B-Vitamin Requirements of *Clostridium autoethanogenum.*

By

Florence J. Annan BSc (Hons, Ebor)

Thesis submitted to the University of Nottingham

For the degree of Doctor of Philosophy

May, 2018

GASCHEM project

Synthetic Biology Research Centre,

University of Nottingham.

I. Declaration

Except where otherwise acknowledged the work presented in this thesis is my own. No part has been submitted for another degree at the University of Nottingham or any other institute of learning.

Florence J. Annan

May, 2018

II. Abstract

Vitamins are micronutrients essential for life in an organism which must be obtained from diet or environment. Standard media often contains ten B-vitamins, which act as cofactors or precursors for co-enzymes. *Clostridium autoethanogenum* and *Clostridium ljungdahlii* are two obligately anaerobic acetogens which can utilize syngas (carbon dioxide, hydrogen and carbon monoxide) as a carbon source, generated from industrial waste gases or gasification of hydrocarbons. *Clostridium autoethanogenum* and *Clostridium ljungdahlii* use the Wood-Ljungdahl pathway to generate Acetyl-CoA from syngas. Acetyl-coA can be used by the bacterium for growth, production of energy and acetate, ethanol and 2,3-butanediol production. To increase the industrial attractiveness of producing chemicals via this route, the process must be as economical as possible and one way to increase the economic viability is to only add essential media components. This study attempted to define the exact B-Vitamin requirements of the two species, to show that the two species require only three "vitamins" – biotin, pantothenate and thiamine. Strains were created which have missing biotin and pantothenate pathway genes added in order to confer prototrophy for the vitamins and to determine the effects the addition of these genes had on viability, growth profile and product profile. A Continuous Stirred Tank reactor experiment was conducted in order to determine the effects of pantothenate, and therefore, Acetyl-CoA limitation on a continuously growing culture which mimicked an industrial reactor. Increased efficiency of the media could lead to a more economically attractive process for the sustainable production of the platform chemical than from fossil fuels leading us one step closer to decoupling our civilisation from oil.

III. Aknowledgements

Thank you to my supervisors Professor Minton and Dr. Klaus Winzer, and especially Dr. Anne Henstra, for their support, guidance, encouragement and supervision that I received, and the opportunity they gave me to undertake this research, here at the University of Nottingham.

The funding for this project was provided by the BBSRC in conjunction with LanzaTech.

My thanks also go to Laudina Safo for her help with the LCMS analysis and to Matthew Abbot for all his help with running my endless HPLC samples, and Bart Pander's undying patience.

In addition, a huge thanks to everyone who worked and put up with me and my inane questions in B60, and to those in CO2, it has been a pretty marvellous few years working with you since the hostile takeover of 2014.

On a personal note, here's to Dad -for shouting at me and making me learn how to balance equations when I was 9, and for giving me my love of Science, and to Mum – for shouting at him, and giving me my enjoyment of learning, and for always being handy with a comma, right up to the midnight hour. To Sam – for making sure I never got above myself, and reminding me it's just a degree in "counting things", to Mr. Miller – thank you for convincing me not to do a degree in English Literature, and for the life changing "which X-Men could you make by genetic engineering" lesson.

Finally to Dr. Samuel Robert Lowe, I am really glad I am your problem.

IV. List of abbreviations

%	Percentage
µl	Microliters
ºC	Degrees Centigrade
µg	Micrograms
5-FOA	5- Fluorooratic acid
AA	Amino acid(s)
ACE	Allele- coupled exchange
ACS	Acetyl-CoA synthase
Acetyl-CoA	Acetyl group bonded to coenzyme A
AMP	Adenosine monophosphate
AOR	Aldehyde: ferredoxin oxidoreductase
ATCC	American Type Culture Collection
ATP	Adenosin-5-triphosphate
Bp	Base pairs
BLAST	Basic Local Alignment Search Tool
C.	Clostridium
cm	Centimetre
Cm	Chloramphenicol
CO	Carbon monoxide
CO_2	Carbon dioxide
CoA	Coenzyme A
CODH	Carbon Monoxide Dehydrogenase
CODH/ACS	Carbon monoxide dehydrogenase and acetyl-CoA synthase in complex
CoFeSp	corrinoid iron-sulphur protein
ddH_2O	Distilled water
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside-5-triphosphate
E.	Escherichia
EAR	Estimated Average Requirement
Erm	Erythromycin
Et al.	Et alteri (and others)
EtOH	ethanol
FAB	Fatty acid Biosynthesis
FAD	flavin adenine dinucleotide
FDA	Food and Drug Administration
Fwd	Forward (generally primer)
Fdx	Ferredoxin
g	Gram
h	hours
H^+	Proton
H_2	Hydrogen
H_2O	Water
Kb/ Kbp	kilo base pair
L	Litre
LB	Luria-Bertani broth media

LHA	Left Homology arm
MES	4-Morpholineethanesulfonic acid
Mg	milligram
ml	millilitres
mM	Millimolar
MW	Molecular weight
N	Nitrogen, normal
Na	Sodium
NAD	Nicotinamide adenine dinucleotide
NAD (P) $^{+}$	Nicotinamidadenindinucleotide-(phosphate)
NADH (P) $^{+}$	Nicotinamidadenindinucleotide-(phosphate), reduced
OD	Optic density
OH^{-}	Hydroxide
ORF	Open reading frame
PABA	paraminobenzoic acid
PBS	phosphate buffered saline solution
PCR	Polymerase chain reaction
pH	negative log of the activity of hydrogen ions in an aqueous solution
PFOR	pyruvate: ferredoxin oxidoreductase
PETC-MES	Media name
Pthl	thiolase promotor
rbs	ribosomal binding side
Rev	Reverse (generally primer)
RHA	Right Homology Arm
RNA	Ribonucleic acid
Rpm	revolutions per minute
RT	Room temperature
SD	Standard Deviation
SE	Standard Error
SEM	Scanning Electron Microscope
SNP	Single nucleotide polymorphism
TE	Tris-EDTA
TEM	Transmission Electron Microscopy
thl	Thiolase gene
TPP	thiamine pyrophosphate
tm	Melting temperature
Tm	thiamphenicol
V	volts
w/v	weight by volume
YTF	Yeast Tryptone Medium

V. Figures

Figure 1 TEM pictures of C. *autoethanogenum* captured as part of this project 26
Figure 2 The Wood-Ljungdahl Pathway 27
Figure 3 Cartoon representation of CODH/ACS complex based on [62] 28
Figure 4 Downstream products produced by the conversion of Acetyl-CoA generated in the Wood Ljungdahl pathway by C. autoethanogenum and C. *ljungdahlii*. 34
Figure 5 Structure and components of Acetyl-CoA 35
Figure 6 pMTL modular plasmid structure. 48
Figure 7 exemplification of the ACE selection system using pyrE as a selectable marker. 51
Figure 8 A representation of SOEing PCR. 84
Figure 9 Growth curve analysis of (A) C. *autoethanogenum* and (B) C. *ljungdahlii* with one vitamin removed from the media, using fructose as a carbon source. 101
Figure 10 Growth Profile comparison of cultures of (A) C. *autoethanogenum* and (B) C. *ljungdahlii*, with individual vitamins removed from the media when grown using CO as a carbon source 103
Figure 11 Graphs of HPLC data for growth of C. *autoethanogenum* using CO as a carbon source. 105
Figure 12 Further growth curve analysis of C. *autoethanogenum* (top) and C. *ljungdahlii* (bottom) with missing vitamins when grown on fructose..... 108
Figure 13 Further growth curve analysis of (top) C. *autoethanogenum* and (bottom) C. *ljungdahlii* with missing vitamins when grown using CO as a carbon source. 109
Figure 14 profiles for acetate and ethanol of C. *autoethanogenum* WT growing using 1 bar CO as a carbon source..... 111
Figure 15 Biosynthetic pathway of Pantothenate and Coenzyme A. 120
Figure 16 Initial and final optical density of (A) C. *autoethanogenum* and (B) C. *ljungdahlii* cultures growing in different concentrations of pantothenate compared to the standard addition (100%). 126
Figure 17 Average number of colonies found on plates containing different concentrations of pantothenate in the media 127
Figure 18 Role of each of the three key missing genes in the pantothenate biosynthesis pathway naturally occurring as a cluster in C. *acetobutylicum*. . 128
Figure 19 Comparison of performance when growing with and without pantothenate in the media, after the transformation of the strains with the plasmid FJAL3. 131
Figure 20 Map of pAnne12 for the deletion of pyrE gene in C. *autoethanogenum*. 135
Figure 21 amplifications using the primers CMH1 and ACE pyrE RSP. 136
Figure 22 Double knockout of pyrE in C. *autoethanogenum* pyrE mutants. .. 137
Figure 23Plasmid map and schematic of unaltered pMTL_CH23 139
Figure 24 Schematic diagram of pMTL-FJA10 plasmid..... 140
Figure 25 Confirmation of pMTL-FJAL10 structure by restriction enzyme analysis, visualised by gel electrophoresis. 140
Figure 26Restoration of C. *autoethanogenum* pyrE mutants. 141

