A new approach to mutagenesis in *Clostridium acetobutylicum*.

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Thesis submitted to the University of Nottingham

for the degree of Master of Research

September 2015

Abstract

Clostridium acetobutylicum has long been of interest to industry due to its solventogenic capabilities through an endogenous acetone-butanol-ethanol (ABE) fermentation pathway. Cooksley *et al*., (2012) carried out a large scale targeted mutagenesis experiment of genes in the *C. acetobutylicum* ATCC 824 ABE pathway. In this study they were unable to knock out several genes central to the pathway, including the main hydrogenase *hydA* and thiolase *thl*; in the case of a third gene phosphotransbutyrylase *ptb*, a knock out mutant was isolated only where a frameshift mutation in the thiolase gene had also occurred. The aim of this study was to use a modified ClosTron with a negative selection marker to: 1) create gene knock down strains of *hydA, thl* and *ptb* respectively in *C. acetobutylicum* ATCC 824. 2) cure these of the ClosTron plasmid to create pure knock out mutant strains using a toxic negative selection marker *codA* (cytosine deaminase). 3) analyse resultant mutant strains produced for potentially 'compensatory' mutations, such was the case in Cooksley *et al.*, (2012) where a double knock-out strain of *ptb* and *thl* was isolated, despite not achieving single knock-outs of either gene individually. If possible, the phenotypes of these mutants would be analysed using GC-MS fermentation analysis.

This study was successful in isolating a ClosTron plasmid cured *thl* knock out mutant, but was unable to carry out wide scale analysis in the timescale available. However, this mutant does serve as proof of the method, using a modified ClosTron, and enables future research into isolation of mutants previously impossible to achieve.

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Acknowledgements

I would like to give thanks to my principal supervisor Professor Nigel Minton, for his personal enthusiasm for this project and enabling me to undertake this MRes year within his research group. To my secondary supervisor Dr Ying Zhang for her early help with practical techniques in molecular microbiology. My bench supervisor Dr Hengzheng Wang, for introducing me to the practicalities of working with *Clostridium acetobutylicum*. To Ryan Hope, for always being willing to give advice, making useful suggestions and his general encouragement throughout my time with the CRG. To Lorna Finch, the ultimate supportive friend.

To my parents, for supporting me through the ups and downs of this year and so much more, I am endlessly and continuously thankful. I literally could not have done it without you both.

1.0 Introduction

The *Clostridium* genus can be generally recognised as obligatory anaerobic rod-shaped bacteria with Gram-positive cell walls that are capable of producing endospores. In practice there is a great deal of phylogenetic heterogeneity between *Clostridium* species which allows for their presence in a diverse variety of habitats. This heterogeneity goes some way to explaining their relevance in multiple avenues of science; as pathogens as well as medically and industrially relevant bacterial species. Pathogenic species include *Clostridium perfringens, Clostridium difficile, Clostridium botulinum* and *Clostridium tetani* which are associated with a range of diseases and conditions in humans and animals (Baron *et al*., 1996). But on the whole, *Clostridium* species are innocuous and ongoing research has even found ways to utilise features of the bacteria, for instance Kubiak & Minton (2015) showed the potential of *Clostridium* spores as a medical delivery agent.

In industrial biotechnology, there has been focus on solventogenic species of *Clostridium* such as *C. acetobutylicum* and *C. beijerinckii.* These species possess a pathway named the 'ABE' pathway which allows the bacteria to produce acetone, butanol and ethanol from various sugar sources at a ratio of 6:3:1. A diagram of this pathway including the major genes involved is shown in Figure 1. The gene annotations in *C. acetobutylicum* are also shown.

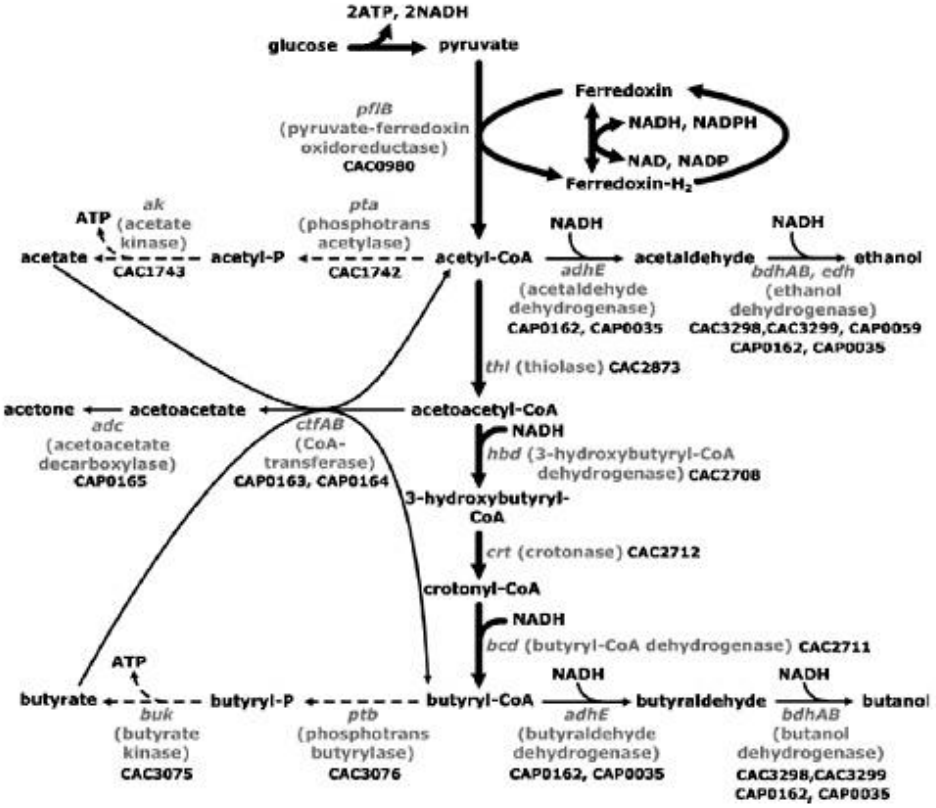


Figure 1. ABE fermentation pathway. Reference Lee *et al.* (2008a). Note, the hydrogenase gene *hydA* is not displayed in this figure, but is linked to this pathway, where it plays a role in recycling NADH and converting ferrodoxin-H_2 to ferrodoxin + H_2, as shown in the top right of the diagram.

Production of these solvents is through a biphasic fermentation with two separate physiological phases. Firstly, acidogenic fermentation takes place while bacteria are in exponential growth phase. This produces butyrate, acetate, CO_2 and H_2, which lower the pH of cell cultures. When the bacteria enter stationary phase, solventogenic fermentation takes place. In this secondary fermentation, the previously produced compounds are utilised and reassimilated to produce acetone, butanol and ethanol. This is a simplistic explanation of a process with a complex underlying control

system which includes genetic regulation, metabolic shift and cellular signal integration (Liao *et al.*, 2015).

ABE fermentation has previously been carried out on a large scale beginning in the 1910s, when it was used for production of acetone, which was used as a solvent in the manufacture of cordite in the First World War (Jones & Woods, 1986). After the war interest in ABE fermentation became focused on production of butanol, which can be used as a fuel as well as having applications in the manufacture of enamels and lacquers (Mascal, 2012). However, most plants were forced to close down in the 1950s as competition from the petrochemical industry, as well as price spikes for feedstock, rendered the process economically inviable, though some plants did remain open in China, Russia and South Africa until the 1980s (Green, 2011). Interest in large scale ABE fermentation has recently been renewed due to the unsustainable nature of fossil fuels and increasing worries about fossil fuel contributions to atmospheric damage and subsequently global warming. Biobutanol is seen as an attractive alternative to petroleum oil, which is currently the world's most utilised fuel. Fossil fuels accounted for 80.3% of energy consumption in 2014, with petroleum oil the biggest single energy source at 34.7%, while renewable fuels from biomass currently account for 4.7% (USA EIA, 2015). Bioethanol is the most commonly used biofuel in the world (US EIA, 2015) as it can be produced in high yields from sugars and starches using microorganisms such as the yeast *Saccharomyces cerevisiae* or the bacterium *Zymomonas*

mobilis through a relatively simple process (Lin & Tanaka, 2006). However, there is strong interest in the production of biobutanol due to the chemical advantages it holds over ethanol. These include: higher energy content (less fuel needed for same energetic output), lower volatility, it is less hydroscopic, less corrosive and can be used in existing engines without modification as a straight swap for gasoline (Lee *et al.*, 2008b). The main disadvantage of the ABE pathway is that butanol production is not currently cost-effective when compared with other bioprocesses producing exclusively ethanol. While the bioprocesses may have similar production costs and issues surrounding the availability and pre-treatment of sustainable feedstocks, bioethanol production has the advantage of the microorganisms involved being moderately well suited to the purpose of production; relatively high yields of ethanol can be achieved. In contrast, the ABE process in clostridia suffers from the bacteria's high sensitivity to increasing solvent levels, thus limiting the yield and titre of solvents that can be produced. Additionally, ABE fermentation causes the production of three different solvents meaning an excess of unwanted solvent is produced. Research is ongoing into how the ABE pathway can be modified to streamline the process to favour butanol production and reduce production of ethanol and acetone, or how these solvents could be reutilised to produce more useful chemicals. For instance, metabolic engineering approaches have been used to express secondary alcohol dehydrogenase genes from *C. beijerinckii* in *C.*

acetobutylicum in order to convert acetone to the more industrially relevant chemical isopropanol (Lee *et al.,* 2012).

In the past, research into *Clostridium* species has been impaired by lack of tools for genetic manipulation. Historically, Clostridia could not be genetically manipulated using the conventional methods of homologous recombination used in many bacteria and generally have poor DNA transformation efficiencies across the genus (Song *et al.*, 2015). This means mutants created were confined to single-crossover knockout mutants, as referenced in Heap *et al.* (2007), which are an unstable form of mutation liable to revert to wild type. Gene knockdown mutants have also been created using antisense RNA technology (Desai & Papoutsakis, 1999), which allows for some level of gene function analysis but is not an effective method for stable strain development due to the continued presence of the gene in the bacteria.

