL21(DE3)/pACYCDuet-1, when the corresponding substrates were fed (Figure 9 a, b, and c). No fold increase in acetyl-CoA levels in BAP1 compared to BL21(DE3)/pACYC or BL21(DE3)/pJWA1 were observed in the acetate, propionate, or butyrate feed conditions. Additionally, increases in methylmalonyl-CoA were observed in both the butyrate and propionate feed conditions (~1.4-8.5-fold) and fold

increases in propionyl-CoA were observed in all feed conditions ranging from ~1.6- to 13.7-fold.

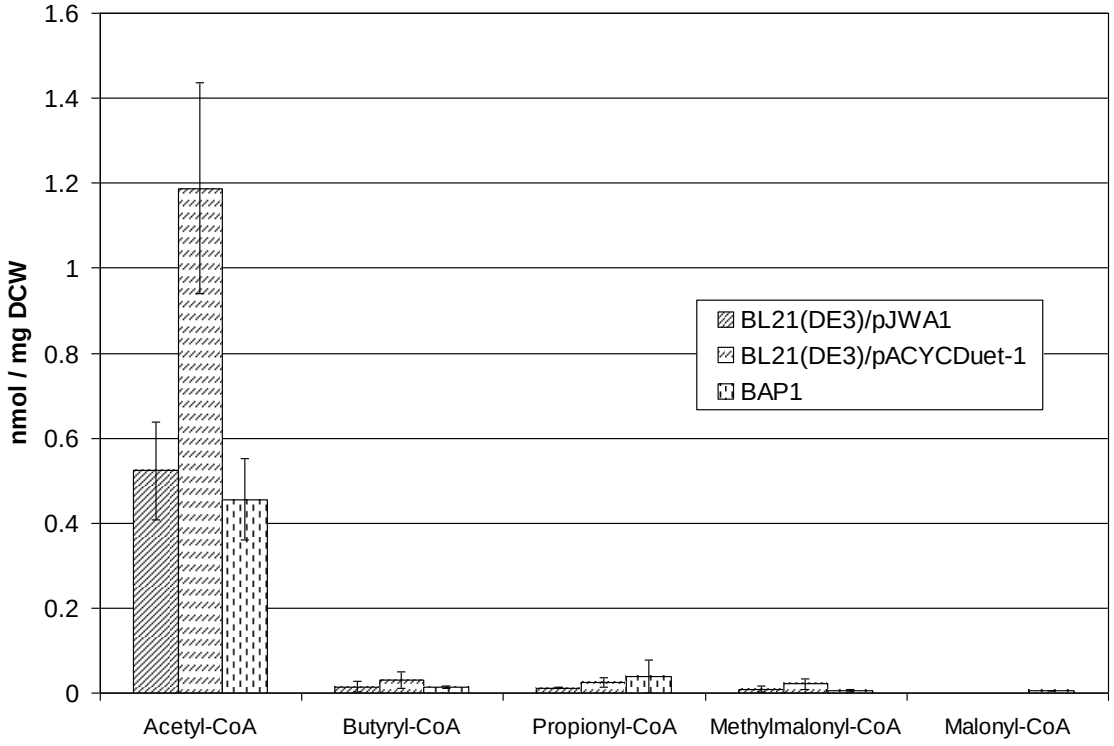
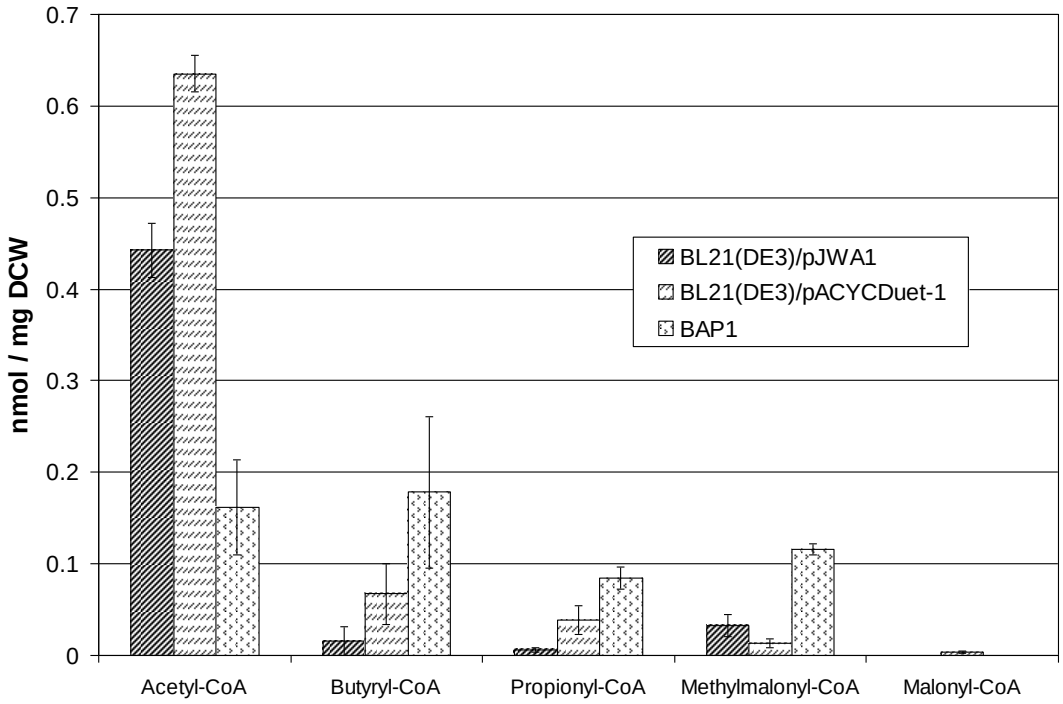
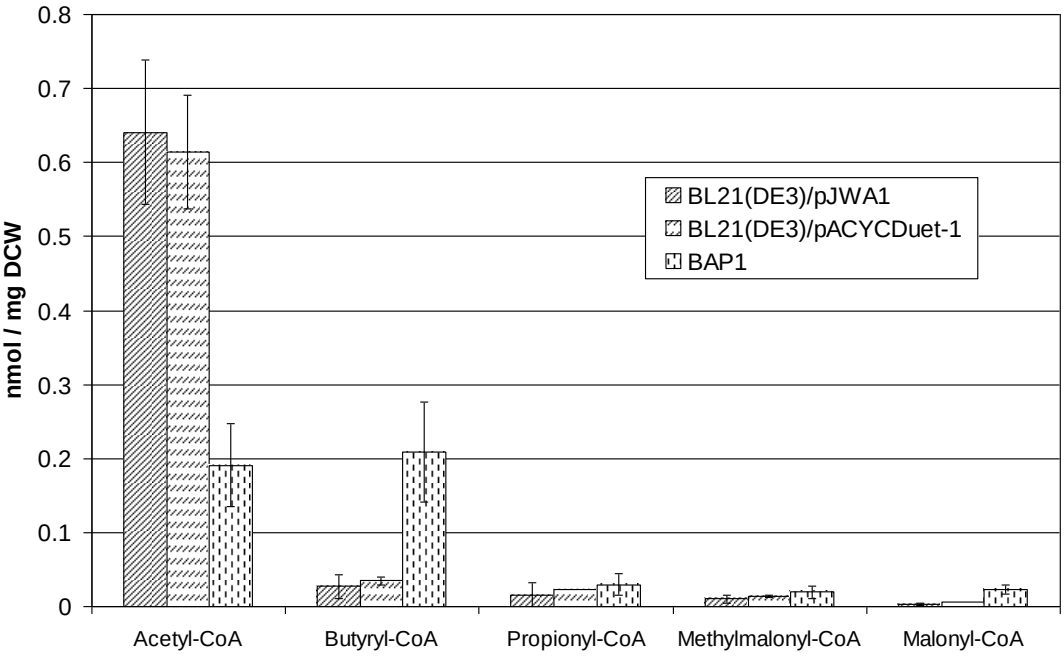