Figure 27 Restoration of C. *autoethanogenum* pyrE mutants. 142
Figure 28 (A) average number of colonies found on plates when wild type and (A) C. *autoethanogenum* and (B) C. *ljungdahlii* +pantothenate strains are grown with and without pantothenate in the media. 143
Figure 29 Comparison of performance when growing with and without pantothenate in the media, after the transformation of the strains with the plasmid FJAL10. 145
Figure 30 HPLC results when a +pantothenate strain and a wild type strain of C. *autoethanogenum* is grown in pantothenate containing and pantothenate free media..... 147
Figure 31pMTL_FJAL11 plasmid map. 148
Figure 32Restoration of C. *autoethanogenum* pyrE mutants whilst bringing in the panBCD genes under the control of a weak promotor. 149
Figure 33 Limited characterisation of FJAL11 (weak pantothenate) strains, . 150
Figure 34 Ketoisovalerate and its downstream products 151
Figure 35 Characterisation of the effects of growth of C. *autoethanogenum* with and without valine and leucine 152
Figure 36 Alternative selection pressure plates. 153
Figure 37 HPLC analysis of samples from +pantothenate strain growing in 100%, 10% and 0% standard pantothenate addition. 155
Figure 38 Different Pathways that lead to the biphasic biosynthesis of biotin. 166
Figure 39Three main routes to the pimelate thioester moiety. 167
Figure 40 Pathway and associated enzymes and genes for the biosynthetic ring formation of the Pimelate thioester to biotin and its activated coenzyme forms. 168
Figure 41Biotin limitation of C. *autoethanogenum*..... 173
Figure 42 Biotin and Pimelate growth experiments 176
Figure 43 Comparison of performance when growing with and without biotin in the media, after the transformation of the strains with the plasmid FJAL22 and 21 180
Figure 44 NEBuilder diagram and plasmid map of FJAL23 containing the bioHCA and BioBDF fragments 182
Figure 45Restoration of C. *autoethanogenum* pyrE mutant whilst carrying in the genes bioBDFHCA. 183
Figure 46 comparison of the average number of colonies grown with or without biotin for C. *autoethanogenum* or +biotin 184
Figure 47 Characterisation of +biotin strain. 185
Figure 48 +biotin strain grown in 100%, 10% and 0% of the standard addition of biotin. 187
Figure 49 +biotin, +pantothenate growth with and without biotin and pantothenate. 189
Figure 50 Graphs to compare growth with and without pantothenate and biotin in the media for wild type C. *autoethanogenum* and C.a::FJAL3+24..... 190
Figure 51 Diagram exemplifying bioreactor setup. 207
Figure 52Overview of total bioreactor data gathered over a period of 120 days. 213
Figure 53 all products from bioreactor over time determined by HPLC. 215
Figure 54Comparison of ethanol and acetate production and the relative ratio of products. 215

Figure 55 Acetate to ethanol ratio. 216
Figure 56GC analysis of Gas in and out of the bioreactor 217
Figure 57 Calculations from GC data of CO up take and Carbon uptake from the bioreactor 217
Figure 58 Comparisons of steady states. 219
Figure 59Pooled Steady state averages in high pantothenate conditions and low pantothenate conditions. 220
Figure 60 2D and 3D PCA of the four steady states of which LC-MS samples were run. 222
Figure 61 Clustered Heat Map analysis of 30 metabolites from C. *autoethanogenum* cultures in high (HP1 HP2) or low (LP1, LP2) cultures. ... 223
Figure 62 VIP scores of the 40 metabolites with the greatest change between the two different conditions. 224
Figure 63 Metabolic flux of CO_2 to ethanol via acetate 233
Figure 64 Blackboard model explanation of product ratio shifts. 235
Figure 65Global ethanol production by country 2007-2016. 241
Figure 66 PanBCD genes amplified from C. *acetobutylicum* for insertion into pMTL_FJAL3. 267
Figure 67 PanCD fragment inserted into pMTL_FJAL4. 268
Figure 68 PanB and PanC fragment inserted into pMTL_FJAL5. 268
Figure 69 *C. acetobutylicum* panB gene sequence from FJAL6 269
Figure 70 C. *acetobutylicum* panC gene sequence from FJAL7. 269
Figure 71 C. *acetobutylicum* panD gene sequence from FJAL8. 269
Figure 72 pairwise sequence analysis of the ΔpyrE strain of C. *autoethanogenum*, when compared to the wild type. 271
Figure 73 pairwise sequence analysis of the transformed strain of C. *autoethanogenum* "+pantothenate" when compared to the wild type. 273
Figure 74 pairwise sequence analysis of the transformed strain of C. *autoethanogenum* "+pantothenate" when compared to the wild type. 274
Figure 75 bioYDA fragment inserted into pMTL_FJAL20. 275
Figure 76 bioBDF fragment inserted into pMTL_FJAL21 275
Figure 77 bioHCA fragment inserted into pMTL_FJAL22 276
Figure 78 pairwise sequence analysis of the transformed strain of C. *autoethanogenum* "+biotin" when compared to the wild type. 278
Figure 79 "big-biotin" fragment inserted into pMTL_FJAL24 279

VI. Tables

Table 1 Strains used in this project 56
Table 2 Plasmids used in this study 57
Table 3 List of Primers used in this Study 59
Table 4 Luria broth recipe 63
Table 5 Yeast Tryptone Fructose Media (YTF) 63
Table 6 PetC- Mes Recipe 64
Table 7 SDMM +/ - uracil 65
Table 8 Initial media recipe for bioreactor. Source: Louise Sewell 65
Table 9 Standard Bioreactor media recipe 66
Table 10 B vitamin stock solution 66
Table 11 trace metal solution 66
Table 12macronutrient solution 67
Table 13 Acid Trace Element solution 67
Table 14Base trace element solution 67
Table 15 supplement table 68
Table 16 standard PCR programme for amplification of DNA fragments. 83
Table 17 Results of bioinformatics research into the presence of genes for biosynthesis of vitamins in the genomes of C. autoethanogenum and C. ljungdahlii. 99
Table 18 Plate viability tests for C. autoethanogenum and C. ljungdahlii when grown with and without different vitamins. 100
Table 19 table showing the results of transformation of different gene combinations of panB, panC and panD in order to determine whether all are essential for biosynthesis of pantothenate. 133
Table 20 table to display viability of both species when grown with genes inserted by plasmid and with an intermediate in the media. 134
Table 21Averages of conditions in each steady state. 214