In the same year, Heap *et al*. (2007) and Shao *et al.* (2007) independently published studies which utilised a technique branded 'TargeTron technology' to create vectors which allow the formation of stable insertion mutations in *Clostridium*. TargeTron vectors are a class of gene targeting vector developed commercially by Sigma-Aldrich, which contain a mobile group II intron and use specific base pairing to recognise DNA target sites. Group II introns are particularly well suited to gene targeting as they can be 'reprogrammed' to target to specific DNA target sites, using an algorithm devised by Mohr *et al*. (2000) to identify the appropriate

base pairs to modify and then synthesising the TargeTron vector appropriately. Additionally, as group II introns by nature are mobile ribozymes (retrotransposons), they are able to self-splice, with the factors for site-specific integration and reverse transcription encoded within intron RNA, allowing them to function without reliance on host factors. Heap *et al*. (2007) and Shao *et al.* (2007) both adapted this system for use in clostridial hosts including *C. acetobutylicum*, using a specific group II intron from the *ltrB* gene of *Lactococcus lactis* (Ll.ltrB). This intron had previously been used for gene inactivation in *C. perfringens* (Chen *et al.*, 2005), however their TargeTron vector was not suitable for use in different species of *Clostridium* due to incompatible replicon and promoter systems. The ClosTron vector plasmid developed by Heap *et al.* (2007) allows for use of the TargeTron in a broader range of *Clostridium spp.*, as well as including a RAM (retrotransposition-activated marker) element for more efficient screening purposes. The RAM element, developed by Zhong *et al.,* (2003) uses a group II intron containing an antibiotic resistance gene, which is interrupted by a group I *td* intron. This group I intron is self-splicing in a reaction coupled to integration of the group II intron in to the genome, linking acquisition of antibiotic resistance to successful integration. A subsequent paper by Heap *et al.* (2010) further improved the ClosTron vector system by use of a modular plasmid format, allowing for exchange of plasmid markers and replicons to increase ease of use in different *Clostridium spp.*

This aim of this masters research project was to serve as an investigation into results gained by Cooksley *et al.,* (2012) in their study 'Targeted mutagenesis of the *Clostridium acetobutylicum* acetone–butanol–ethanol fermentation pathway' and the possible implications of this. In their study, a number of ClosTrons were synthesised targeted to genes identified as part of the metabolic pathway for ABE fermentation in *C. acetobutylicum.* This pathway is shown in Figure 1. The aim of their work was to study the phenotypes of each of these gene defective strains, primarily through the analysis of fermentation products, in order to advance knowledge on solvent production of *C. acetobutylicum* and apply this to development of the strain for industry. The study found two of their target genes could not be inactivated using conventional ClosTron technology, despite repeated attempts using different intron insertion sites into the targets. These genes were the main hydrogenase gene *hydA* and thiolase *thl*. Further research carried out by members of the Clostridia Research Group at the University of Nottingham were unable to inactivate *thl* using an alternative gene knock out method- the allelic exchange method (Heap *et al.,* 2012; N.P. Minton, Personal Communication). In fact, this group found that they were unable to inactivate any of the genes in the ABE pathway which encode the conversion of acetyl-CoA to butyryl-CoA, including *thl*, *hbd* (3-hydroxy-butyryl-CoA dehydrogenase) and *bcd* (butyryl-CoA dehydrogenase). These data suggest that inactivation of these genes lead cells to become non-viable under the conditions tested.

The inception of the present study was brought about by the isolation, experimentation and subsequent genome sequencing of a *ptb* (phosphotransbutyrylase) knock out mutant produced by Cooksley *et al.* (2012). The isolation of a successful intron insertion event into this gene was extremely difficult, requiring the design of three different retargeting ClosTron plasmids before a single putative mutant was obtained. During solvent analysis of the mutants created, this *ptb* knock out mutant exhibited an unexpected phenotype whereby ethanol became the primary fermentation product by far, with little butanol and no acetone produced. This was in conflict with what would be expected based upon current models of ABE fermentation where the knock out of the *ptb* gene was forecasted to positively impact butanol production due to knock out of the butyrate production 'arm' shown in the bottom left of Figure 1. Further analysis of the mutant found that genetic complementation could not be achieved using a vector containing the wild type *ptb* gene. Genome sequencing of the strain revealed that it contained a single nucleotide polymorphism (SNP) in the *thl* gene, leading to a frameshift mutation in the *thl* gene and resulting in a *ptb*-*thl* double knock out strain. The creation of this strain was surprising as the *thl* gene was one of several genes previously mentioned where knock out mutants could not be isolated singularly; posing a question as to how these two mutations were viable in combination but not alone. The work presented here aimed to investigate two alternative hypotheses relating to the viability of this strain. Namely, whether the isolation of a *ptb* mutant was possible

because the cell in which the insertion event took place had acquired a secondary *thl* mutation, or alternatively that the acquisition of a *thl* frameshift was the result of the intron insertion into *ptb*. Reframed, these hypotheses present a 'which came first, the chicken or the egg' conundrum as to whether the frameshift mutation in the *thl* gene was present in the strain prior to transformation with the ClosTron targeted to *ptb*, for example due to a random mutation in the wild type *C. acetobutylicum* stock used for the transformation, or if this *thl* mutation came about as a compensatory mutation in tandem with the knockout of *ptb*, as an adaption of the mutant strain to allow viability. Crucially, this study wished to continue to attempt isolations of gene knock out mutants which it has previously not been possible to isolate, namely those of *hydA* and *thl,* and then analyse these for secondary mutations that may have arisen and contributed to their viability.

In this study, an unconditional *thl* mutant was achieved in *C. acetobutylicum*, however due to time constraints of the project genomic DNA sequence data could not be gained. In addition, when carrying out fermentation analysis profiles, glycerol stocks of the plasmid cured *thl* mutant could not be revived.

2.0 Materials and Methods

2.1 Bacterial strains and media

The bacterial strains used in this project are listed in Table 1. *C. acetobutylicum* ATCC 824 and its mutant derivatives were grown in 2* yeast tryptone medium (Sigma-Aldrich, US) with additional glucose to a 2% w/v final concentration. The use of this medium is referenced as 2*YTG. As for Cooksley *et al.* (2012), supplemented *Clostridium* basal medium (CBMS) was used for fermentation assays in an anaerobic cabinet (A95 TG Anaerobic Workstation, Don Whitley Scientific Ltd) containing an atmosphere of 80% nitrogen, 10% hydrogen and 10% carbon dioxide at a temperature of 37^{o}C. Screening experiments were undertaken in anaerobic conditions using either Clostridial Growth Medium (CGM) (Hartmanis & Gatenbeck, 1984) or non-supplemented *Clostridium* basal medium (CBM) (O'Brien & Morris, 1971). *Escherichia coli* strains were grown in Luria-Bertani medium at 37^{o}C**.** Antibiotics were used at the following concentrations: thiamphenicol, 15µg/ml for *C. acetobutylicum*; chloramphenicol, 25µg/ml, tetracycline 10µg**/**ml for *E. coli*. 5-fluorocytosine was used at a concentration of 100µg/ml.

2.2 Plasmids, primers, DNA techniques

Table 1. List of bacterial strains and plasmids.

Strain or plasmid	Relevant Characteristics	Source
Bacterial strains		
Clostridium acetobutylicum ATCC 824	Wild type	ATCC
Escherichia coli XL1-blue	DH5α™ derivative, 5-alpha F'I^q Competent	New England Biolabs
Cac-*thl*406s::CT	Uncured *C. acetobutylicum* 824 *thl* mutant	This Study
Plasmids		
pMTL007C-E2	Clostridial expression vector containing ERM RAM. Cm^R	Heap *et al.* (2010)
pMTL007C-E2::cac-ptb-505a	ClosTron plasmid retargeted to *C. acetobutylicum ptb* gene	Cooksley *et al.* (2012)
pMTL007C-E2::cac-ptb-87s	ClosTron plasmid retargeted to *C. acetobutylicum ptb* gene	Cooksley *et al.* (2012)
pMTL007C-E2::cac-adhE2-696s	ClosTron plasmid retargeted to *C. acetobutylicum adhE2* gene	Cooksley *et al.* (2012)
pMTL007C-E2::cac-bdhA-459s	ClosTron plasmid retargeted to *C. acetobutylicum bdhA* gene	Cooksley *et al.* (2012)
pMTL007C-E2::cac-hydA-936s	ClosTron plasmid retargeted to *C. acetobutylicum hydA* gene	Cooksley *et al.* (2012)
pMTL007C-E2::cac-thl-406s	ClosTron plasmid retargeted to *C. acetobutylicum thl* gene	Cooksley *et al.* (2012)
pMTL007CC-E2::cac-ptb-505a	ClosTron plasmid retargeted to *C. acetobutylicum ptb* gene	This Study
pMTL007CC-E2::cac-ptb-87s	ClosTron plasmid retargeted to *C. acetobutylicum ptb* gene	This Study
pMTL007CC-E2::cac-adhE2-696s	ClosTron plasmid retargeted to *C. acetobutylicum adhE2* gene	This Study
pMTL007CC-E2::cac-bdhA-459s	ClosTron plasmid retargeted to *C. acetobutylicum bdhA* gene	This Study
pMTL007CC-E2::cac-hydA-936s	ClosTron plasmid retargeted to *C. acetobutylicum hydA* gene	This Study
pMTL007CC-E2::cac-thl-406s	ClosTron plasmid retargeted to *C. acetobutylicum thl* gene	This Study
pMTL007CC-E2	ClosTron plasmid with *catP* replaced by *catP-codA*	Hengzheng Wang (CRG)
pMTL-SC7515	In-frame deletion vector, *repL*, Cm^R, *codA*, *adc*-recombinant allele	Culture Collection (CRG)
PMTL-ME2	In-frame deletion vector, repL, Cm^R, *pyrE*	Culture Collection (CRG)
pAN2	Plasmid containing Φ3T I methyltransferase gene of *B. subtilis* phage Φ3*t*I	Heap *et al.* (2007)

Table 2. List of primers used, including their purpose.