Figure 9. Acyl-CoA profile comparing BL21(DE3)/pJWA1, BL21(DE3)/pACYCDuet-1, and BAP1 in LB media supplemented with:

a.) 20 mmol l^{-1} sodium acetate and induced with 100 μmol l^{-1} IPTG. Acyl-CoA concentrations normalized to mg/DCW.



b.) Acyl-CoA profile when supplemented with 20 mmol l^{-1} sodium propionate and induced with 100 µmol l^{-1} IPTG.



c.) Acyl-CoA profile when supplemented with 20 mmol l^{-1} sodium butyrate and induced with 100 µmol l^{-1} IPTG

3.4 AtoAD expression and analysis

To help alleviate substrate uptake limitations and improve intracellular acyl-CoA levels, a propionate transporter was overexpressed in BAP1/pJexpress411-*prpE-RS* with the gene *atoAD*. The strain BAP1/pAtoAD/pJexpress411-*prpE-RS* was compared to BAP1/pACYCDuet-1/pJexpress411-*prpE-RS*. Fold increases of 1.44, 1.34, and 0.51 were observed for butyryl-, acetyl-, and propionyl-CoA, respectively, when fed with the corresponding substrate at 20 mmol l^{-1} and induced with 100 μmol l^{-1} IPTG. Only the increase in acetyl-CoA was determined to be statistically significant with a student t-test p value <0.05 (Figure 10).