Table of Contents

I. Declaration **1**

II. Abstract **2**

III. Aknowledgements **3**

IV. List of abbreviations ..... **IV**

V. Figures **6**

VI. Tables **9**

1 Introduction **17**

1.1 Vitamins 17

1.2 Gas Fermentation 19

1.3 *Clostridium autoethanogenum* and *Clostridium ljungdahlii* 23

1.3.1 The Clostridium Genus 23

1.3.2 Acetogens 24

1.4 Wood-Ljungdahl Pathway 26

1.4.1 Wood-Ljungdahl pathway and the production of Acetyl-CoA 28

1.4.2 Energy conservation and production in the Wood-Ljungdahl Pathway 32

1.4.3 Acetyl-CoA 34

1.4.4 CoEnzyme A 35

1.4.5 ACS 35

1.4.6 Using Acetyl-CoA for growth 37

1.4.7 Acetogenesis and Solventogenesis 38

1.4.8 Alternative carbon source metabolism 42

1.5 Genetic Tools 43

1.5.1 Genome annotation 43

1.5.2 Genetic manipulation of *Clostridium autoethanogenum* and *C. ljungdahlii* 45

1.5.3 Plasmid transfer in *C. autoethanogenum* 46

1.5.4 pMTL system 47

1.5.5 Homologous Recombination and Allelic Coupled Exchange 48

1.6 Aims 54

2 Materials and Methods **55**

2.1 Bacterial Strains and Plasmids 55

2.1.1 Bacterial Strains used in this Study 55

2.1.2 Plasmids used in this study 56

2.2 Oligonucleotide primers 58

2.2.1 Oligonucleotide primers 58

2.3 Chemicals and suppliers 59

2.4 Bioinformatics tools. 60

2.4.1 GenDB 60

2.4.2 DNA Sequence Analysis 61
2.4.3 Protein Structure Analysis 61
2.4.4 Plasmid Design and visualisation. 61
2.4.5 Oligonucleotide design 61
2.4.6 Data analysis and presentation 62
2.5 General Microbiological techniques 62
2.5.1 Growth Media 62
2.5.2 Stock solutions 66
2.5.3 Individual Vitamin stocks 68
2.5.4 Aseptic technique 69
2.5.5 Growth Conditions 69
2.5.6 Strain storage and revival 70
2.5.7 Preparation of competent cells 71
2.5.8 Transformation 73
2.6 Development of the *ΔpyrE* strain 75
2.6.1 *pyrE* selection. 75
2.6.2 Complementing a *ΔpyrE* strain and bringing in a payload 76
2.7 Microscopy. 77
2.7.1 Phase contrast microscopy. 77
2.7.2 Transmission Electron Microscopy 77
2.8 General Molecular Techniques. 79
2.8.1 Preparation of Genomic DNA from Clostridial species 79
2.8.2 Plasmid DNA from *E. coli* 79
2.8.3 Restriction Enzyme Digests 80
2.8.4 Dephosphorylation of linear DNA 80
2.8.5 Plasmid DNA Ligation 80
2.8.6 Hi-Fi or Gibson's Assembly 81
2.8.7 Agarose Gel Electrophoresis 81
2.8.8 DNA Extraction from Agarose gel 82
2.8.9 Polymerisation Chain Reaction 82
2.8.10 DNA Fragment Sequencing 85
2.9 Characterisation Methods 85
2.9.1 Plate viability tests 85
2.9.2 Serial dilution tests 85
2.9.3 Growth Experiments 86
2.10 Measurement Techniques 86
2.10.1 Optical Density 86
2.10.2 HPLC 87

3 Chapter Three: Elucidation of Vitamin Requirements for Two Gas Fermenting Clostridia Species. 88

3.1 Introduction 88
3.1.1 Thiamine 89
3.1.2 Riboflavin 89
3.1.3 Niacin 90
3.1.4 Pantothenate 91
3.1.5 Pyridoxine 92
3.1.6 Biotin 92
3.1.7 Folic Acid 93
3.1.8 Paraminobenzoic Acid 93
3.1.9 Cobalamin 94
3.1.10 Lipoic Acid 94
3.1.11 Chapter Aims 95
3.2 Chapter Specific Materials and Methods 96
3.2.1 Bioinformatics 96
3.2.2 Bacterial Strains 96
3.2.3 Media 96
3.2.4 Inoculum 97
3.2.5 Growth Experiments 97
3.2.6 HPLC 98
3.3 Results 98
3.3.1 Bioinformatics 98
3.3.2 Plate Viability 99
3.3.3 Growth Profile with Fructose as a Carbon Source 100
3.3.4 Growth profile with CO as a carbon source 102
3.3.5 HPLC results 104
3.3.6 Pantothenate, Biotin and Thiamine 107
3.4 Discussion 111
3.5 Chapter Summary 115

4 Creation of Pantothenate Prototrophic Strains of *Clostridium autoethanogenum* and *Clostridium ljungdahlii.* 116

4.1 Introduction 116
4.1.1 CoEnzyme A and Phosphopantetheine 116
4.1.2 Biosynthesis of CoEnzyme A 117
4.1.3 Genetic capacity for production of CoA and Pantothenate in *C. autoethanogenum* and *C. ljungdahlii* 120
4.1.4 Methods for conferral of traits by plasmid 120
4.1.5 Methods to confer traits into the genome 121

4.1.6 Chapter Aims 122
4.2 Chapter specific methods and conditions 123
4.2.1 Bacterial Strains 123
4.2.2 Bioinformatics 123
4.2.3 Media 123
4.2.4 Plate viability tests 124
4.2.5 Growth Experiments 124
4.3 Development and Characterisation of Strains. 125
4.3.1 Further characterisation of the Wild Type and elucidation of limitation by pantothenate 125
4.3.2 Restoration of Prototrophy for Pantothenate Biosynthesis in *C. autoethanogenum* and *C. ljungdahlii* using plasmids. 127
4.3.3 Determining gene importance 131
4.3.4 Insertion of *panBCD* into the genome of *C. autoethanogenum* 134
4.3.5 Characterisation of *panBCD* Cluster into the Genome 142
4.3.6 Development of non-antibiotic based selection system for plasmids 152
4.3.7 Effects of the Reduction of Pantothenate to 10% 154
4.4 Discussion 155
4.4.1 Defining requirements for the wild type strains 155
4.4.2 Restoration of prototrophy by plasmid 156
4.4.3 Long term conveyance of prototrophy by augmenting the genome 157
4.4.4 Weak vs. Strong Promotors 159
4.4.5 Valine and Leucine interference 159
4.4.6 Part fixing system 160
4.4.7 Similarity of results in the two organisms 161
4.4.8 Reduction in pantothente addition to 10% 161
4.4.9 Pantothenate production 162
4.4.10 Conclusion 162
4.5 Chapter summary 163
5 Creation of Biotin Prototrophic Strains of *Clostridium autoethanogenum* 164
5.1 Introduction 164
5.1.1 CoEnzyme R 164
5.1.2 Biosynthetic pathway of Biotin 165
5.1.3 Regulation of biotin production 169
5.1.4 Genetic capacity of Biotin Biosynthesis in *C. autoethanogenum* 169
5.1.5 Chapter Aims 169

5.2 Chapter Specific Methods 170
5.2.1 Bacterial Strains 170
5.2.2 Serial dilution tests 170
5.2.3 Growth Experiments 170
5.2.4 Genome insertions 170
5.3 Strain Development and Characterisation 171
5.3.1 Further elucidation of Limitation by Biotin 171
5.3.2 Addition of missing genes in the pathway by synthetic biology to restore prototrophy for Biotin biosynthesis in *C. autoethanogenum* 173
5.3.3 Introduction of genes for the full biosynthetic pathway of biotin 177
5.3.4 Conferring Biotin Synthesis genes into the genome of *C. autoethanogenum* 180
5.3.5 Effects of Biotin Reduction to 10% 186
5.3.6 Biotin and pantothenate strain. 187
5.4 Discussion 190
5.4.1 Determination of requirement for biotin by the Wild Type 191
5.4.2 Introduction of the *bioYDA* cluster of genes from *C. acetobutylicum* 192
5.4.3 Introduction of the genes *bioBDF* and *bioHCA* from *D. nigrificans* 193
5.4.4 Double Prototrophy 195
5.5 Chapter Summary 197
6 A Study of the effects of Pantothenate Limitation on *Clostridium autoethanogenum* grown in a Continuous Stirred Tank Reactor. 198
6.1 Introduction 198
6.1.1 Batch cultures 198
6.1.2 Continuous culture 200
6.1.3 Steady states definition 201
6.1.4 Product shifts 202
6.1.5 Pantothenate limitation in bioreactor 203
6.2 Methods and materials 204
6.2.1 Media recipes 204
6.2.2 Gas composition 205
6.2.3 Bioreactor operation 206
6.2.4 Inoculation 207
6.2.5 Sampling methods 208
6.3 Results 211
6.3.1 Overview 211
6.3.2 Effects of Limiting Pantothenate Concentration 211

6.3.3 Economically Valuable Product Changes 217
6.3.4 LC-MS 221
6.3.5 Genomics 225
6.4 Discussion 225
6.4.1 Reactor conditions 226
6.4.2 Overview and steady states comparison 227
6.4.3 Redox Equivalents 232
6.4.4 Metabolic flux 233
6.4.5 Blackboard model Explanation for product ratio changes 235
6.4.6 LCMS results 237
6.4.7 Conclusion 238
6.5 Chapter Summary 239
7 General Discussion 240
7.1 Introduction 240
7.1.1 Ethanol 240
7.1.2 Acetate 242
7.1.3 2,3-Butanediol. 242
7.1.4 Production by *C. autoethanogenum* 243
7.1.5 Aims of the project 244
7.1.6 Further work 250
7.2 Concluding remarks 253
8 Bibliography 255
9 9 Supplementary Material 267
1.1 Chapter Four 267
1.1.1 Sequencing comparison of insert into pMTL_FJAL3 267
1.1.2 Genes inserted into pMTL_FJAL4 267
1.1.3 Sequencing Comparison of insert into pMTL_FJAL5 268
1.1.4 Insert sequence for pMTL_FJAL6 268
1.1.5 Insert sequence for pMTL_FJAL7 269
1.1.6 Insert Sequence for pMTL_FJAL8 269
1.1.7 *pyrE* deletion 269
1.1.8 *C. autoethanogenum* +*pantothenate* strain insertion at *pyrE* locus 271
1.1.9 *C. autoethanogenum weak pantothenate* strain insertion at *pyrE* locus 273
1.2 Chapter Five Sequencing 274
1.2.1 Genes inserted into pMTL_FJAL20 274
1.2.2 Genes inserted into pMTL_FJAL21 275

1.2.3 Genes inserted into pMTL_FJAL22 276
1.2.4 *C. autoethanogenum* +*Biotin* strain insertion at *pyrE* locus . 276
1.2.5 "Big-biotin" Fragment insert for pMTL_FJAL24 278

1 Introduction

Clostridium autoethanogenum is an obligately anaerobic acetogen, which is capable of growing and using carbon monoxide as its sole energy and carbon source[1]. The organism can generate several industrially attractive compounds such as acetate, ethanol and 2,3-butanediol, and several companies are attempting to use the organism commercially. In current media compositions, a "B-vitamin" solution is added to facilitate growth, and the exception of this solution from the media results in a complete lack of viability for the culture.