Primer	Sequence 5'-3'	Purpose
Hpa-pyrE /F	AATATTGTTAACTAAGGAGAAGATATAAA TGAGTAATATAAATGT	Isolate *pyrE* fragment
Hpa-pyrE/R	AATATGTTATCTTATTTTTGTTCTCTACTAC CTGGTTTTACAAAA	
ClosTron RT/F	ATTTTGTAGATGTAGATAGGATAATAGAA TCC	Presence of intron DNA
ClosTron RT/R	CTCTGTTATTGTTCGTTCGTAAAAATTC	
AdhE2/2F	GGAGCAGGAAATACACCAGC	Presence of ClosTron in *C. acetobutylicum adh*E2 gene
AdhE2/2R	CGTGTGTCCACTTCCACCTATAGGTGGAA GTGGACACACG	
Thl/2F	GGTTCAGGACTTAGAACAGTTAGC	Presence of ClosTron in *C. acetobutylicum thlA* gene
Thl/2R	CTGCTGTTATTCCCATGTGGTACCACATGG GAATAACAGCAG	
HydA/F	GGCTCCATCAGTAAGAACTGC	Presence of ClosTron in *C. acetobutylicum hydA* gene
HydA/R	CCATAGCAGGATCAACTTCACC	
Ptb/F	AAGGGAGTGTACGACCAGTG	Presence of ClosTron in *C. acetobutylicum ptb* gene
Ptb/R	GTCCGTCAACTACACAACCTTT	
BdhA/F	GGGAGGAGTGCAATAGACTG	Presence of ClosTron in *C. acetobutylicum bdhA* gene
BdhA/R	GCACCTTCAACACCACTAAGT	
CatP/F	CGTTGAACGAAACTTGAATTTGAGAGGGA ACTTAG	Presence of *catP* gene
CatP/R	CCGGCGCGCCGTTTAAACAAGTTAACTATT TATCAATTCCTGCAATTCGTTTAC	

Genomic DNA preparation was carried out using the Qiagen DNAeasy blood and tissue kit or by phenol:chloroform:isoamyl alcohol extraction (Cartman & Minton, 2010). Plasmid isolation was carried out either using Sigma GenElute Plasmid Miniprep kit or Qiagen QIAprep Miniprep kit. Purification of DNA fragments from agarose gels was carried out using

Sigma GenElute DNA extraction kit. Restriction enzymes were supplied by New England Biolabs and ThermoScientific and used according to the manufacturer's instructions. For ligation reactions Promega LigaFast Rapid DNA ligation system was used. Protocols for agarose gel electrophoresis and transformation of *E. coli* were carried out as described in Sambrook *et al.* (1989). PCR amplifications were carried out using ThermoScientific Dream Taq green PCR master mix (2X); the reaction programme used for PCR reactions was: samples heated to 95^{o}C for 3 minutes (10 minutes for colony PCRs), then cycles were as follows: denaturation at 95^{o}C 30 seconds, annealing at the primer melting temperature minus 5 for 30 seconds and elongation at 72^{o}C for 30 seconds/kb product. The final step was a hold at 72^{o}C for 10 minutes. Nucleotide sequencing was done by Source BioScience LifeSciences Ltd, Nottingham.

2.3 Transformation of *C. acetobutylicum*

Plasmid DNA was methylated by purifying the plasmid from *E. coli* containing plasmid pAN2, in order to protect DNA from *C. acetobutylicum* Cac8241 DNA restriction activity (Heap *et al.,* 2007). DNA was transformed into *C. acetobutylicum* competent cells using the electroporation protocol 2.0kV, 2525 µF and ∞Ω program on a Biorad Genepulser. The transformation protocol was as described by Mermelstein & Papoutsakis (1993).

2.4 Transformation of *E. coli*

E. coli XL1-blue was transformed using chemically competent cells (New England Biolabs). The protocol for using chemically component cells was as follows: 3µL ligation reaction mixture was added to 5µL *E. coli* cells. This was incubated at 4^{o}C for 30-40 minutes, followed by heat shock at 42^{o}C for 45 seconds and then 2 minutes on ice. Immediately following this 500µL recovery SOC medium (Sigma Aldrich, UK) was added. The mixture was shaken in an Eppendorf tube for 1 hour at 37^{o}C before being plated at varying concentrations on LB medium supplemented with appropriate antibiotics for plasmid selection.

2.5 Construction of ΔRAM ClosTrons with *codA* negative selection marker

Targeted ClosTron plasmids pMTL007C-E2::cac-ptb-505a, pMTL007C-E2::cac-ptb-87s, pMTL007C-E2::cac-adhE2-696s, pMTL007C-E2::cac-bdhA-459s, pMTL007C-E2::cac-hydA-936s, pMTL007C-E2::cac-thl-406s previously designed and synthesised in the study Cooksley *et al.* (2012) were utilised as donors of the retargeted introns. These were digested with restriction endonuclease enzymes *Mlu*I and *Hin*dIII to excise a fragment containing the retargeted intron, which was cloned into ΔRAM pMTL007CC-E2 (Hengzheng Wang, Figure 2) which had been cut with the same enzymes to excise the RAM element and untargeted intron. pMTL007CC-E2 contains *codA* from the plasmid pMTL-SC7515 cloned next to the *catP* gene, as well as the *ltrA* gene. Clones were firstly confirmed

using PCR (Table 2, ClosTron RT primers) and then by restriction digest with *Nde*I and *Hin*dIII.

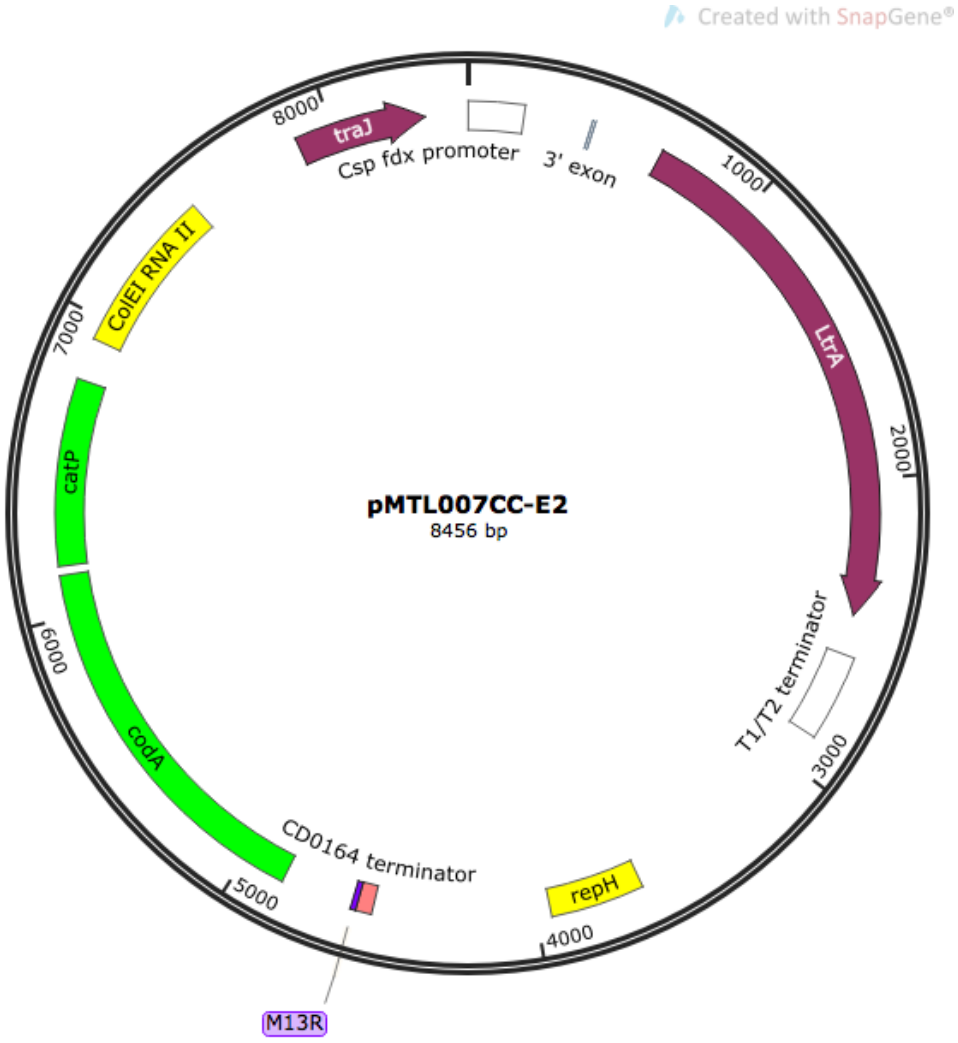


Figure 2. Plasmid map of pMTL007CC-E2. The map was created using SnapGene software.

2.6 Incorporation of *pyrE* negative selection marker

Attempts were made to clone the *pyrE* gene for 5-fluoroorotic acid resistance into the retargeted pMTL007CC-E2 vectors using a similar methodology of excising the RAM element with *Mlu*I, removing the *catP* gene with *Pme*I and *Fse*I and cloning in a *catP-pyrE* fragment from the

vector pMTL-ME3 into the ΔRAM Δ*catP* pMTL007CC-E2 vector also cut with *Pme*I and *Fse*I. However, restriction digestion analysis with *Hind*III and *Nde*I showed cloning had not been successful therefore *pyrE* directed cloning was discarded in favour of *codA,* although the original project proposal had planned to have both negative selection markers in the final plasmid.

2.7 Gene knockout protocol using pMTL007CC-E2 in *C. acetobutylicum*

The final plasmids pMTL007CC-E2::cac-ptb-505a, pMTL007CC-E2::cac-ptb-87s, pMTL007CC-E2::cac-adhE2-696s, pMTL007CC-E2::cac-bdhA-459s, pMTL007CC-E2::cac-hydA-936s and pMTL007CC-E2::cac-thl-406s were methylated using pAN2 and then transformed in to *C. acetobutylicum* by electroporation and plated on to CGM containing thiamphenicol. Colonies that grew were patch plated 50 colonies per plate in duplicate onto thiamphenicol supplemented CGM to retain the plasmid. Patch plates were screened after 18 hours growth by washing the 50 patch colonies into 2ml 2*YTG and centrifuging for 10 minutes at 21, 000 x *g*. Supernatant was discarded and the pellet retained for analysis. Mixed colony genomic DNA was isolated from the pellet and analysed by PCR using specific primers for the gene targeted by the ClosTron being used (Table 2). The presence of a mutant band in the PCR analysis was used as evidence that at least 1/50 patches from the colony PCR contained a successful mutant where the ClosTron had spliced into the correct gene. To isolate the correct mutant patch, patches were

screened in pools of 10 from the original 50 and then streaked individually to single colonies and screened until a single mutant isolate was obtained.

2.8 Plasmid curing

Mutants successfully isolated containing correct genomic insertion of the ClosTron were re-streaked several times on antibiotic free medium before plating on medium containing appropriate antibiotics (e.g. thiamphenicol) in order to show plasmid loss before confirmation with appropriate primers (Table 2). This is the conventional method of plasmid curing used when using ClosTron technology.

If plasmid could not be lost using this conventional re-streaking after 7 re-streaks, the process was repeated using 5-fluorocytosine supplemented CBM medium at a lethal concentration (10mg/ml), in order to force the loss of the plasmid. The mutant colony was spread onto CBM plates containing 5-FC over a 10-fold serial dilution and any colonies growing were spread onto both CGM (to retain stock) and CGM supplemented with thiamphenicol to confirm plasmid loss. Genomic DNA was also prepared from the colonies and screened using two separate PCR reactions to confirm plasmid loss and continued intron presence in the target gene.