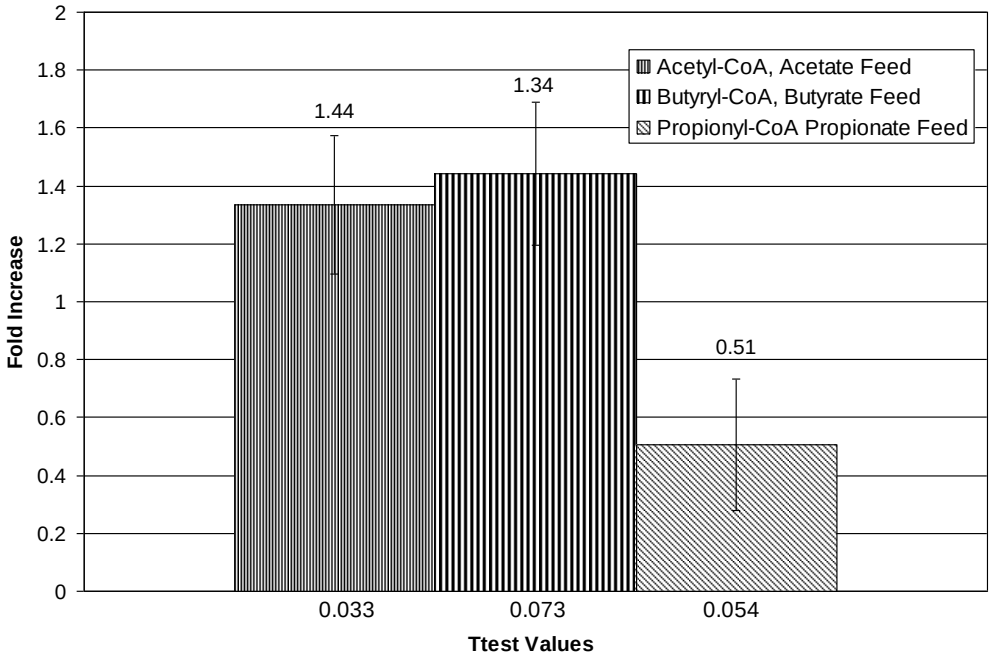


Figure 10. Fold increases in acyl-CoAs comparing BAP1/pAatAD/pJexpress411-*prpE-RS* : BAP1/pACYCDuet-1/pJexpress411-*prpE-RS* in LB media supplemented with 20 mmol l^{-1} sodium acetate, propionate, or butyrate and induced with 100 μmol l^{-1} IPTG.

3.5 6-deoxyerythronolide B analog production

The production of 6dEB remained between 30-50 mg/l for TB3/pJWA1/pBP130/pBP144 and TB3/pACYC/pBP130/pBP144 when either acetate and propionate or butyrate and propionate were fed. Titers of acetate and butyrate analogs remained below 5 mg/l for each condition and no appreciable analog titer increase was observed when *prpE-RS* was expressed using pJWA1 (Figure 11).

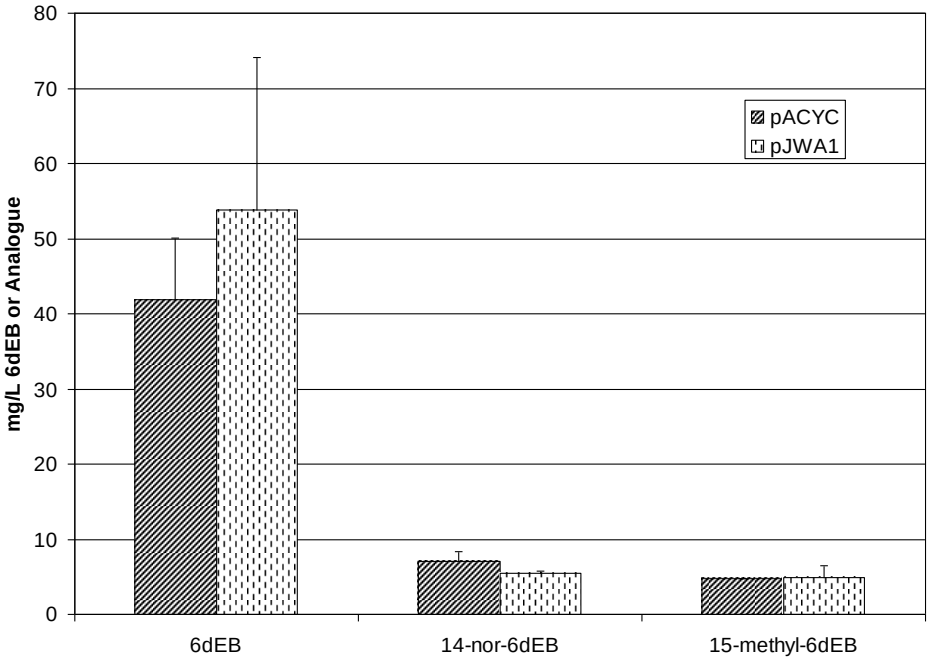
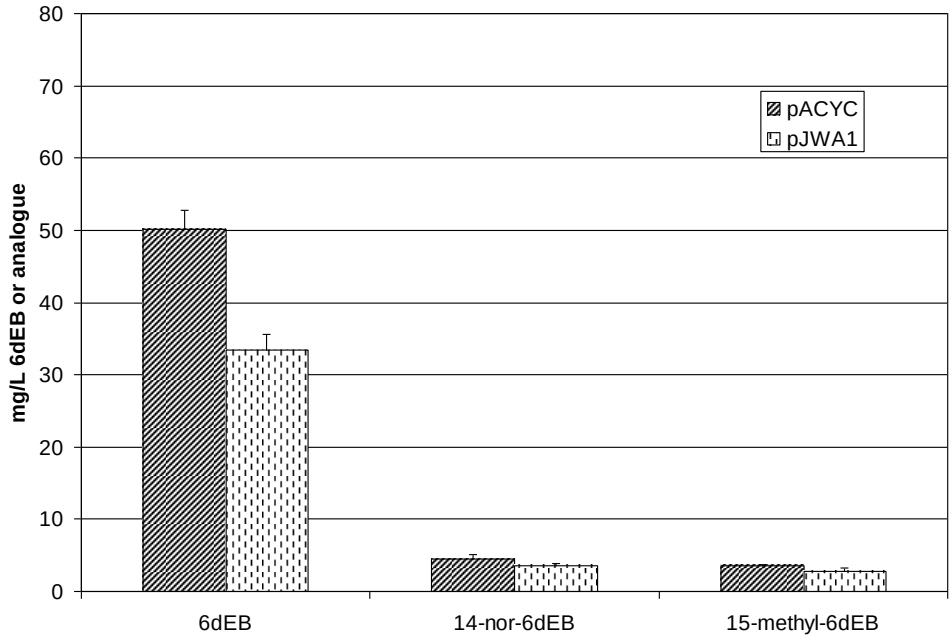