1.1 Vitamins

Vitamins are micronutrients essential for life in a particular species, which cannot be biosynthesised by an organism and must be obtained from its diet or environment. Two of the primary functions of vitamins are to enable the creation of enzymes and to allow enzymes to function by acting as co-enzymes or cofactors.

B-vitamins are a class of water soluble vitamins, all of which act as co-factors or coenzymes for enzymes involved in cell metabolism. Commonly, but not exclusively, added B-vitamins to media for bacteriological or yeast fermentation are Thiamine, riboflavin, niacin, pantothenate, pyridoxine, biotin, folic acid, cobalamin, Paraminobenzoic acid (PABA) and lipoic or thioctic acid, and these may be added individually or as part of a complex containing all of them[13]. All of these act as cofactors or precursors to co-enzymes[9].

A cofactor is a chemical component separate from a protein, without which the protein cannot function. When the protein in question is an enzyme, B-vitamins which are complex organic molecules, can act as specific cofactors or co-

enzymes for a particular type of enzymatic reaction[14]. Unlike the enzyme, the coenzyme is often consumed by the reaction and so a constant supply is required. Lack of availability of a B-vitamin can reduce enzyme kinetic rate and prevent functionality of the enzyme. A co-enzyme which is bound to a protein in the course of the reaction can be denoted as a "prosthetic group," but many are released through the course of the reaction they are aiding in, and termed "co-substrates", but maybe altered irreparably during the course of the reaction they are facilitating[14].

The Global Vitamin market is currently estimated at around $25 billion and is set to increase as greater demands for nutritional requirements are caused by increasing populations, and demands increase by the growing biotechnology market who require them for growth and functionality of their organisms, as well as the increasing demand for supplementation of vitamins in livestock farming and agricultural sectors. Due to their essential nature for viability for not only our own but all species, it is essential that the anthropogenic usage of vitamins is as sustainable, secure and "future-proof" as possible. If costs for biotechnological enterprises are driven up by demand for vitamins, then the processes may become less profitable, competitive or feasible, as vitamins can represent a large cost share of the feedstock used. Increasing avenues for production of synthetic vitamins by organisms modified by synthetic biology to be either sufficient in their production of the chemicals to not require supplementation in the media or by the development of hyper producing strains could result in decreased costs of media and lead to an increase in competitiveness of the process[11].

1.2 Gas Fermentation

Crude oil is used as a platform chemical for a diverse range of products, from fuels to pharmaceutical components, dyes to artificial sweeteners, acids to plastics. It is speculated that 85% of the volume of an average barrel of crude oil is used for the production of fuels, whilst the rest goes into a platforms for fine chemicals, plastics, explosives, and thousands of other products[17]. It has been estimated that crude oil is converted into over 30,000 chemicals. Despite recent discoveries, and efforts made to discover new sources for non-renewable hydrocarbons such as shale gas, crude oil supplies are a dwindling finite resource. The global population's demand for these derivatives from petrochemicals is increasing, and the cost of the production of these chemicals from petrochemical feedstocks is rising[18]. In order to remove our dependence from crude oil, scientists need to not only focus on the generation of biologically synthesised fuels, but also find alternatives and different pathways to all the other chemicals which currently stem from non-renewable sources.

Large amounts of work has been undertaken to find biotechnological routes to the production of these essential chemicals using both unmodified and genetically engineered microorganisms [19]. However, much of this research has been focussed on primary and secondary generation feedstock sources –food parts of crops and the non-food part of a plant such as straw. Despite the gluts of food production in the west and implementation of "set-aside" by the EU, many people object to the concept of using sugar, starch and other "food" feedstocks for fuel production when there are people starving in the World[20]. One renewable technology which has emerged which aims to tackle this issue is Gas Fermentation.

Gas Fermentation is a biological process in which bacteria ferment "synthesis gas" into transport fuels and platform chemicals. Three companies are currently utilizing gas fermentation as a biotechnology for the production of renewable chemicals and these are LanzaTech, INEOS Bio and Synata Bio. LanzaTech is in the process of building a commercial scale plant capable of the production of 34 million gallons of ethanol per year[21] [22], in conjunction with China Steel Corporation, and a 9.8 million gallon per annum plant in Ghent, using syngas as a waste product from the steel mills. Recently, news reports have detailed a link between LanzaTech biofuels and Virgin Airlines and in 2016 the first test flight using Jet fuel produced by gas fermentation was reported[23].

Syngas is a mixture of hydrogen, CO, and CO_2 generated from the gasification of hydrocarbons[24]. Syngas is generated in four main stages. In the first stage the feedstocks are gasified at temperatures between 800-1500^{O}C. This can be done by conventional external heating, pyrolysis or by partial oxidation. In the second stage of gasification the feedstock reacts with limited oxygen, water and CO_2, this results in an impure "raw" syngas, which needs to be purified to remove contaminants like methane, sulphur compounds, tar and ash. After this the proportions of hydrogen and carbon monoxide are adjusted, resulting in a synthesis gas which is ready to be catalysed into complex molecules[25]. Many microorganisms, such as; acetogens, carboxytrophs and methanogens are able to utilise the single carbon (C1) gases present in syngas as their carbon source[26], and use it for the production of energy for growth, and also generate anthropogenically useful chemicals from this carbon source.

Syngas fermentation is advantageous compared to primary or secondary stage feedstocks[27] because it uses all of the biomass regardless of quality. Due to the

production method by pyrolysis, virtually any hydrocarbon can be used in the production of syngas. Non-food biomass can be used for gasification such as straw, agricultural waste, household waste, rapidly growing energy crops, sewage, biomass from forestry and other industrial hydrocarbon waste can be heterogeneically converted into synthesis gas [28-32]. In 2005, it was estimated that the energy potential from human cultivated biomass was globally around 250EJ per year, but less than four percent of this was used for energy generation[29]. The ability for syngas to be produced from current pollutants and waste streams such as municipal waste and sewage is a further advantage, as the generation of syngas for the fermentation of the carbon within, actually alleviates potential pollution issues, and they are available at attractively low costs. Non-biomass hydrocarbon sources can also be used for gasification to produce syngas such as coal, tar sands, non-useful fractions from the oil refining process, kerogen, reformed natural gas and shale gas, which are often used in the Fischer-Tropsch process but can be repurposed for syngas production [33, 34]. Industries such as steel manufacturing, Iron smelting, corn-to-ethanol plants, many fermentation processes, ammonia production etc., all produce large amounts of CO and/or CO_2 which can be captured and recovered for use as syngas, which would otherwise be released into the atmosphere and act as greenhouse gases. Using gas fermentation as a biotechnological process removes these waste streams and converts them into valuable bulk and fine chemicals.

Although the process of bulk and fine chemical production through gas fermentation can be slower than traditional chemical production (such as Fischer-tropsch synthesis) the process has several advantages. Due to the nature of the microbes involved in the process, gas fermentation can occur at relatively low

temperatures and atmospheric pressures which negates two of the common major costs of chemical production as often they occur at high temperature and pressure. Biological production of chemicals also offers a second key advantage as the specificity of enzymatic conversion results in high yields and low by-product formations, and can result in homosteroisotropic products[33]. Furthermore, the nature of enzymatic reactions can avoid thermodynamic equilibrium relationships and avoid degradation of the product back into its substrate components [33, 35]. Due to the adaptable nature of microbes, the range of appropriate ratios of CO_2 and H_2 in the gas supply is reasonably flexible, whereas in traditional chemical processes in order to get desired products a specific gas composition is very important [30]. Furthermore, biocatalysts and microbial cultures have a much higher sulphur tolerance than inorganic catalysts so are less susceptible to poisoning by sulphur compounds potentially present in the gases if improperly "scrubbed" [30, 36].