2.9 Fermentation and analytical techniques

Batch fermentation experiments were carried out in triplicate 25ml volumes of CBMS broth in 100ml conical flasks. Cultures were inoculated to a starting OD_{600} of approximately 0.05 or 0.1 (slow growing mutant

cultures) using an overnight starter culture. The fermentations were carried out for 96h in an anaerobic cabinet. 1ml samples were taken every 90 minutes for 12h and every 24h following that. At each time point growth was also measured using OD_{600}. Samples taken were centrifuged at 16,000 x *g* for 5 minutes. Supernatants were stored at -20^{o}C before analysis by gas chromatography. As described by Cooksley *et al*. (2012) acetone, butanol, ethanol, acetate and butyrate were quantified using a Thermo Focus GC with a 30m TR-FFAP column and a flame ionization detector, the protocol used for GC analysis was identical to that referenced. Samples were prepared for GC analysis using an in-group protocol as follows: samples were thawed at room temperature, 500µL of the sample was added to a 1.5mL Eppendorf tube, 5 µL 10M H_2SO_4 was added and vortexed briefly. 500 µL propyl propionate containing 50mM valeric acid was added for use as an internal standard. Samples were vortexed for 10 seconds before centrifuging at 21, 000 x *g* for 5 minutes. 300µL was carefully pipetted from the top layer into a GC sample vial containing a glass insert and the lid quickly secured before loading on to the machine. Solvent analysis was carried out on *C. acetobutylicum* wild type and an uncured *thl* knock out mutant.

3.0 Results and Discussion

3.1. Mutagenesis using a modified ClosTron in *C. acetobutylicum*

The first phase of this work was to clone gene 'knock-down' ClosTron plasmids into *C. acetobutylicum*. These knock-down plasmids were each targeted to one of 5 different genes in the ABE pathway: *adhE2, bdhA, hydA, ptb* and *thl*. Of these the first two genes *adhE2* (alcohol/aldehyde dehydrogenase 2) and *bdhA* (butanol dehydrogenase) had been successfully knocked out in *C. acetobutylicum* previously by Cooksley *et al*., (2012) using conventional ClosTron plasmids. The latter three genes *hydA, ptb* and *thl* had not previously been knocked out in the bacterium, despite repeated attempts using different intron target sites.

In order to isolate knock outs of these potentially essential genes, a RAM-less ClosTron with a negative selection marker was developed. The use of a RAM-less ClosTron allows the initial production of gene knock down mutants, where expression of the targeted gene is seen at a variable impaired rate in comparison to the wild type. This is due to the functionality of the intron whereby once it has inserted into the sense orientation of a gene, the presence of an *ltrA* intron-encoded protein (IEP) in the ClosTron plasmid will lead to splicing of the intron from transcribed mRNA, resulting in translation of a functional gene product (Lambowitz & Zimmerly, 2011). Therefore, production of conditional knock out mutants, where the target gene still has some activity, should allow survival of cells in which the intron has spliced into a gene essential for cell viability.

However, the splicing activity of the *ltrA* IEP is impaired by the presence of the large RAM element, therefore this element was excised from the ClosTron plasmid. The nature of the knock down mutants created also led to the necessity of a negative selection marker. This is because conditional mutants of essential genes are maintained by the presence of the *ltrA* gene splicing action allowing gene activity from the target gene, without which the cell would be rendered inviable. Therefore, the cell cannot lose the plasmid containing *ltrA* unless containing the plasmid was also lethal to the cell. The use of a negative selection marker theoretically would allow for selection of cells that have incurred secondary, compensatory mutations which allow the survival of unconditional mutants with knock out of 'essential' genes. The negative selection marker used in this study was the *codA* gene encoding cytosine deaminase which converts the innocuous compound 5-flurocytosine (5-FC) into the highly toxic 5-flurouracil (5-FU). Attempts were also made to include a second negative selection marker, *pyrE,* encoding phosphoribosyltransferase, which allows selection using 5-fluoroorotic acid (FOA). A schematic of this process is shown in Figure 3.

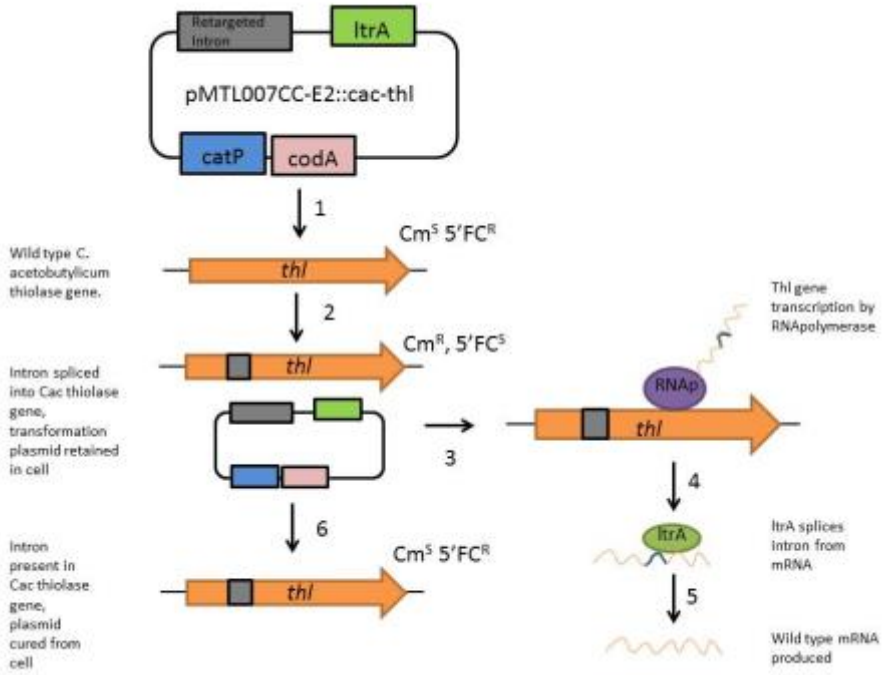


Figure 3. Schematic of experimental logic, using RAMless ClosTron with negative marker to isolate rare mutants in *C. acetobutylicum*, demonstrated with the thiolase gene. 1) ClosTron containing retargeted intron to the thiolase gene, *ltrA* gene (intron encoded protein), *catP* gene for chloramphenicol/thiamphenicol resistance, *codA* gene for negative selection with 5-flurocytosine (5-FC), transformed into wild type *C. acetobutlicum*. 2) intron splices into thiolase gene, disrupting the gene. The transformation plasmid is retained in the cell, causing a thiamphenicol resistant, 5-FC sensitive phenotype. 3) RNA polymerase transcribes mRNA from the intron interrupted thiolase gene. 4) Retained presence of *ltrA* gene in the cell causes the *ltrA* intron encoded protein to splice the intron from the thiolase gene at an unknown frequency. 5) Wild type *thl* mRNA is produced, allowing for the translation of functional thiolase gene, at a frequency proportional to intron splicing. 6) *C. acetobuylicum* cell from which the plasmid has been cured, reversion to thiamphenicol sensitive, 5-FC resistant phenotype. *ltrA* gene no longer present therefore intron is not spliced from transcribed mRNA, resulting in a non-functional gene product after gene translation.

3.1.1 Transformation of *C. acetobutylicum* with retargeted pTML-007CC plasmid pMTL007CC-E2::cac-adhE2-696s

Retargeted pTML-007CC plasmids were cloned in *E. coli* as described in the Materials and Methods section. The plasmid pMTL007CC-E2::cac-

adhE2-696s was used as a control plasmid for proof of method, the *adhE2* gene having been knocked out previously by Cooksley *et al.,* (2012) using conventional ClosTron technology. The plasmid was cloned into *C. acetobutylicum* with 100% rate of intron insertion into the target gene. The PCR screen for this is shown in Figure 4, along with a screen for pMTL007CC-E2::cac-thl-406s which shows a 0% insertion rate. The primers used in this screen are shown in Table 2. Each set of primers was designed to amplify part of the target gene, therefore presence of the ClosTron successfully integrated into the gene showed a band larger than the size of the wild type gene. In the case of *adhE2* successful integration events show bands at 1.2kbp while the wild type displays a band obtained from the unmodified gene, which is 468bp, as shown in Figure 4. These PCR screens were done using the mixed genomic DNA of a number of colonies from transformation plates, as it was expected that insertion rates would be low and therefore a mixed colony PCR would be advantageous for screening purposes, as opposed to screening hundreds of colonies individually for rare insertion events.

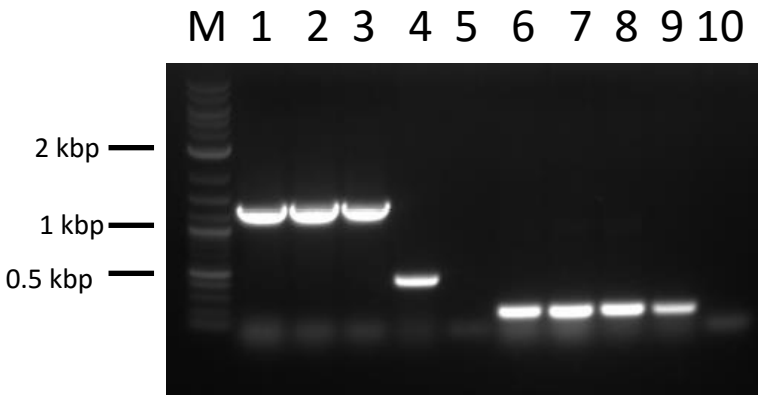
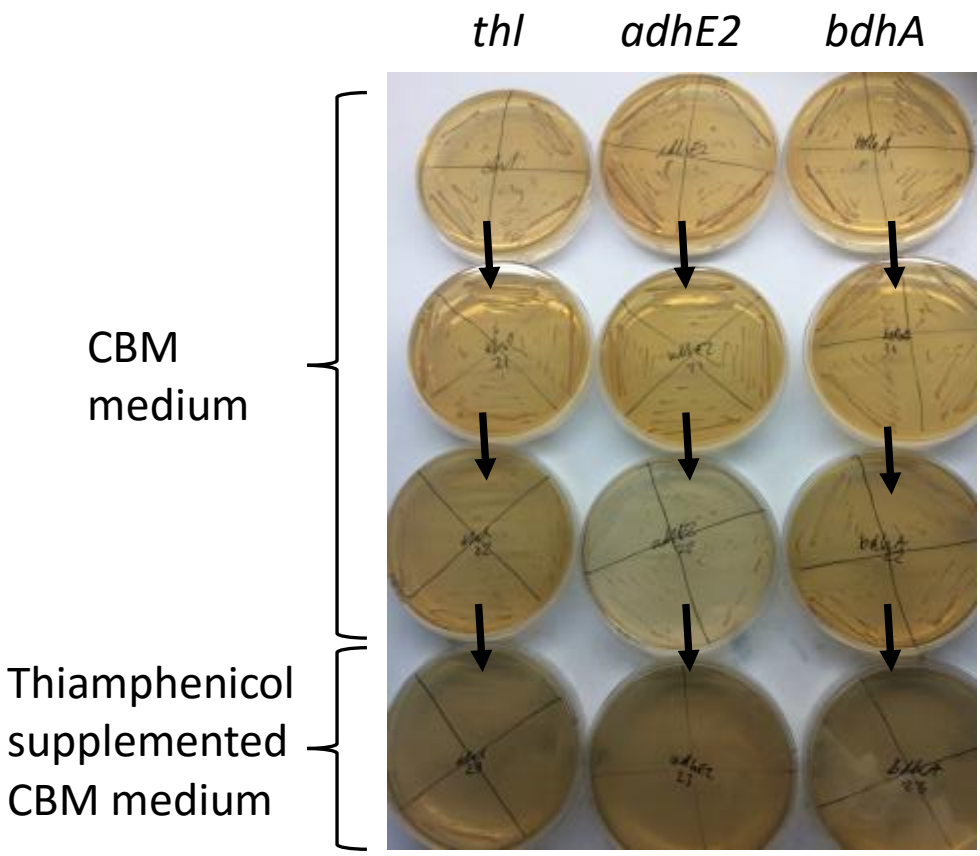