Figure 11 Production of 6dEB, 14-nor-6dEB (acetate analog), and 15-methyl-6dEB (butyrate analog) **a.)** 20 mmol l^{-1} sodium acetate and 2 mmol l^{-1} sodium propionate by TB3/pACYC/pBP130/pBP144 and TB3/pJWA1/pBP130/pBP144 in LB media after 72 hours culture at 22˚C



b.) Production of 6dEB and analogs supplemented with 20 mmol l^{-1} sodium butyrate and 2 mmol l^{-1} sodium propionate in LB media after 72 hours culture at 22 ˚C.

4. Project Summary and Discussion

The project presented demonstrated robust method development for the analysis of acyl-CoA molecules via quantitative LC-MS/MS, in particular, for use with isolated *E. coli* cell extracts. Because of the low intracellular concentration of acyl-CoAs in bacterial cells and inherent dilution of sample during extraction with many targeted metabolite analysis protocols, tandem mass spectrometry was used as a sensitive and selective tool to profile a range of acyl-CoA molecules to better understand the metabolic dynamics of engineered *E. coli* strains. Additionally, several heterologous enzymes involved in acyl-CoA production were tested to diversify and increase the acyl-CoA profiles in engineered *E. coli* hosts.

The acyl-CoA profiles observed in this study suggest that the modifications present in the engineered strains BAB2, BAP1, and TB3 provide better support for polyketide production as increased levels of both propionyl- and methylmalonyl-CoA were observed upon induction and propionate feeding (Figure 5). By improving substrate availability for polyketide production, the potentially rate-limiting step of substrate loading may be alleviated, but not necessarily eliminated. Increased substrate availability may only eliminate one bottleneck in the polyketide biosynthesis pathway, and additional metabolic and genetic modifications may be necessary to reach the next level of productivity in *E. coli.* One potential improvement for novel analog production may be protein engineering of the DEBS loading domain to accept more diverse acyl-CoAs (Long 2002).

The observed increases in acyl-CoA concentration confirms the previously suggested metabolic engineering approaches for increased 6dEB production (Zhang et al 2010, Pfeifer et al 2001). The modifications include the overexpression of the native *prpE* in BAP1 (which converts propionate to propionyl-CoA), the *sbm-ygfDGH* deletion in BAB2 (which reduces the conversion of propionyl-CoA to propionate), and the *ygfH* deletion in TB3 (Zhang et al 2009, Pfeifer et al 2001). The engineered strains revealed increased levels in required substrates for 6dEB production and also improvements in overall 6dEB titers (Zhang et al 2009). Induction alone improved the overall increases in propionyl- and methylmalonyl-CoA but some improvement was also observed when only propionate was fed. The latter observation may suggest lax regulation of the T7 expression machinery or possibly innate metabolic capabilities for the production of propionyl-CoA in the given cell lines.

Additionally, the acetyl-CoA levels for the engineered strains were lower when propionate was fed under both induced and uninduced conditions compared to non-engineered controls (i.e., YW22 and BL21(DE3)). The decrease was not apparent when no propionate was fed under both induced and uninduced conditions (Figure 5c and 5d). The most plausible explanation for this result is the propionate- and engineered *prpE*-dependent coupling of free CoA molecules to alternative acyl units. The observation of this trend without the addition of IPTG again suggests leaky expression of the native *prpE* gene, supported by the reduced differences between acetyl-CoA levels (Figure 5b) when compared to the condition with both propionate and IPTG present (Figure 5a). It is also interesting to note the increase in acetyl-CoA levels within the engineered strains with only the addition of IPTG, suggesting an additional capability of the native PrpE in the absence of its preferred propionate substrate.

In an attempt to further improve the availability and diversity of the acyl-CoA substrate pool in engineered *E. coli*, the flexible PrpE enzyme from *Ralstonia solanacearum* (PrpE-*RS)* was expressed in BAP1. PrpE-*RS* had previously shown broad in vitro flexibility by converting acetate, propionate, and butyrate into their corresponding CoA thioesters with variations in specificity and efficiency for each substrate (Rajashekhara et al. 2004). The preferences reported indicated strength of activity as acetate>propionate>butyrate but greater k_{cat}/K_m values for propionate>acetate>butyrate. When tested in BAP1, improvements in all three intracellular substrates were observed ranging from 1.5- to 15-fold, with the greatest increase in butyryl-, followed by propionyl-, and then acetyl-CoA. However, these

improvements could not be confirmed in the context of a key control. The intracellular activity could not be confirmed for PrpE-*RS* when compared to an empty pACYCDuet-1 vector in BAP1 (Figure 8). Induction of BAP1/pJWA1 vs. BAP1/pACYCDuet-1 only revealed small fold increases in acyl-CoA compounds between 1.2 and 2.1. This result suggests that PrpE-*RS* may either not have full activity in BAP1 or, more interestingly, the native PrpE enzyme in *E. coli* may be demonstrating its own substrate flexibility.