The key disadvantages of gas fermentation are; issues in gas-to-liquid mass transfer, slow cell growth, media costs, low key product production rate, potential product inhibition and difficult fine chemical recovery (recovery of bulk chemicals such as ethanol is relatively easy)[28, 37, 38]. Environmental concerns such as energy footprint and water usage also need to be considered but industrial scale efforts to recycle water usage, and increase overall efficiency are in development.

1.3 *Clostridium autoethanogenum* and *Clostridium ljungdahlii*

1.3.1 The Clostridium Genus

One of the largest bacterial genera, *Clostridium* is comprised of over 300 species. Whilst they are diverse, the genus is bound by some major similarities –obligate anaerobic metabolisms (some more tolerant to oxygen presence than others), gram positive cell wall structure, rod shaped, motile and in many cases, but not universally, the ability to form spores[39]. The genus is much maligned due to some of its notorious members, who are able to produce some of the most deadly cytotoxins known to man, which can kill either by flaccid paralysis or muscle contraction spasms (*Clostridium botulinum* and *Clostridium tetani* respectively), and cause gas gangrene (*Clostridium noyvi* and *Clostridium histolyticum*) abscesses (*Clostridium butyricum, Clostridium cadaveris, Clostridium glycolicum, Clostridium hastioforme, Clostridium oroticum*) and cause huge issues as hospital borne infections for patients with dysbiosis caused by use of broad spectrum antibiotics (*Clostridium difficile*)[40]. However, due to the vast range of species within the genus, the metabolic processes the genus is capable of, and diversity of environments the genus occurs in, many species play a vital part in carbon cycle regulation, production of useful chemicals, bioremediation and is even being developed as a method for drug delivery for anti-tumour drugs, and the development of new antibiotics [41-44].

Clostridia are found in a wide range of anaerobic environments and due to the somewhat inhospitable climates these environments can provide, are capable of using a wide range of feedstocks as carbon sources[40]. Many species of

Clostridia are capable of fermenting classic sugars like glucose, arabinose, xylose, fructose, and rhamnose. Saccharolytes like *C. thermocellulum* are able to degrade cellulose and hemicellulose into sugars they are capable of fermenting such as glucose, xylose and rhamnose[40]. Efforts have been made in recent years to combine cellulose hydrolysis and fermentation into one process using sugar fermenting species and cellulolytes to produce anthropogenically useful products from waste products which are high in recalcitrant lignocellulose, such as straw or sugar cane bagasse, which is difficult to breakdown for many organisms and represents a large amount of carbon biomass [45, 46], which is often burnt in order to dispose of it causing the release of black carbon (a pollutant) into the atmosphere. Conventional processing of the biomass involves complicated pre-treatment steps which can be very expensive. As well as the use of sugars and converted sugars as substrates, some acetogenic clostridia are able to grow chemolithotrophically using CO_2 and H_2 or CO in the form of syngas as previously mentioned. Acetogens fix roughly 20% of global CO_2 and produce a billion tonnes of acetate per year[47] and some Clostridia are capable of fixing nitrogen such as *C. acetobutylicum* [48].

1.3.2 Acetogens

Acetogens are microorganisms which use the Wood-Ljungdahl pathway to produce Acetyl-CoA[49] and can produce acetate, ethanol, butanol and butyrate from Acetyl-CoA, as well as a range of other chemicals such as 2,3-butanediol[50] and are obligate anaerobes. They constitute the bulk of the most industrially relevant microorganisms which can utilise carbon monoxide or dioxide as a carbon source and hydrogen as an energy source[51]. The majority of acetogens are part of the phylum “firmicute,” but acetogens are reasonably

diverse with members which are spirochaetes, δ-proterobacteria and acidobacteria. Acetogens play an important role in the ecosystems of soil, water courses and bodies, and have been discovered in a multitude of natural environments, such as the intestines of animals, the continuously submerged soil in rice paddies, salt water, and soil to name a few[52]. In less natural environments, acetogens have also been discovered in ammunition manufacturing runoff, industrial waste water treatments and are even being developed as a mechanism of dechlorination for bioremediation[52].

Two Clostridial acetogens able to grow chemolithotrophically on syngas are *Clostridium autoethanogenum (*Figure 1*)* and *Clostridium ljungdahlii*[53]. In order to grow, *C. ljungdahlii* and *C. autoethanogenum* rely on the operation of the Wood-Ljungdahl pathway to generate Acetyl-CoA from carbon monoxide or hydrogen and carbon dioxide[26]. *Clostridium autoethanogenum* and *C. ljungdahlii* are very genetically similar organisms[54] due to their phylogenetic proximity, 100% sequence identity when using 16s RNA and high similarity on a genomic level [55]. *Clostridium autoethanogenum* was discovered in 1994 by Abrini and isolated from rabbit faeces [56]. *Clostridium ljungdahlii* was isolated from chicken yard waste and first published in 1988 by Barrick et al [57]. Both species were reported to be capable of growth on syngas as the sole carbon source.

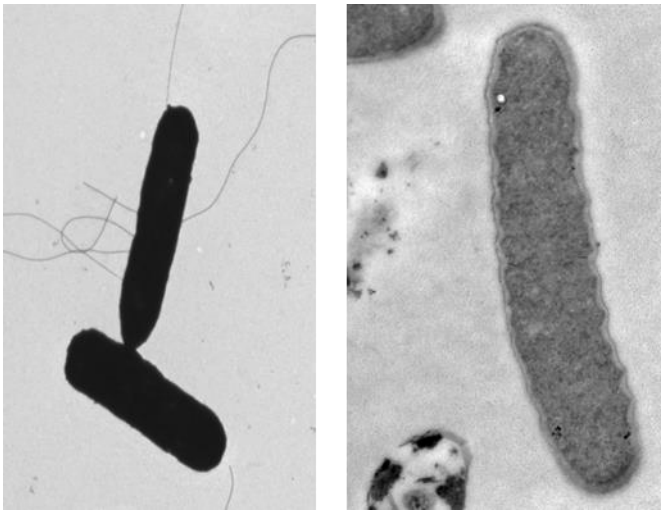


Figure 1 TEM pictures of C. autoethanogenum captured as part of this project

1.4 Wood-Ljungdahl Pathway

The Wood-Ljungdahl pathway (Figure 2) named for its co-discoverers Lars Ljungdahl and Harland Wood[58], is also known as the reductive Acetyl-CoA pathway and allows for the reduction of CO_2 to the Acetyl component of the platform compound Acetyl-CoA which can be used as the carbon currency of the cell [47]. The Wood-Ljungdahl pathway is found in many organisms and within several phyla. At least 23 genera have been discovered to have the pathway present in some of its species and are capable of the conversion of CO_2 into cellular carbon[47]. These species are able to use the aforementioned Wood-Ljungdahl pathway to ferment syngas to produce acetate and ethanol using the following reactions:

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O$$

$$2\ CO_2 + 6\ H_2 \rightarrow C_2H_5OH + 3\ H_2O$$

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2$$

$$6\ CO + 3\ H_2O \rightarrow C_2H_5OH + 4\ CO_2$$

Clostridium autoethanogenum and *Clostridium ljungdahlii* are able to ferment syngas in order to grow and yield useful products by using the Wood-Ljungdahl pathway. The pathway can be used in both the oxidative and reductive directions. In acetogens (such as *C. autoethanogenum* and *C. ljungdahlii*) the pathway is used in the reductive manner in order conserve energy and assimilate carbon[59], however, when methanogens grow on hydrogen and carbon dioxide they can use the pathway in both directions – reductive to form cell carbon and conserve energy and oxidative to break down acetate and form methane[60].