Figure 4. Mixed colony PCR screen for successful intron insertion into *adhE2* and *thl* respectively. Each experimental lane represents the DNA of 50 colonies picked from a transformation plate. M, 2 log DNA ladder. Lane 1-3, successful integration of intron into *adhE2* gene. Lane 4, *C. acetobutylicum* wild type *adhE2* gene. Lane 5, deionised water. Lane 6-8, unsuccessful integration of intron into *thl* gene. Lane 9, *C. acetobutylicum* wild type *thl* gene. Lane 10, deionised water.

In the case of pMTL007CC-E2::cac-adhE2-696s, successful insertions of the intron into the *adhE2* gene occurred at a rate of 100%. This was observed over 3 separate transformations, and insertions were confirmed by DNA sequence analysis. Additionally, in each of the transformations 5-10 colonies were picked and tested independently, showing the same rate of 100% insertion. The same frequency was seen for pMTL007CC-E2::cac-bdhA-459s (results not shown). The *bdhA* gene was also successfully knocked out in *C. acetobutylicum* by Cooksley *et al.,* (2012) using conventional ClosTron technology. In the case of both of these genes, it was possible to cure the bacteria of the targeted pMTL007CC-E2 plasmid without use of the negative selection marker. This was done by re-streaking the transformed bacteria twice on antibiotic free medium (CBM) and then streaking on to thiamphenicol supplemented CBM. The plasmid

was naturally lost by the bacteria by re-streaking, with no growth seen on the antibiotic supplemented medium, since the gene for thiamphenicol resistance was found in the plasmid (Figure 5). As shown in Figure 6, it



was not possible to cure a plasmid from a latterly achieved insertion of pMTL007CC-E2::cac-thl-406s into *C. acetobutylicum* this way.

Figure 5. Curing *C. acetobutylicum* of successfully integrated pMTL007CC-E2 plasmids. The top three plates show successful transformations of pMTL007CC-E2::cac-thl-406s, pMTL007CC-E2::cac-adhE2-696s and pMTL007CC-E2::cac-bdhA-459s in *C. acetobutylicum* on CBM medium. Below each is a re-streak from the above plate. The bottom three plates show the bacteria streaked on thiamphenicol supplemented CBM medium. pMTL007CC-E2::cac-adhE2-696s and pMTL007CC-E2::cac-bdhA-459s have both been successfully cured from the bacteria (no growth). Growth is seen on the bottom left hand plate, indicating pMTL007CC-

E2::cac-thl-406s is still present within the cells and this plasmid has not been successfully cured. A close up of this plate is shown in Figure 6.

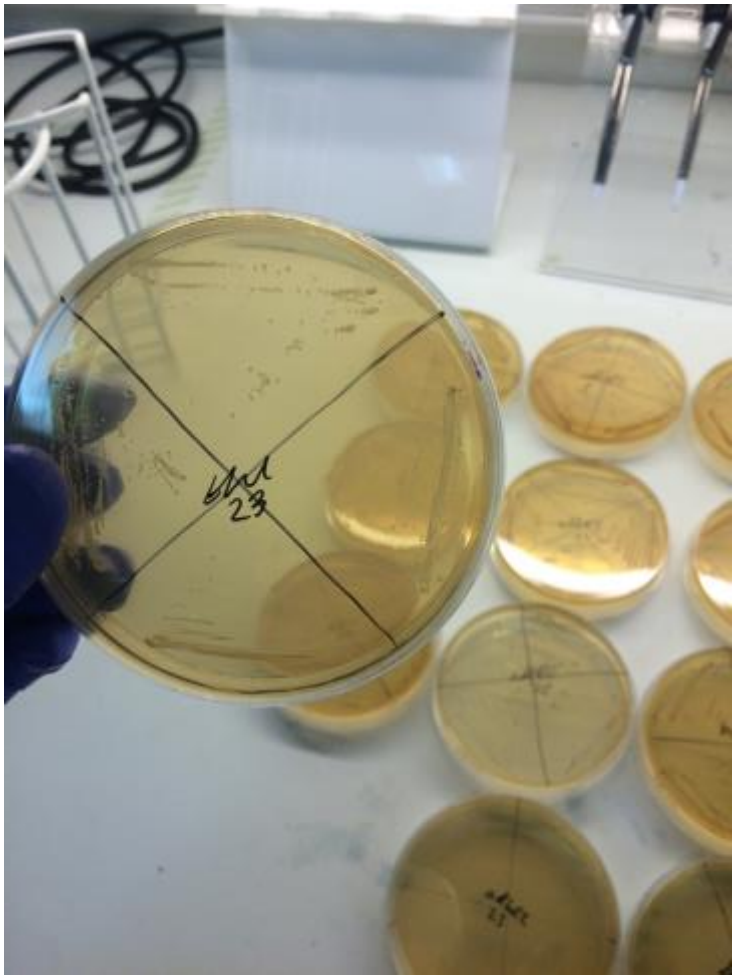


Figure 6. Photograph showing growth of *C. acetobutylicum* containing pMTL007CC-E2::cac-thl-406s plasmid on thiamphenicol supplemented CBM medium.

The high rate of intron insertion into *adhE2* and *bdhA* is interesting in itself, as despite their use in the study as a proof of method, it was unexpected that a 100% knock out rate should be achieved. This result suggests that the inactivity of these genes is advantageous for growth of *C. acetobutylicum*. This could be because transformation of the knock out plasmid into the cell occurs before the cell is undergoing solventogenic growth where these genes are utilised, therefore their activity in the wild

type cell may be continued at the expense of other essential gene expression, resulting in at least an initial growth advantage in knock out mutants of these genes. Additionally, Cooksley *et al*. (2012) found that in terms of alcohol dehydrogenase genes, only knock out of the *adhE1* gene resulted in almost complete solvent eradication, whereas *adhE2, bdhA* and *bdhB* mutants exhibited wild type solvent formation profiles under the conditions used. This suggests that these genes have a relatively minor contribution to the ABE process and further research in strain development should consider whether the removal of the genes could be used to promote *C. acetobutylicum* growth and streamline the ABE process without negatively affecting solvent production.

3.1.2 pMTL007CC-E2::cac-ptb-87s

Three separate transformations were undertaken using pMTL007CC-E2::cac-ptb-87s and in total 1050 colonies were screened for insertion, of which none were successful. A PCR screen for ClosTron insertion into the *ptb* gene is shown in Figure 7.

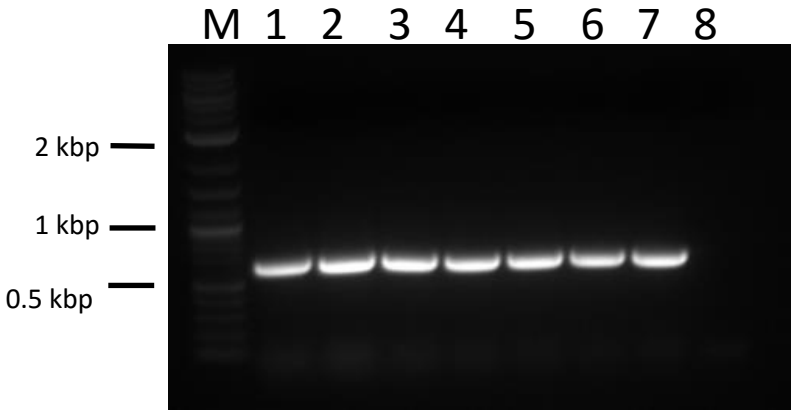


Figure 7. Mixed colony PCR screen for successful intron insertion into *ptb*87s. Each experimental lane represents the DNA of 50 colonies picked from a transformation plate. M, 2-log DNA marker ladder. Lane 1-6,

unsuccesful insertion of intron into *ptb* gene. Lane 7, *C. acetobutylicum* wild type *ptb* gene. Lane 8, deionised water.

Following this, a transformation with a differing target was undertaken using pMTL007CC-E2::cac-ptb-505a and 250 colonies were screened for intron insertion, with no successful insertions isolated (Figure 8).

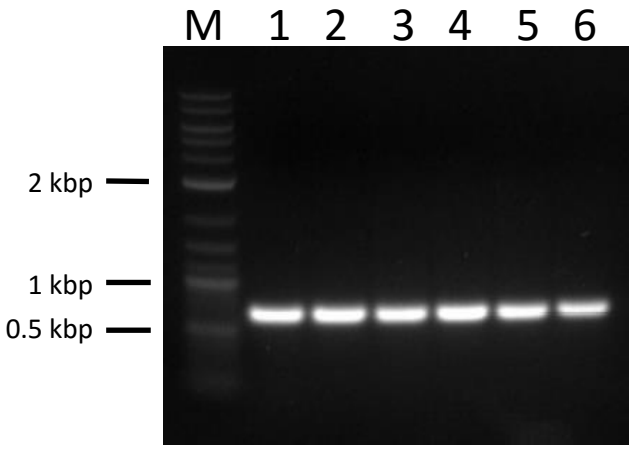


Figure 8. Mixed colony PCR screen for successful intron insertion into *ptb*505a. Each experimental lane represents the DNA of 50 colonies picked from a transformation plate. M, 2-log DNA marker ladder. Lane 1-5, unsuccesful insertion of intron into *ptb* gene. Lane 6, *C. acetobutylicum* wild type *ptb* gene.