To further confirm this hypothesis, pJWA1 and pACYCDuet-1 were transformed into BL21(DE3) and compared to BAP1 alone. As above, acetate, butyrate, and propionate were fed, and the induced acyl-CoA profiles were compared between each strain. BAP1 showed increased levels of butyryl- and propionyl-CoA, while BL21(DE3)/pJWA1 did not show increased levels with respect to the control BL21(DE3)/pACYCDuet-1. Additionally, increases in methylmalonyl-CoA were observed for BAP1 when propionate was fed. The increase in methylmalonyl-CoA in the absence of a dedicated propionyl-CoA synthetase points to native metabolism capable of producing this compound, as we have observed previously in the context of 6dEB formation (Pistorino et al 2009, Zhang et al 2010).

This result demonstrates the flexibility associated with the native *E. coli* PrpE enzyme, as little activity from the heterologous PrpE-*RS* was measured and cannot account for the flexibility observed. Such information may then be used directly by applying protein engineering to expand or improve upon these native capabilities. In addition, the analytical method developed here will be applied in the context of future metabolic engineering attempts to improve the availability of acyl-CoA molecules for

heterologous biosynthetic attempts and to probe the introduction of new pathways to further diversify this important pool of metabolites.

A second strategy was attempted to further improve acyl-CoA levels in *E. coli* by increasing the flux of propionate from the culture medium into the cell by way of the acetoacetyl-CoA transferase, AtoAD. A combination of both AtoAD and PrpE-*RS* within BAP1 were compared to an empty vector control. However, under the conditions tested here, AtoAD showed only slight positive impact on intracellular acyl-CoA levels. As demonstrated by Jenkins et al, overexpression of AtoC activates the expression of the *ato* operon and in particular the genes *atoA* and a*toD*. The transporter subunits AtoA and AtoD improve the uptake of fatty acid, and in theory should improve production of the respective -CoA moiety (Jenkins 1987). Hanai et al, additionally demonstrated that expression of the *atoAD* operon improved isopropanol production which can convert acetoacetyl-CoA to acetyl-CoA and, with substrate flexibility, butyryl-CoA. This may explain the increases observed in acetyl- and butyryl-CoA with expression of the AtoAD enzyme (Figure 10). Additionally Kennedy et al applied expression of the *atoAD* operon to improve production of the 6dEB analog 15-methyl-6dEB by increasing intracellular butyryl-CoA levels. The AtoAD transporer has substrate flexibility and is able to activate acetyl-CoA and other short chain fatty acids (Kennedy et al 2003). In the current work, production studies of 6dEB and 6dEB analogs, fed with propionate and butyrate, showed approximately 8 fold more 6dEB was produced compared to 15-Me-6dEB, the butyryl-CoA analog. This observation was similar to those observed by Kennedy et al and Pieper et al who observed a 10 fold ratio in vivo and an 8 fold preference in vitro, respectively (Kennedy et al 2003, Pieper et al 1996). The differences in magnitude of observed acyl-

CoA levels in our results from previously reported expression of the *atoAD* gene suggests that including additional genes from the *ato* operon may be required for proper expression and activity of the AtoA and AtoD transporter subunits. Alternatively, with overexpression of the *prpE* gene, the observed increases with *atoAD* expression may not account for a significant portion of acyl-CoA level improvements, which may be overshadowed by the action of the PrpE enzyme.

With the improved intracellular acyl-CoA levels observed, the production of 6dEB and 6dEB analogs was tested in the host TB3/pJWA1/pBP130/pBP144 versus TB3/pACYCDuet-1/pBP130/pBP144. Comparing the two systems revealed no significant increase in 6dEB or 6dEB analog production contrary to previously observed reports (Pieper 1996, Kennedy 2003, Brown 1995, Luo 1996). One strategy for improvement in an *E. coli* system may include introducing a pathway to provide the methylmalonyl-CoA extender unit without the need to feed propionate into the system, thus decreasing the competition at the loading domain when acetate or butyrate is fed and supplemented with propionate. Alternatively, protein engineering may be applied to broaden the specificity selectivity of the loading domain to accept more unnatural substrates.

5. Future Directions

The findings from these studies present several opportunities for future research and analysis. The LC-MS/MS method and acyl-CoA extraction provides a platform for future metabolic evaluations of engineered *E. coli* hosts used for the production of polyketide products. Beyond the previously developed analytical techniques for detecting the final product, 6dEB, the analysis of intracellular metabolites used in the biosynthesis of polyketides provides an additional and valuable method of quantifying future improved metabolic engineering approaches. Additionally, quantification of these important substrates may direct efforts of identifying bottlenecks in the production of novel analogs. Although, a number of acyl-CoAs were quantified in this study, the similarity in neutral loss fragmentation of all acyl-CoAs, allows the method to be easily adapted to analyze a more diverse acyl-CoA pool with little effort or chromatographic optimization.