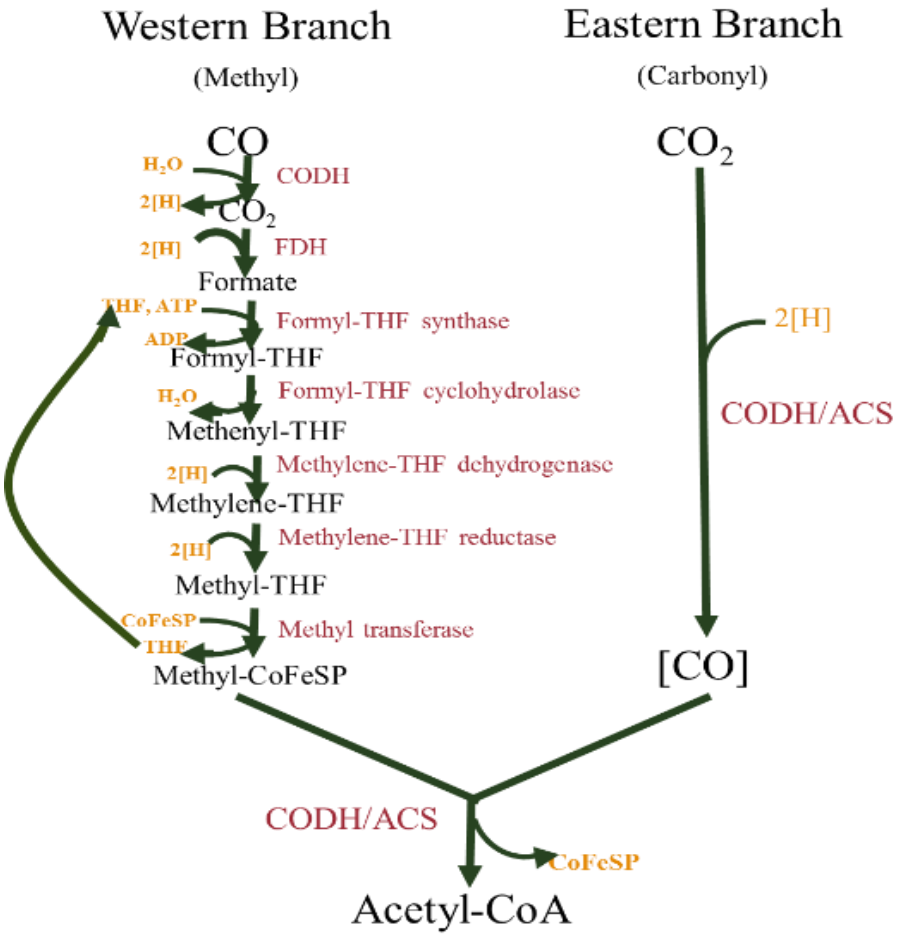


Figure 2 The Wood-Ljungdahl Pathway
Used by Acetogens to generate Acetyl-CoA which can be used by the organism to produce a range of downstream products. ACS, Acetyl-CoA synthase; CODH, carbon monoxide dehydrogenase; CoFeSP, corrinoid iron sulfur protein; FDH, formate dehydrogenase; Pta, phosphotransAcetylase; THF, tetrahydrofolate.

1.4.1 Wood-Ljungdahl pathway and the production of Acetyl-CoA

The Wood-Ljungdahl pathway acts as a terminal electron accepting process which allows for the conversion of CO_2 into cellular carbon[58]. As exemplified in figure 2, the Wood-Ljungdahl pathway consists of two branches- a carbonyl and a methyl. The methyl branch reduces CO and produces the methyl part of Acetyl-CoA called methyl-coFeSP. In the carbonyl branch an enzyme complex -carbon monoxide dehydrogenase/Acetyl-CoA synthase (CODH/ACS) reduces CO_2 to CO. The two branches are fused together by the CODH/ACS enzyme complex to bind the CO and the methyl group to generate Acetyl-CoA[26].

The methyl branch uses an enzyme cascade to enable the six electron reduction of CO to the methyl moiety of Acetyl-CoA. CO is reduced to CO_2, which is converted to formate by formate dehydrogenase. This formate is then condensed with tetrahydrofolate to form 10-formyl-THF, using formyl-THF synthase and further converted to 5,10-methenyl-THF by formyl-THF cyclohydrolase. Methylene-THF dehydrogenase then reduces the methenyl group to methylene, followed by methyl- THF reductase reducing the methylene further to a methyl group [61]. A cobalamin dependent methyltransferase then confers the methyl group to the corrinoid iron sulphur protein (CoFeSP) component of the bi-functional CODH/ ACS complex.

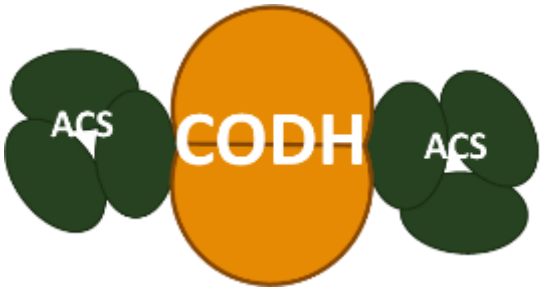


Figure 3 Cartoon representation of CODH/ACS complex based on [62]

The CODH/ACS complex shown in Figure 3 is comprised of two separate proteins [63]. The CODH part of the enzyme causes the reduction of CO_2 to CO, and the terminal product of the pathway – Acetyl-CoA is formed when CO, the methyl group which is bound to CoFeSP, and CoA are condensed together by Acetyl-CoA synthase. CODH disulphide reductase and NADP or ferredoxin are also required to convert CoA into Acetyl-CoA [64].

1.4.1.1 Reducing Equivalents and Hydrogenases

The oxidation of hydrogen, reduction of ferredoxin and NADH provide the reducing equivalents required for the Wood-Ljungdahl pathway to convert syngas into Acetyl-CoA[49]. Hydrogenases are the enzymes responsible for the catalysis of hydrogen through the reversible reaction:

$$H_2 \leftarrow\rightarrow 2H^+ + 2e^-$$

The reaction flows to the left hand side of the equation with the generation of hydrogen when electron donors are present, and the availability of electrons is plentiful. The reaction tends to the right hand side of the equation when an electron acceptor is present leading to the oxidation of hydrogen and the production of hydrogen ions (also just referred to as protons)[65]. Hydrogenases are metalloenzymes and there are three main types; nickel-iron ([NiFe]), iron-iron ([FeFe]) and iron ([Fe]) [66]. Ech hydrogenases (part of the [NiFe] clan of hydrogenases) have been shown to be present in many species of Clostridia and have been shown to enable the reduction of ferredoxin, at the expense of hydrogen gas and ATP, but in different conditions can also oxidise ferredoxin in order to generate hydrogen[67]. The genus of Clostridia have been shown to have

many different Iron-iron ([Fe-Fe]) hydrogenases which allow them to rapidly adapt to different conditions[66]. Hydrogenases in this class, within the genus, have been shown to be capable of transferring electrons from reduced ferredoxin to form hydrogen, and further were shown to be capable of oxidising formate to generate hydrogen. A hydrogenase from one species was demonstrated to both oxidise previously reduced ferredoxin and NADH in order to generate hydrogen in low hydrogen conditions and it was supposed that in high hydrogen conditions that the NADH could potentially be oxidized to form ethanol[67]. Homologs of this enzyme were shown to exist in *C. acetobutylicum* a very closely related species to *C. autoethanogenum* and *C. ljungdahlii*.

1.4.1.2 CODH

C. autoethanogenum and *C. ljungdahlii* are able to grow either using CO_2 as a carbon source and hydrogen as an electron source or using CO as a carbon and energy source. Of the two options, carbon monoxide is favoured for growth and solventogenesis, as the use of carbon monoxide as an electron donor is more energetically favourable than hydrogen[68]. Some studies have shown that if both hydrogen and carbon monoxide are present, hydrogen will not be used as an energy source [68, 69].

Carbon monoxide conversion to carbon dioxide (the first step in the methyl branch of the Wood-Ljungdahl pathway) is effective at generating very low potential electrons. The reduction potential engendered by the oxidation of carbon monoxide can then reduce electron carriers such as ferredoxin, reduce sulphur species to produce H_2S, generate hydrogen from protons, reduce CO_2 into acetate or reduce aldehydes into alcohols such as ethanol[47, 58, 70, 71]. Many organisms cannot exclusively use carbon monoxide as an energy and

carbon source, this has been hypothesized to be because of inhibition of metalloproteins and hydrogenases by CO[71].

CODH or carbon monoxide dehydrogenase is an enzyme capable of acting in the reversible conversion of:

$$CO + H_2O \leftrightarrow CO_2 + 2[H] + 2e^-$$

Also known as the biological water-gas shift reaction. CODH is capable of two distinct roles within the Wood-Ljungdahl pathway and is present in a monofunctional and a bifunctional form (when in complex with ACS). The enzyme is involved in the oxidation of carbon monoxide to carbon dioxide and when in complex with ACS, catalyses the reduction of CO_2 to CO which is coupled with the generation of Acetyl-CoA from the resulting CO in the carbonyl Branch[72] which can later be converted into cellular carbon for growth. The CODH enzyme has been well characterised and studied – particularly in the case of *Moorella thermoacetica*[72]. The active site of the enzyme has a [3Fe-4S] cluster which is bonded to a Ni-Fe component. It has been suggested that a lysine and a histidine are next to the active site and facilitate the reaction[72].

1.4.1.3 Oxidation of CO

As stated, CODH is capable of enabling the conversion of CO to CO_2. Infrared spectra suggested the existence of a Nickel-CO complex which implied the first step in the oxidation of CO to CO_2 was the binding of Carbon monoxide to the nickel$^{(2+)}$ and the simultaneous binding of the $Fe^{(2+)}$ component to H_2O[73, 74]. The complexing of these two compounds forms a conformational shift in the shape of the active site bringing the CO closer to the hydroxyl group of the water molecule to allow for a nucleophilic attack on the Carbon monoxide by the iron

bound hydroxyl group and a decarboxylation results in the generation of carbon dioxide and the reduction of the cluster. The electrons in the reduced cluster are shifted to nearby [4Fe-4S] clusters and ultimately ferredoxin[75]. This oxidises the nickel-iron complex in the active site.