This study was unsuccessful in creating a *ptb* knock out strain using pTML007C-E2::cac-ptb-505a or pTML007C-E2::cac-ptb-87s. Cooksley *et al*. (2012) were successful in creating a *ptb* knock out strain using pTML007C-E2::cac-ptb-505a, although the strain produced was shown to have a mutation in the *thl* gene when genotyped. A *ptb* knock out strain made in a separate study by Lehmann *et al.* (2012) using pTML007C-E2::cac-ptb-87s was subsequently shown to have a SNP in the gene *hbd* (unpublished data). This gene encodes 3-hydroxybutyryl-CoA dehydrogenase and is also

present in the central metabolic pathway of *C. acetobutylicum*, where it is part of the butyrate metabolism pathway. In both cases these mutants exhibited butyrate negative phenotypes that are at odds with previously published results by Desai & Papoutsaki (1999) who produced a knock down mutant for the *ptb* gene in *C. acetobutylicum* using asRNA. This mutant had impaired solvent production but a similar butyrate fermentation profile to the wild type strain. The fact that the knock out mutant strains of Δ*ptb C. acetobutylicum* both incur secondary compensatory mutations in separate genes in the ABE fermentation pathway does not seem coincidental, especially when the difficulties attached to acquiring a *ptb* knock out mutant are also considered. The reasons for this remain to be elucidated, but could reasonably be linked to fluctuations in redox potentials in the cell leading to the survival of cells that incur secondary gene disruptions in order to achieve cellular redox balance.

3.1.3 pMTL007C-E2::cac-hydA-936s

Two separate transformations were undertaken using pMTL007C-E2::cac-hydA-936s. Transformations using this plasmid had poor efficiencies, leading to screening of only 200 colonies from the two insertion attempts, a significantly lower number than the other ClosTron plasmids used. From these no successful insertions were isolated. Figure 9 shows a PCR screen for intron presence in the *hydA* gene.

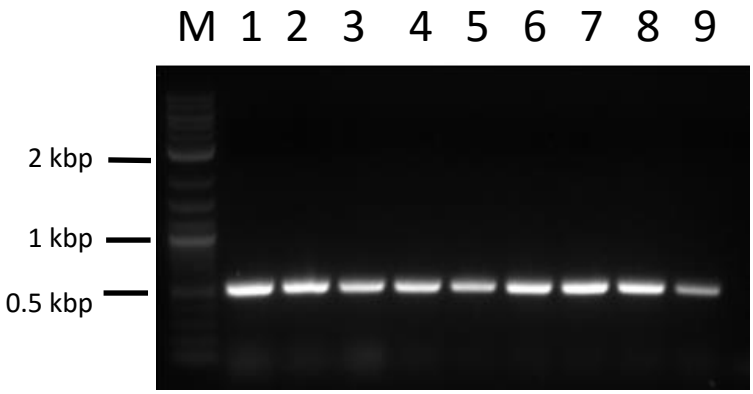


Figure 9. Mixed colony PCR screen for successful intron insertion into *hydA*. Each experimental lane represents the DNA of 50 colonies picked from a transformation plate. M, 2-log DNA marker ladder. Lane 1-8, unsuccesful insertion of intron into *hydA* gene. Lane 9, *C. acetobutylicum* wild type *hydA* gene.

Cooksley *et al.* (2012) suggested that the requirement for balanced cellular redox stoichiometry may be the reason that a *hydA* knock out mutant has not yet been obtained in *Clostridium*. Jang *et al.*, (2014) claimed to be successful in knocking out *hydA* from a quadruple *C. acetobutylicum* knock out mutant which was *pta*$^{-}$, *buk*$^{-}$, *ctfB*$^{-}$ and *adhE1*$^{-}$ to create a quintuple knock out mutant, although the methodology used is open to question as the strain does not seem to have been plasmid cured, therefore continued *ltrA* presence in this strain would designate the effects on these five intron containing genes as knock down (assuming mutants are in the sense orientation) as opposed to a stable knock out mutations. Soucaille (2006) also described the deletion and/or attenuation of *hydA* in a multiple knock out strain, in order to control hydrogen fluxes in the strain resulting from other gene knock outs purportedly including *ptb.* This is the only published report of an intron

with no RAM element being used to create a knock down mutation, where the *hydA* gene was reported to have 16% of wild type expression with the intron contained within the gene and *ltrA* still present within the cell.

The existence of *hydA* knock out mutants only in combination with knock outs of other genes seems to mirror what has been seen with *ptb* knock out strains. In this case, the reasons for this can be more readily attributed to redox stoichiometry as this is a hydrogenase gene and therefore could be considered essential for redox balance to support cell viability, without which excessive metabolic burden may be placed on the cell.

3.1.4 pMTL007CC-E2::cac-thl-406s

In the first screen for intron insertion into the *thl* gene using pMTL007CC-E2::cac-thl-406s, 150 colonies were screened from the transformation of which none were successful (Figure 4). In the second transformation, 200 colonies (4 pools of 50 colonies each) were screened by mixed colony PCR (Figure 10, Lanes 1-4). In lane 3 of this PCR, a faint band was visible at the correct point for ClosTron insertion into the *thl* gene, suggesting that some colonies within this screen had achieved successful insertion of the intron into the gene.

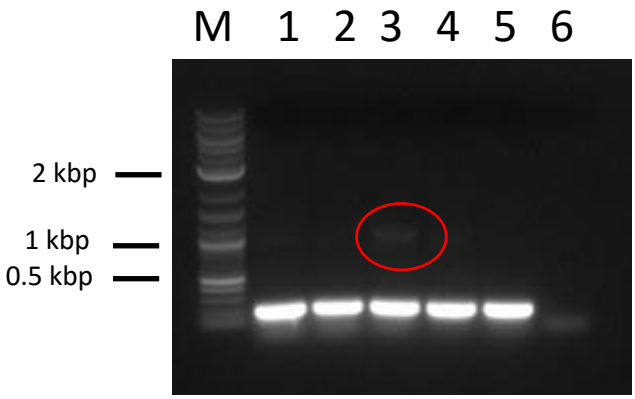


Figure 10. Mixed colony PCR screen for successful intron insertion into *thl*. Each experimental lane represents the DNA of 50 colonies picked from a transformation plate. M, 2-log DNA marker ladder. Lane 1-4, screen for intron insertion into *thl* gene. Lane 5, *C. acetobutylicum* wild type *thl* gene. Lane 6, deionised water. A faint band visible in lane 3 is circled, the band suggets insertion of the ClosTron into *thl.*

The screening process for these colonies, as described in the Materials and Methods, involved picking a number of colonies from the transformation plate and patch plating these in duplicate. One of these patch plates was retained while the colonies on the other were resuspended in 2*YTG and used for the DNA analysis in this PCR. A limitation in this methodology meant that having found that bacteria on this plate contained the plasmid, it was difficult to isolate a pure colony of *C. acetobutylicum* containing pMTL007CC-E2::cac-thl-406s from the patch plate. Figure 11 shows a screen of 9 separate plates, where patches from the original transformation had been streaked out to achieve single colonies. The single colonies on these plates were washed into solution and analysed using the same primers as previously. As can be seen in this

PCR screen, four of these plates contain a mixture of wild type and mutant colonies.

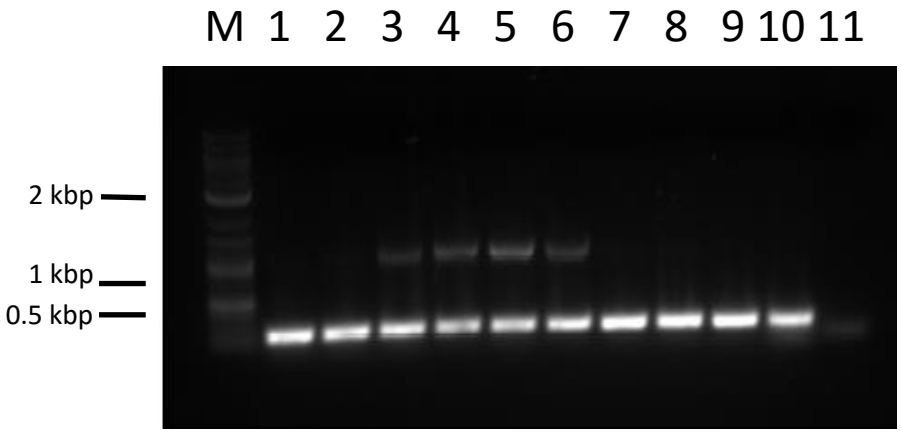


Figure 11. Single colony PCR using patch colonies from Lane 3 of screening PCR in Figure 10. M, 2-log DNA marker ladder. Lane 1-9, screen for intron insertion into *thl* gene. Lane 10, *C. acetobutylicum* wild type *thl* gene. Lane 11, deionised water. Lanes 3-6 show mixed PCR products indicative of both wild type DNA and transformant DNA, where the ClosTron is present in the *thl* gene.

In order to acquire a plate containing purely mutant colonies, all 23 individual colonies that were analysed in lane 4 of Figure 11 were re-streaked again to single colonies. A screen of DNA from these plates is shown in Figure 12 (6 out of 23 colonies screened are shown). Overall, 22 out of 23 plates were found to be a pure culture of transformed *C. acetobutylicum* with the intron contained within the *thl* gene. Colonies on one plate, shown in Lane 2 of Figure 12, had reverted to the wild type.

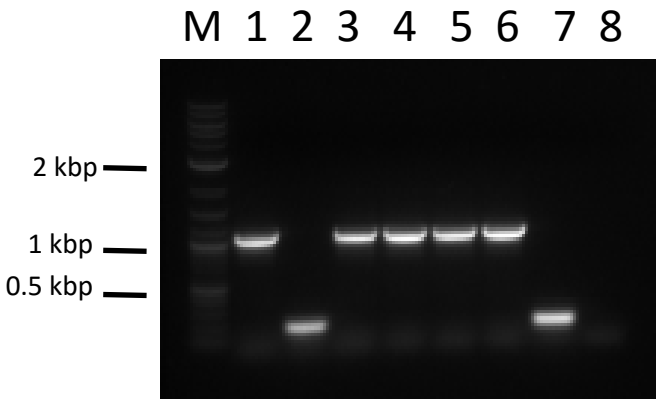


Figure 12. PCR showing the isolation of single colonies with intron inserted into the *thl* gene of *C. acetobutylicum.* M, 2-log DNA marker ladder. Lane 1-6, screen for intron insertion into *thl* gene. Lane 7, *C. acetobutylicum* wild type *thl* gene. Lane 8, deionised water.