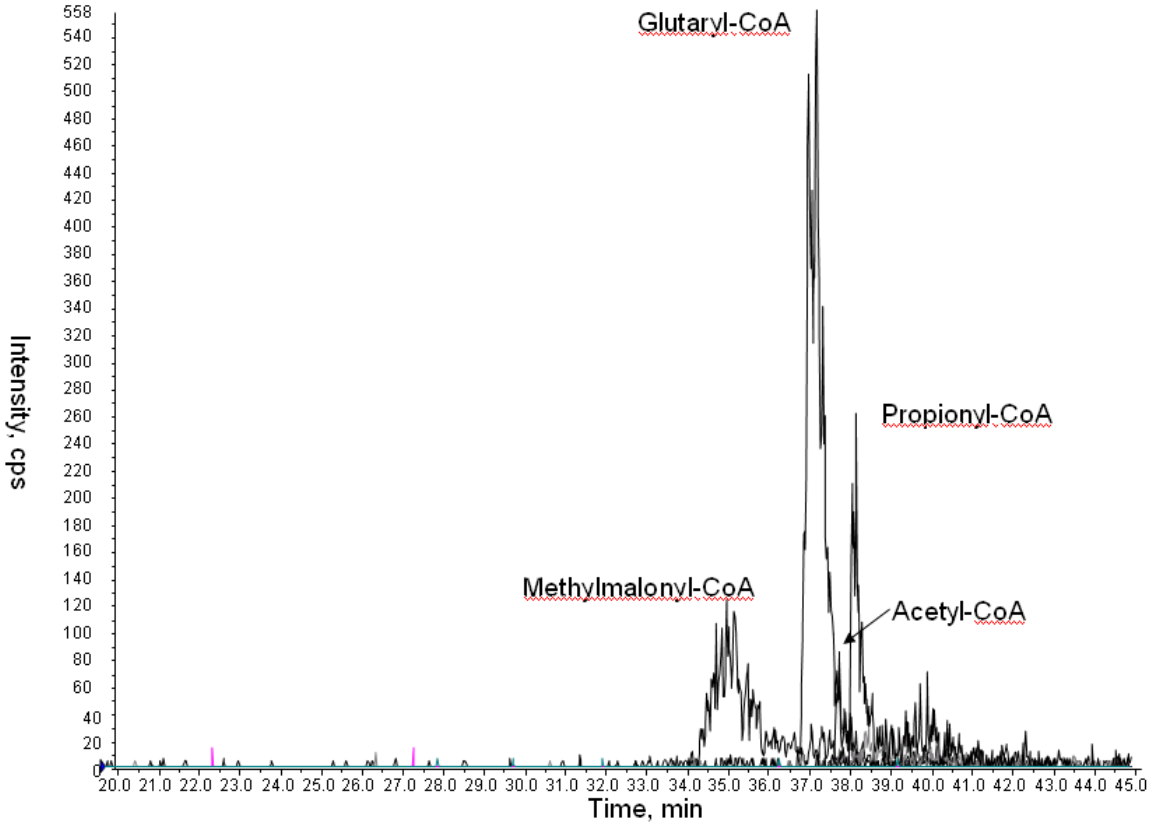
Although sensitive and selective enough for our purposes, additional method improvements may be made to increase sensitivity or selectivity of acyl-CoA detection by varying elution buffer, MS spray conditions, and the extraction procedure. Additionally, alternative analytical techniques may be tested including NMR and GC-MS which have also been used extensively for metabolomic studies, although not to a great extent for analyzing acyl-CoAs. The extraction method provided sufficient cell lysis and preservation of acyl-CoAs, as previously demonstrated (Bennet et al 2009), but additional optimization and analysis may provide more insight into improving efficiency and

uniformity in extraction of *E. coli* culture, while remaining compatible with downstream analytics..