1.4.1.4 Reduction of CO_2

When the CODH enzyme is involved in the reductive direction of the reversible reaction i.e., the generation of CO from CO_2 (carbonyl phase of the Wood-Ljungdahl pathway), the process occurs by the principal of "Microreversibility" in which the active site is initially reduced rather than oxidised to allow the formation of a Nickel- carbon dioxide bond instead of a nickel-carbon monoxide bond [61, 76].

1.4.2 Energy conservation and production in the Wood-Ljungdahl Pathway

When growing chemolithotrophically, it is essential for organisms such as acetogens utilising the Wood-Ljungdahl pathways to not only generate cellular carbon from the conversion of the resulting product – Acetyl-CoA but the cells must also be able to conserve energy. It has been reported in anaerobes that roughly 100mol of ATP is necessary for the production of 1kg of dried biomass (for anaerobes)[70]. The Acetyl-CoA produced by the Wood-Ljungdahl pathway is an energy dense compound from which an ATP can be generated by substrate level phosphorylation (SLP) with the use of the enzymes acetate kinase and phosphotransacetylase (Ack and Pta respectively)[69]. However, if we look at the pathway shown in Figure 2 we can see that the generation of Acetyl-CoA consumes a molecule of ATP during the conversion of formate to 10-formyl-THF, so the net gain of ATP is zero[52]. The reduction of CO_2 to form CO in the eastern branch of the Wood-Ljungdahl pathway also requires energy and it has

been estimated that the requirement is roughly 1/3 of an ATP equivalent[58]. It has been suggested that electron transport phosphorylation is involved in the energy conservation of acetogens and that the movement of protons and sodium ions across a membrane (chemiosmosis) could also play a role. Some closely related acetogens have been shown to possess a membrane associated electron transport system involving menaquinones, cytochromes and oxioreductases which transport protons across the membrane and out of the cell [47], however, these so far seem to be absent in *C. autoethanogenum* and *C. ljungdahlii.*

The Rnf complex is a membrane bound ferredoxin: NAD^+- oxidoreductase which has been shown to be essential in the energy conservation of *C. autoethanogenum and C. ljungdahlii* [77-81]. The complex utilises reduced ferredoxin from the oxidation of CO (to produce CO_2) and reduces NAD^+ with the associated transport of sodium ions or protons across the membrane. The ion gradient is then used to generate ATP by H^+ ATP synthase or Na^+ ATP synthase[82].

As previously discussed, an energy conservation strategy for anaerobes using the bifurcation of electrons by hydrogenases as a mechanism has been proposed [82] and demonstrated for a range of different enzymes such as NADH-dependent reduced ferredoxin:NAD oxidoreductase Nfn and a similar mechanism has also been suggested for the methylene-THF reductase during the conversion of methylene-THF to methyl-THF which would allow the step to be coupled with an Rnf complex and ensure further energy conservation during this very exergonic reaction [79, 83, 84].

1.4.3 Acetyl-CoA

Acetyl-CoA is a key central metabolite for all life. In acetogens, Acetyl-CoA produced from the Wood-Ljungdahl pathway can be used by acetogens and more specifically *Clostridium autoethanogenum* and *C. ljungdahlii* as a precursor for anabolic and catabolic processes which can form different chemicals as shown in Figure 4.

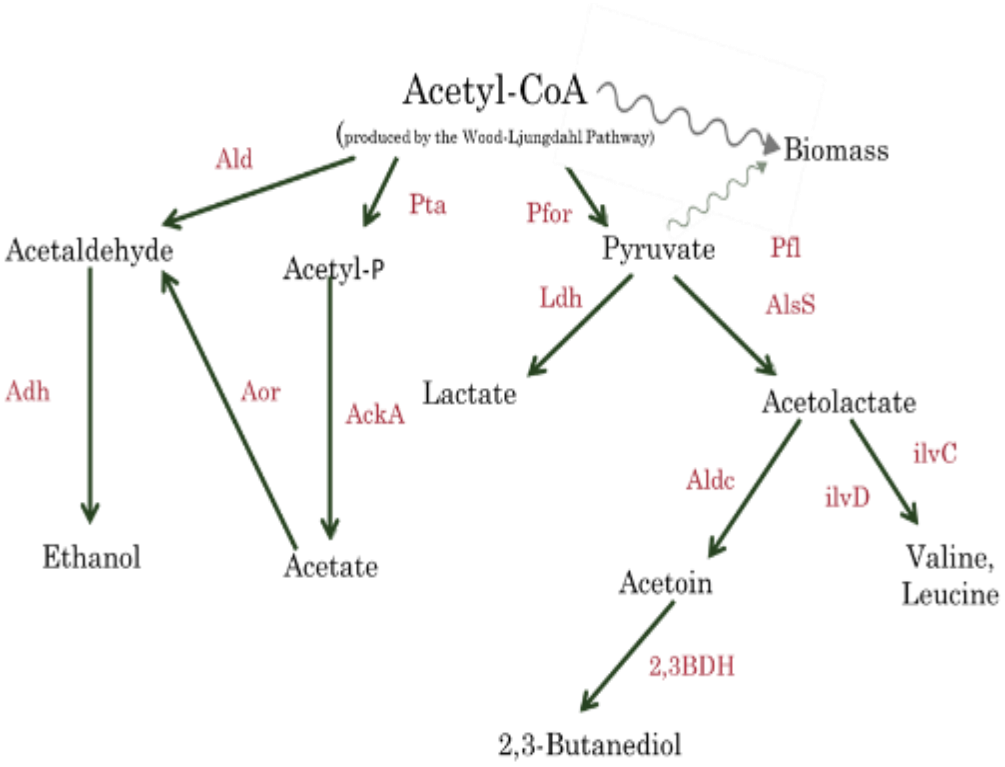


Figure 4 Downstream products produced by the conversion of Acetyl-CoA generated in the Wood Ljungdahl pathway by C. autoethanogenum and C. ljungdahlii. .

Acetyl-CoA is an energy dense molecule which is involved in a diverse range of reactions within a cell. The structure of the molecule is shown in Figure 5. The Acetyl group is linked to a sulfhydryl component of CoA by a strong thioester bond which is very high in energy and highly reactive. Hydrolysis of the bond is exergonic.

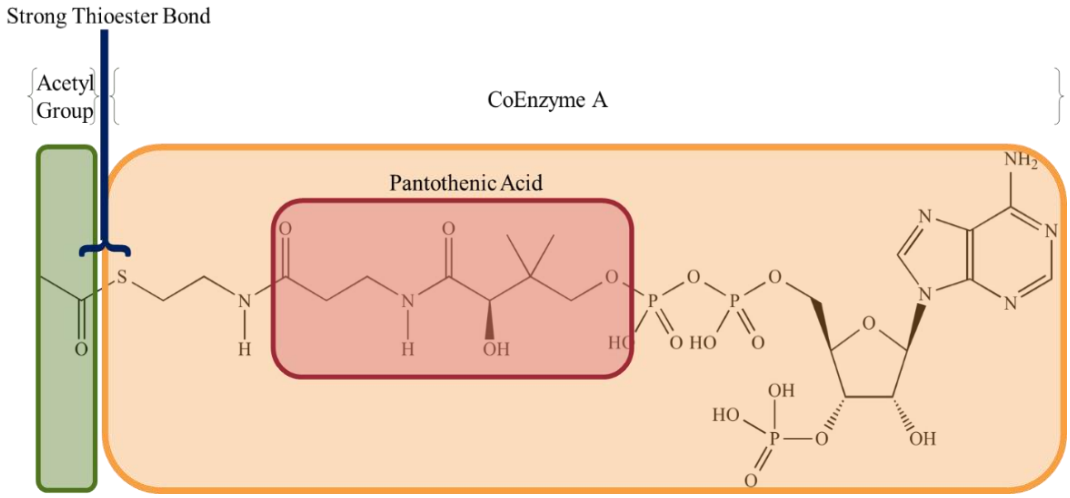


Figure 5 Structure and components of Acetyl-CoA

1.4.4 CoEnzyme A

CoEnzyme A, as will later be detailed, is produced from the essential vitamin pantothenate, and cysteine by a five phase pathway using four ATPs [85]. Coenzyme A is involved in the generation and oxidation of fatty acids and cholesterols, and in the generation of Acetyl-CoA where it makes up a large component of the molecule. Every organism possesses genes for the generation of enzymes which require CoA as an essential component of the reaction and it has been suggested that 4% of enzymes use either CoA or a thioester such as Acetyl-CoA as a substrate[86]. CoA in its capacity as a co-enzyme exists in order to transfer acyl and Acetyl groups, and can act to transport carbon atoms around the cell, and activate or deactivate enzymes.