As predicted, the plasmid pMTL007CC-E2::cac-thl-406s could not be cured from *C. acetobutylicum* using conventional re-streaking despite the knock out plasmids being cured easily from *adhE2* and *bdhA* knock out mutants with two re-streaks (Figures 5 and 6). This suggests that the hypothesis the plasmid containing the *ltrA* gene cannot be lost from the cell while the intron is spliced into an 'essential' gene such as the thiolase gene is correct.

Following this, negative selection using 5'flurocytosine was employed as per the Materials and Methods section. Using this it was possible to cure *C. acetobutylicum* of pMTL007CC-E2::cac-thl-406s to give the strain Cac-*thl*406s::CT. This strain was confirmed using PCR for intron presence in the genome and a second PCR, using primers to isolate the *catP* gene (Figure 13) to show the plasmid is no longer present to confirm phenotypic characteristics.

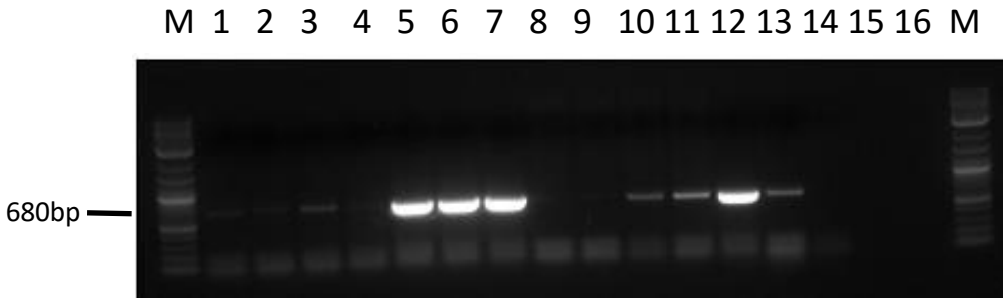


Figure 13. PCR screen for presence of *catP* in mutant *thl* colonies. M, 2-log DNA marker ladder. Lane 1-14, mutant *thl* colonies, band indicates *catP* is present. Lane 15, *C. acetobutylicum* wild type. Lane 16, deionised water.

These colonies were all picked as they exhibited growth on medium containing 5'FC and no growth on medium containing thiamphenicol. However this PCR shows that *catP*, and therefore the plasmid, is still present in the majority of *thl*::intron colonies which brings into doubt the efficacy of the plasmid. Particularly the functionality of the *codA* gene should be questioned as no colonies containing this gene should be able to grow at such a lethal concentration of 5'flurocytosine. However, several colonies in which *thl* had been successfully knocked out were acquired, as shown in lanes 8, 9 and 14 of Figure 13, where *catP* is no longer present.

3.1.5 Further analyses of Cac-*thl*406s::CT *thl* knock out mutant

Unfortunately, after freezing the Cac-*thl*406s::CT strain in a 20% glycerol stock at -80^{o}C, the strain could not be revived by plating on to minimal medium despite repeated attempts. Therefore, no further experimentation on the strain could be completed within the timeframe of the project. That the strain could not be revived suggests the *thl* gene

knockout severely impairs the growth and life cycle of *C. acetobutylicum*, as would be expected if this was an essential gene. However, the confirmation of the strain does show a knockout can be achieved, although without analysis of the genomic DNA of the strain it is difficult to elucidate whether additional SNPs may have contributed to this. It was also unfortunate that the strain could not be subjected to further analysis such as full solvent analysis, complementation and Southern blot to confirm and examine the phenotype of the strain.

3.2 Analysis of fermentation products

An experiment was designed in which to analyse the solvent profile of the Cac-*thl*406s::CT *thl* knock out mutant, however due to its failure to be revived from the freezer stock, fermentation analysis was carried out on an uncured *C. acetobutylicum Δthl* mutant still containing the ClosTron plasmid with *ltrA* gene; therefore results should be treated as analogous to a knockdown mutant. Fermentation analysis was carried out for the production of major products in the ABE fermentation pathway: acetone, acetate, butanol, butyrate and ethanol. A wild type control was run alongside the experimental uncured *thl* mutant, annotated as 'uncured *C. acetobutylicum thl* mutant' and referred to in the text as '*thl1* or *thl2*' depending on the inoculation time of the flasks. A growth curve for the these can be seen in Figure 14.

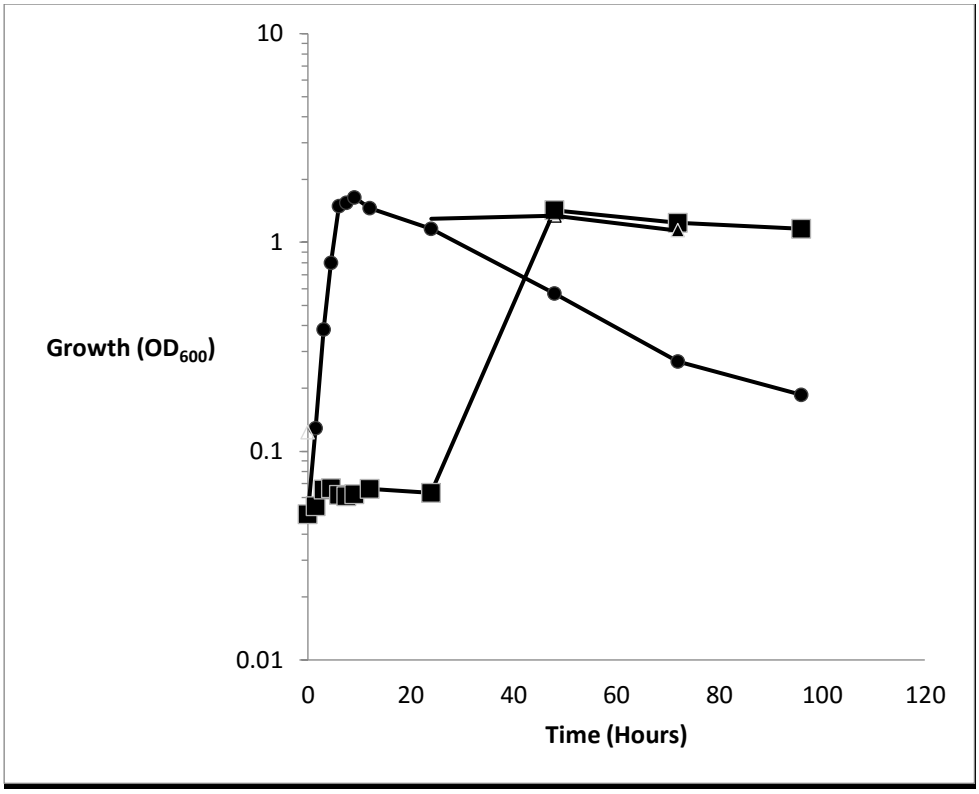


Figure 14. Growth curve of *C. acetobutylicum* ATCC 824 (●), Uncured *C. acetobutylicum thl* mutant 1 (▪), Uncured *acetobutylicum thl* mutant 2 (▲).

This mutant underwent a long lag phase before beginning growth as can be seen in the growth curve (Figure 14), therefore for this reason three additional flasks were inoculated to give a higher starting OD_{600} of approximately 0.1, the average results of these flasks are labelled 'thl2'. Analysis of these results is dubious due to the wild type cultures having not had a solventogenic switch. This means the bacteria did not move from the acidogenic phase to the solventogenic phase, where the compounds produced in the acidogenic phase are assimilated to produce alcohols such as butanol, this was likely due to the age of the inoculation culture. This can be seen in Figure 16, where butanol production by wild type *C. acetobutylicum* remains constant over time, with no peak to

indicate that the bacteria had entered the solventogenic phase. Without a reliable fermentation profile for the wild type profile it is difficult to analyse the differences in fermentation profile of the mutant Cac-*thl*406s::CT. However due to time constraints attached to this study, fermentation and subsequent gas chromatography analysis could not be repeated, therefore results of this experiment have been included for reference.

3.2.1 Gas chromatography results for butyrate production

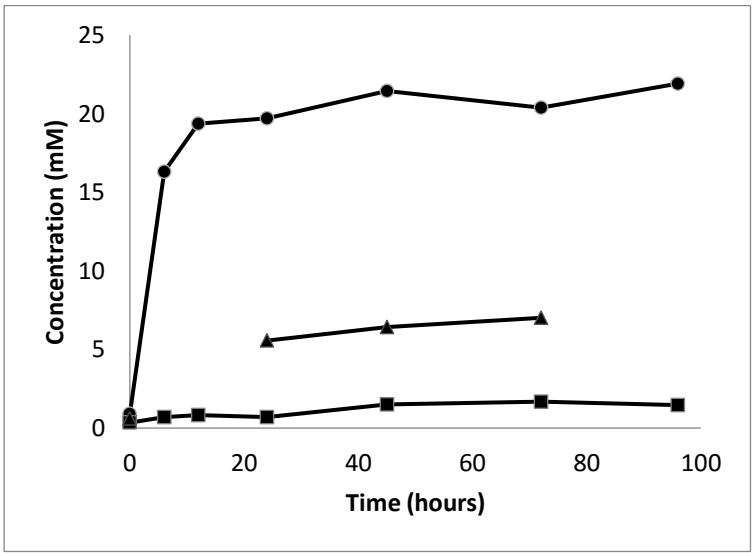


Figure 15. Gas chromatography results for butyrate. *C. acetobutylicum* ATCC 824 (●), Uncured *C. acetobutylicum thl* mutant 1 (▪), Uncured *acetobutylicum thl* mutant 2 (▲).

In the wild type *C. acetobutylicum* cultures, concentration of butyrate was raised significantly in the first 6 hours of growth, presumably in line with

the log growth phase of the cultures as growth peaked at around this time (Figure 15). In comparison the *thl*1 strain did not produce any significant levels of butyrate, with concentrations remaining low over the entire fermentation profile, and only a slight rise where the cultures have peaked in growth at around 48 hours. The data for the *thl*2 cultures were more difficult to analyse as a growth curve was not completed for these cultures, and samples for solvent analysis were first taken at 24 hours. This means some degree of theory must be applied to results. As for the other cultures, butyrate was not present in the starting cultures, however after 24 hours when it can be assumed the culture is at a point around peak growth, according to growth data available, butyrate was present at higher levels than for t*hl*1, but at lower levels than the wild type. The difference between the two sets of Δ*thl* cultures could potentially be attributed to the length of the lag phase for *thl*1 contributing to a poor overall fermentation of the *thl*1 cultures. However, it should be noted that gas chromatography data for butyrate were poor due to technical problems in solvent preparation, therefore these results cannot be seen as reliable or accurately quantitative.