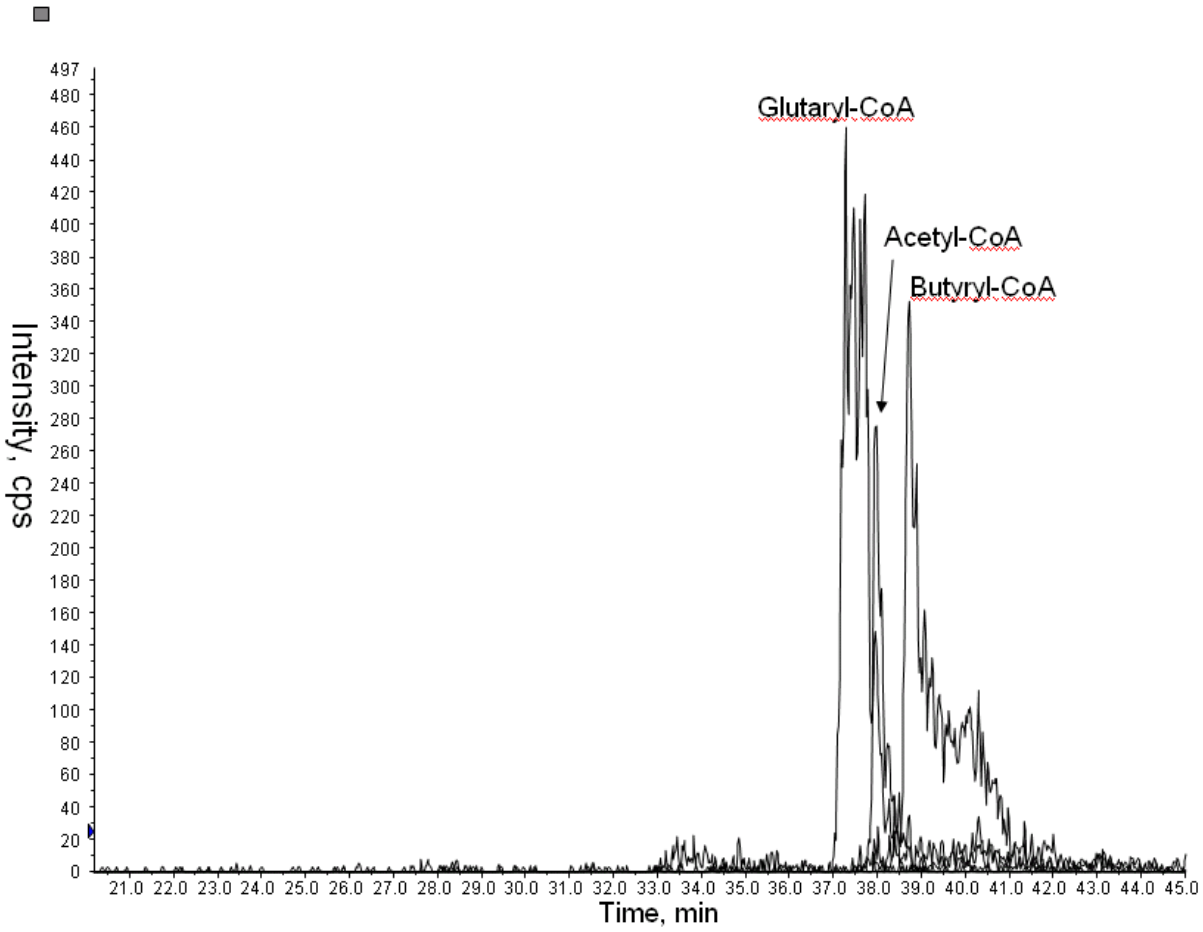
To further confirm the flexibility observed by the native PrpE, in vitro kinetic studies may provide an additional method of demonstrating flexibility of the native PrpE. With confirmation, more focus can be applied towards optimizing the native PrpE for improving intracellular acyl-CoA levels, thus eliminating the need for expression of heterologous PrpEs.

Finally, although improved intracellular acyl-CoA levels did not significantly increase the production of novel analogs in this study, additional techniques including protein engineering or pathway optimization in *E. coli* may better reveal the effects of improved intracellular acyl-CoA levels on analog production. Increases in acyl-CoA concentrations may eliminate the bottleneck of substrate loading, but bottlenecks further downstream in polyketide biosynthesis may limit analog production in the current system. Previous improvements have been observed with a DEBS 1 mutant in *Streptomyces coelicolor* (Brown 1995), and similar applications may be amenable to *E. coli* systems.