3.2.2 Gas chromatography results for butanol production

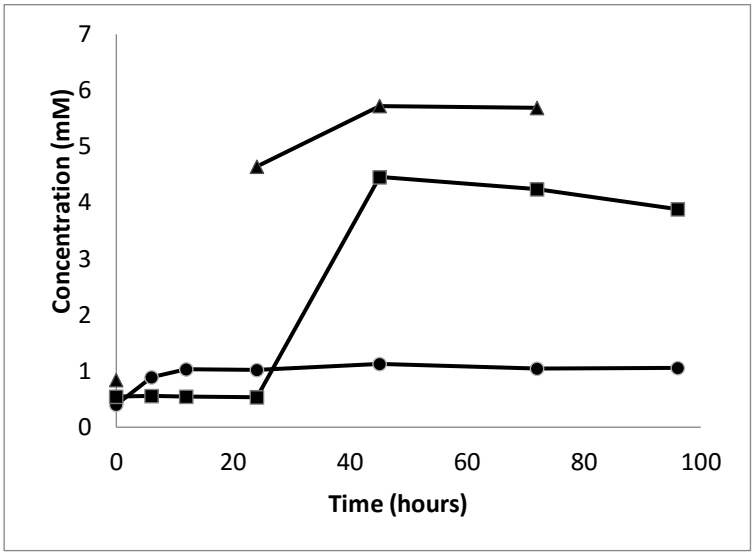


Figure 16. Gas chromatography results for butanol. *C. acetobutylicum* ATCC 824 (●), Uncured *C. acetobutylicum thl* mutant 1 (▪), Uncured *acetobutylicum thl* mutant 2 (▲).

For the wild type cultures, little butanol production was seen, indicating that they had not undergone a solventogenic switch (Figure 16) In both *thl* knock down fermentations, butanol levels were much higher than the wild type. The wild type had fairly consistently low butanol levels over the fermentation, while the *thl* knock down fermentations exhibited a rise in butanol concentration linked to growth, suggesting these cultures did undergo a solventogenic switch. Particularly for *thl*1 cultures it can be seen that peak butanol levels coincided with the highest recorded growth of the cultures, suggesting that butanol was being produced at a high level during stationary phase, as would be normally expected during ABE

fermentation. In both sets of *thl* knock down cultures the peak butanol level was recorded at 48 hours.

3.2.3 Gas chromatography results for acetate and acetone production

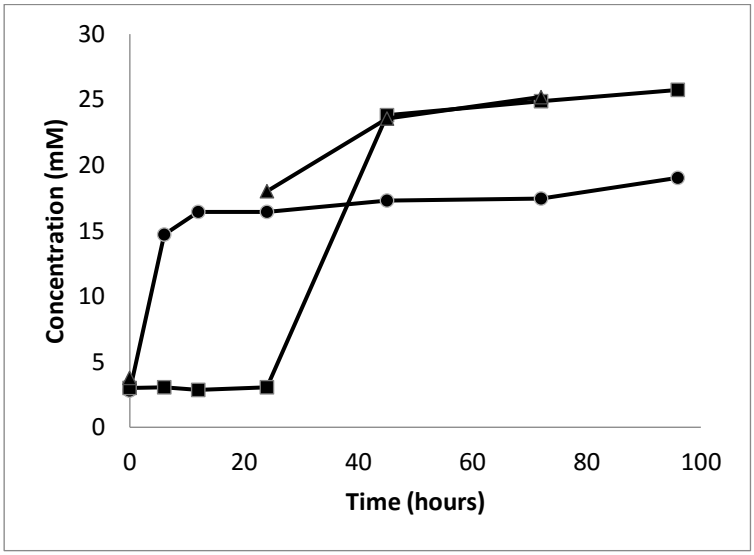


Figure 17. Gas chromatography results for acetate. *C. acetobutylicum* ATCC 824 (●), Uncured *C. acetobutylicum thl* mutant 1 (▪), Uncured *C. acetobutylicum thl* mutant 2 (▲).

Acetate production showed a marked link to the growth patterns of the cultures (Figure 17). In both the wild type and *thl*1 cultures, concentration of acetate produced peaked in conjunction with peak growth. After cultures reached stationary phase, acetate levels were fairly consistent with a slight rise over time. Peak acetate levels were similar between the two sets of *thl* knock down fermentations, with these cultures producing more acetate in comparison to the wild type.

Attempts to measure acetone production resulted in very poor gas chromatography data therefore disappointingly no reliable analysis of these results could be made. Raw gas chromatography data (not included) gave inconsistent peaks and troughs of acetone concentration over the fermentations, with large differences between the individual flasks in each triplicate. This is unfortunate as knocking out/decreasing acetone generation is one of the main aims of strain development in solventogenic *Clostridium*.

3.2.4 Gas chromatography results for ethanol production

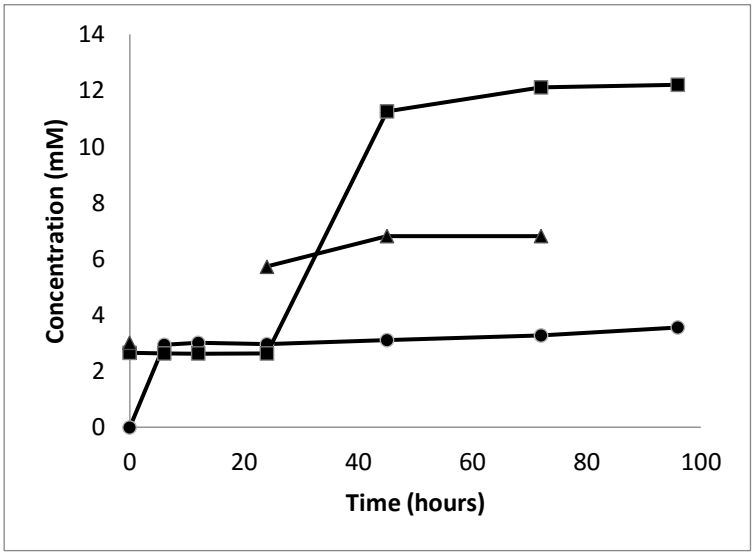


Figure 18. Gas chromatography results for ethanol. *C. acetobutylicum* ATCC 824 (●), Uncured *C. acetobutylicum thl* mutant 1 (▪), Uncured *acetobutylicum thl* mutant 2 (▲).

Ethanol production showed a similar pattern to butanol production (Figure 18). The wild type cultures generated fairly consistent ethanol concentrations over time, again likely due to lack of a solventogenic switch occurring. Whereas the Δ*thl* cultures produced much higher levels of ethanol in line with their growth phase. Ethanol production reached peak levels when cultures could be presumed to be in stationary phase where the concentrations then remained fairly constant.

4.0 Conclusion

In this study knock out of *adhE2* and *bdhA* genes respectively in *C. acetobutylicum* was carried out as a control for use with the modified ClosTron, as these knock out strains had previously been created using conventional ClosTron technology by Cooksley *et al*. (2012). However it is important to highlight that in this study these genes were knocked out with 100% efficiency, suggesting that their loss may incur a growth advantage to *C. acetobutylicum* or at the very least be considered non-essential. This is concurrent with fermentation profiling carried out by Cooksley *et al*. (2012) where profiles of these knock out strains showed no difference to wild type.

This study was successful in isolating a *thl* knock out mutant in *C. acetobutylicum*. This was previously attempted by Cooksley *et al.* (2012) but not achieved. No previous examples of a *thl* knock out in *C. acetobutylicum* have been published. This is likely due to its central role in the ABE fermentation pathway and it has not been considered as a potential target for defective gene analysis. Unfortunately, although a plasmid cured strain was produced in this study, the strain could not be subsequently revived from glycerol freezer stocks. A fermentation profile of an uncured *C. acetobutylicum*::*thl::CT* mutant could be considered to be analogous to a knock down mutant as with the *ltrA* gene still functional in the cell, the *thl* gene is likely to have some level of gene expression. The overall phenotype of this strain is a low butyrate production, with high

acetate concentrations produced. Ethanol production by the *thl* mutants was increased in comparison to the wild type strain, with comparatively low amounts of butanol produced. However as described in the results section, the wild type strain did not undergo a solventogenic switch during this fermentation analysis; therefore it is difficult to make comparisons to the wild type strain. The phenotype of a cured *thl* mutant could be expected to be similar to that of the *hbd* (3-hydroxybutyryl-CoA dehydrogenase) mutant produced by Lehmann & Lütke-Eversloh, (2011) as this mutant exhibited reduced thiolase enzyme activities compared to the wild type *C. acetobutylicum* strain. In this strain no butyrate or butanol production was seen, with a strong counter production of ethanol instead. However, the strain analysed by Lehmann & Lütke-Eversloh (2011) could be considered questionable as no complementation data or genome sequencing of the strain has been published, while members of the Clostridia Research Group at the University Of Nottingham have found they cannot create knock out strains of this gene, even using two different methods (N. P. Minton, Personal Communication). Nevertheless, these results hold some similarity to the phenotypes of the *thl* mutants in this study, particularly the *thl*1 cultures, which produced approximately three times more ethanol than butanol, though low level production of butanol and butyrate were still seen.

The inability to isolate *C. acetobutylicum* strains where the intron had spliced into the *ptb* or *hydA* genes respectively is not entirely unsurprising

due to the predicted essentiality of the genes and unknown level of *ltrA* splicing activity in the cell, causing isolation of mutants to be a rare event. The requirement for the use of a RAMless ClosTron also made it difficult to screen for successful insertions, an issue found in other studies where the RAM element was excised, such as by Dong *et al.* (2010) who screened 900 colonies in order to isolate one successful mutant where the intron had spliced into their target gene.

In the case of *hydA*, this is particularly disappointing as other researchers have been successful in this, albeit using an unknown method in order to create *hydA* knock down mutants in multiple knock out strains, whereby the intron has spliced into the gene but *ltrA* is also still retained in the cell to allow low level function of *hydA* (Soucaillle, 2006). However for this gene, the least number of colonies were able to be screened due to poor transformation efficiencies, therefore it is possible that a future transformation and subsequent screening may be more successful in obtaining an intron containing *hydA* mutant strain.

The results from this study were inconclusive in regards to addressing the original hypothesis that intron splicing may increase the incidence of compensatory mutations. The secondary *thl* mutation found in the genome of a *ptb* mutant isolated by Cooksley *et al.* (2012) may have been an extremely rare event related to intron splicing, or in fact a spontaneous mutation incurred during the cloning process.

Future work is suggested to focus upon gaining knock down mutants in *ptb* and *hydA* genes, measuring gene expression of knock down mutants created, and regaining a cured *C. acetobutylicum thl* gene knock out strain for further analysis, crucially including genome sequencing to identify any compensatory mutations that may have led to the survival of this strain. Solvent analysis of any mutants identified should also be repeated with sufficient replicates to allow statistical analysis of any changes observed.

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