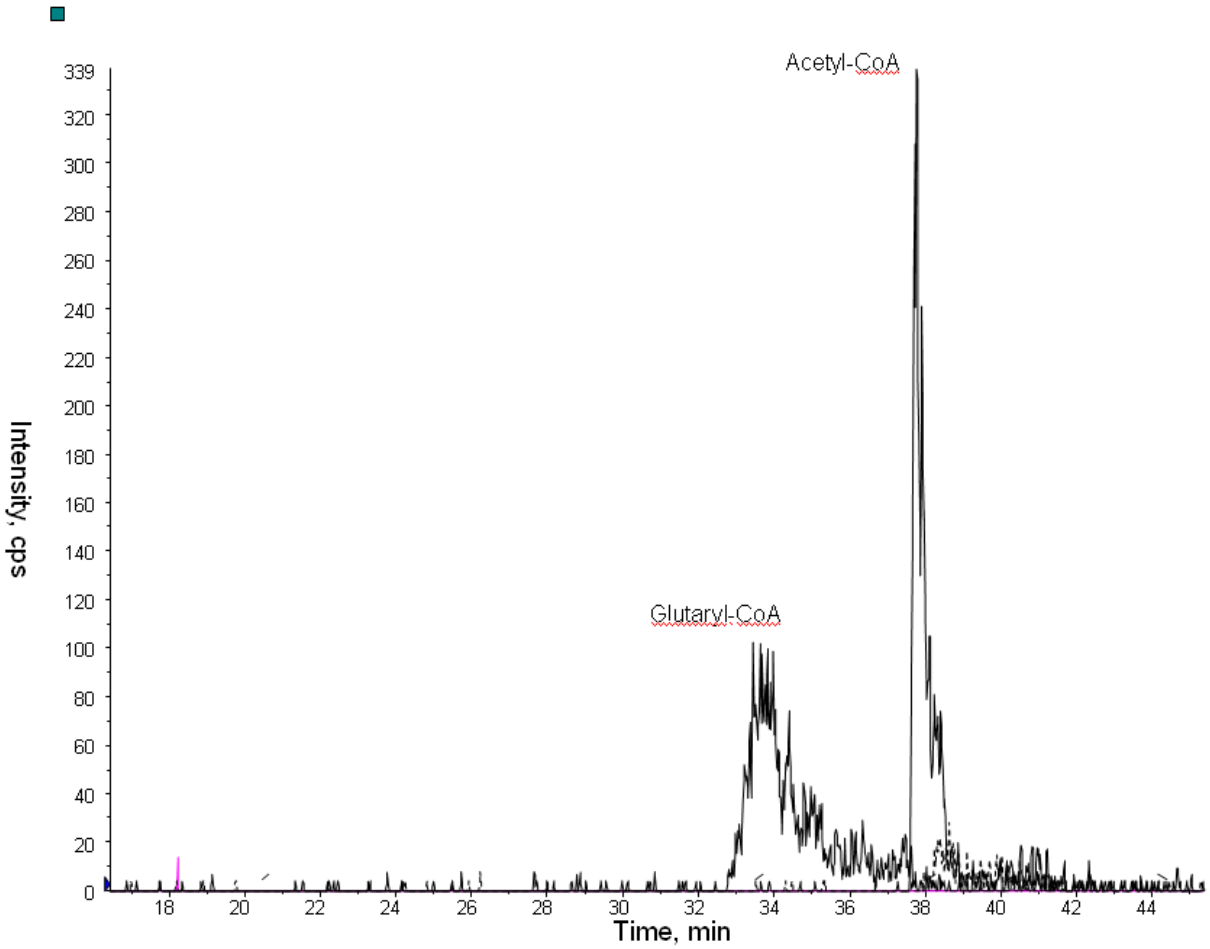
Appendices



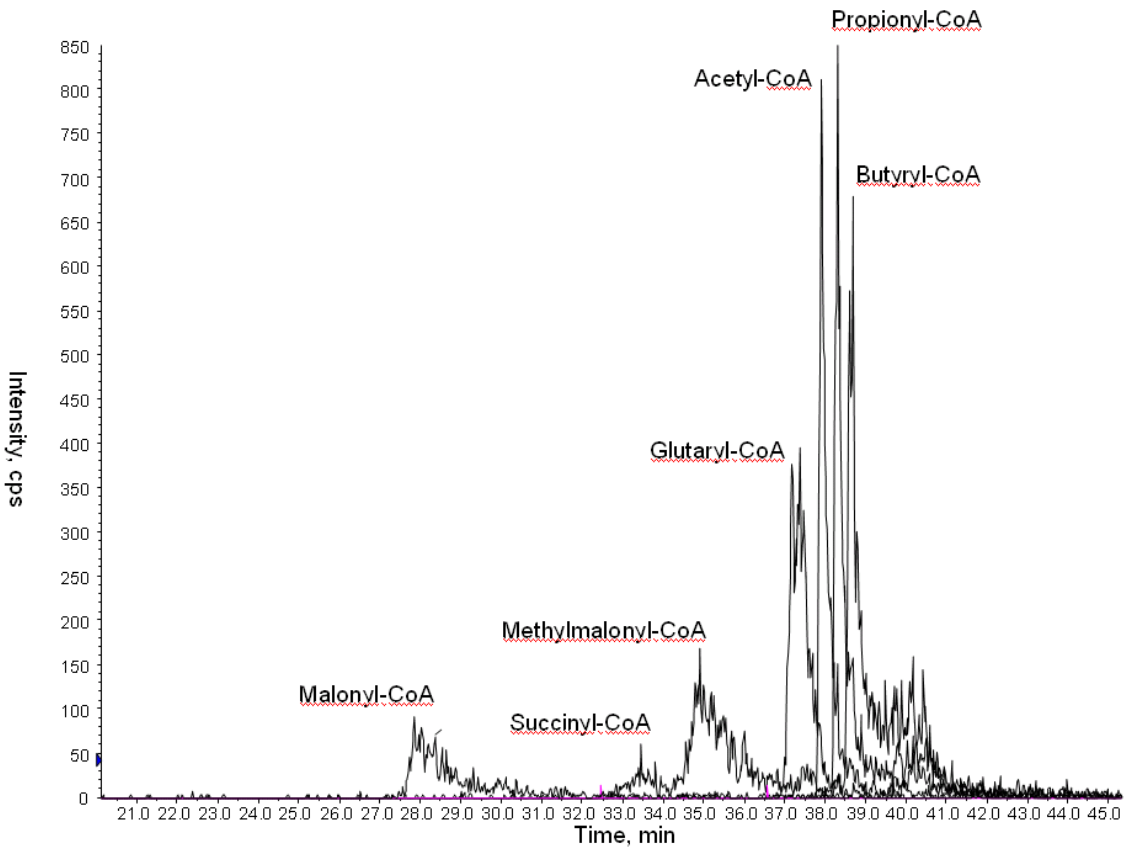
Appendix 1. Representative chromatogram of BAP1/pJWA1 with 20 mmol l^{-1} sodium propionate and 100 µmol l^{-1} IPTG.



Appendix 2. Representative chromatogram of BAP1/pJWA1 with 20 mmol l^{-1} sodium butyrate and 100 µmol l^{-1} IPTG.



Appendix 3. Representative chromatogram of BAP1/pJWA1 with 20 mmol l^{-1} sodium and 100 µmol l^{-1} IPTG.



Appendix 4. Representative chromatogram of 10 μmol l^{-1} standard acyl-CoAs

Parameters Tested for Method Optimization

Temperature:	130˚C, 250˚C, 350˚C
Curtain Gas:	30, 50 psi
Nebulizer Gas:	20, 30, 50 psi
Auxiliary Gas:	10, 20, 30 psi

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