1.4.5 ACS

Acetyl-CoA Synthase or ACS is the second component enzyme in the bifunctional CODH/ACS complex. Another name for this enzyme is CO-methylating Acetyl-CoA synthase. The enzyme catalyses the following reaction:

CO (generated by CODH) +CH_3-CoFeSP+ CoA → Acetyl-CoA and CoFeSP

Where the methyl CoFeSP had been generated by the western branch of the Wood-Ljungdahl pathway.

The ACS enzyme functions because the active site (also known as the A-cluster) contains a nickel^{2+}-nickel^{2+} cluster. An [Fe_4S_4] cluster is also near the active site with two cysteine molecules nearby[87]. The space generated by the clusters allows for the formation of an active site and can allow substrates and products to bind[88]. The exact mechanism by which the enzyme works is yet to be determined but the enzyme has been isolated in both a "closed" and an "open" configuration [73, 88]. It has been predicted that the catalysis phases begin in the open phase whereby the active site interacts with the Corrinoid iron sulphur protein in the methyl transfer step and then the site "closes" allowing the transfer of CO from the CODH through the linked unit. The two positions are opposite and one cuts off the other by burying the subunit. The A-cluster then rotates to bind CoA and release the product[73].

The A-cluster allows for the Carbon-carbon and carbon-sulphur bonds to be formed which allow the condensation of the Acetyl group and the coenzyme A. The enzyme is comprised of three units – the A-cluster which acts as the active site, a unit which interacts with the CODH enzyme and may bind with a ferredoxin to activate the transferral of CO from CODH to the ACS unit, and a subunit which binds CoA[87].

The proximity of the ACS and CODH enzymes allows for the rapid transfer of electrons. It has been suggested that the CODH is at the centre of the complex with two ACS subunits framing it as shown in Figure 3[74].

1.4.6 Using Acetyl-CoA for growth

1.4.6.1 Pyruvate

Pyruvate is an essential platform chemical within the cell as it can be used in order to generate cellular carbon and a range of other products. Pyruvate can also be converted into lactate, formate or acetolactate. Acetolactate can then either be used to generate amino acids or be converted by AldC into acetoin and then to 2,3-butanediol by 2,3-butanediol dehydrogenase[89].

Pyruvate: ferrodoxin oxidoreductase or PFOR is an essential enzyme for growth. It catalyses the reaction of Acetyl-CoA with CO_2 to form pyruvate and oxidise ferredoxin. This occurs in the following reversible reaction:

$$\text{Pyruvate} + \text{CoA} + \text{Fd (ox)} \leftrightarrow \text{CO2} + \text{Acetyl-CoA} + \text{Fd (red)}$$

PFOR enzymes have been discovered in many acetogens, but *Moorella thermoacetica* has been the best studied and characterised[61]. PFOR is involved in the oxidation of pyruvate by acetogens as it catalyses the first step in metabolism of pyruvate, and also couples the Embden-Myerhof-Parnas pathway to the Wood-Ljungdahl pathway. PFOR also acts as a pyruvate synthase to convert Acetyl-CoA to pyruvate so that it can enter the incomplete reductive tricarboxylic acid cycle (TCA)[52].

In order to function PFOR requires TPP (thiamine pyrophosphate). PFOR forms an intermediate which can form a hydroxyethyl adduct between the pyruvate and the thiazolium component of TPP[90, 91]. This adduct allows the transfer of one electron to one of the three [4Fe-4S] clusters present in the active site of PFOR which produces a radical intermediate. The [4Fe-4S] cluster the radical is bound to can then react with Coenzyme A to produce an electron and Acetyl-CoA.

Microreversibility allows this enzyme to function to catalyse both directions of the reaction and Acetyl-CoA, carbon dioxide and an electron can be used as substrates to form pyruvate and regenerate CoA as well.

In many other organisms pyruvate is metabolised by the enzyme pyruvate dehydrogenase (PDH), however, anaerobes preferentially use PFOR as it uses low-potential electron transfer proteins such as ferredoxin which increase the feasibility of pyruvate synthesis as the electron donor for PDH as NADH is not a strong enough electron donor to reduce Acetyl-CoA to pyruvate (NAD→NADH is 200mV more positive than Acetyl-CoA→ pyruvate)[52].

1.4.7 Acetogenesis and Solventogenesis

Alongside the previously mentioned reactions to generate ATP, Acetyl-CoA is also involved in the generation of biomass and some anthropogenically useful products (such as acetate, ethanol and 2,3-butanediol). *C. autoethanogenum* and *C. ljungdahlii* produce acetate, which can undergo substrate level phosphorylation to replenish energy which was used during the Wood-Ljungdahl pathway. Acetate is produced (with ATP as a by-product) by Pta and Ack, which are encoded by two genes in the same operon (*pta* and *ack*) [92]. If the organism can only access carbon dioxide and hydrogen as its substrates then only acetate is produced as a major product with very low expression of ethanol [1, 93, 94]. If CO is (also) available as a substrate then ethanol, 2, 3-butanediol, lactate and formate can also be potential products [50, 95]. Historically, in solventogenic clostridia, the sugar or starch metabolising species have been the best industrial producers of solvents and been used in ABE fermentation, [40] and *C. acetobutylicum* remains the most well studied and industrially used solventogenic species [96].

Product formation in Clostridia generally exist in two phases – acidogenesis in which acetate is produced and solventogenesis in which solvents such as ethanol are produced. The acidogenic phase generally occurs in the exponential growth phase with the generation of carboxylic acids, hydrogen and carbon dioxide, followed by solventogenesis in which the acids are reduced or converted into solvents, which generally occurs when the culture is in stationary phase [97]. This product shift has been shown to be manipulated by gas flow, pH shifts, limitations in sulphates or phosphates, high concentrations of acetic acid, changes in ATP:ADP and NAD(P)H ratios and other conditions in the reactor [98-101] [102].

As shown in Figure 4, Acetyl-CoA can be converted into a range of downstream products. The most important of these in industrial terms are ethanol, acetate and 2,3-butanediol.

1.4.7.1 Ethanol formation

Ethanol is formed from Acetyl-CoA in a two-step process. Initially, aldehyde dehydrogenase (ALDH) converts Acetyl-CoA into acetaldehyde which is reduced by alcohol dehydrogenase (ADH) to form ethanol.

ALDH catalyses the following reaction:

$$CH_3COO- + NADPH + 2H^+ \rightarrow CH_3CHO + NADP^+ + H_2O$$

Alcohol dehydrogenase reduces acetaldehyde in the following reaction:

$$CH_3CHO + NADPH + H^+ \rightarrow CH_3CH_2OH + NADP^+$$

Gene knockouts and Enzymatic assays have demonstrated both enzymes are required for the production of ethanol from Acetyl-CoA[103].

As well as direct production from Acetyl-CoA, ethanol can also be formed indirectly from acetate by conversion using aldehyde: ferredoxin oxidoreductase (AOR) which has been shown to reduce carboxylic acids into their corresponding aldehydes which can then be converted into ethanol by Ad [104-106]. In conditions where ATP is limited, the conversion of acetate to acetaldehyde by AOR (and subsequent conversion to ethanol) is essential for ethanol production, as this mechanism allows an ATP to be generated during the synthesis of acetate by substrate level phosphorylation, whereas production of ethanol via Acetyl-CoA does not[107]. *C. ljungdahlii* and *autoethanogenum* both possess the AOR gene[55]. AOR has also been suggested to couple reduced ferredoxin which has been generated by CODH during CO oxidation to the reduction of acetate into acetaldehyde [55, 84]. Deletion of AOR in hyper-ethanol production strains has been shown to drastically reduce the yield of ethanol [108]. Ethanol generation via this route as opposed to the direct production from Acetyl-CoA results in the production of 1.2 mol of ATP[103].

1.4.7.2 Acetate formation

Acetate is formed from Acetyl-CoA with the help of two enzymes – Pta or phosphotransacetylase and Ack or acetate kinase.

Pta catalyses the conversion of Acetyl-CoA to Acetyl-P in the following reaction:

$$CH_3COSCoA + HPO_4^{2-} + H^+ \rightarrow CH_3COOHPO^{-}{}_3 + CoASH \quad (10)$$

Ack catalyses the conversion of Acetyl-P to acetate in the following reaction:

$$CH_3COOHPO^{-}{}_3 + ADP^{3-} \rightarrow CH_3COO^- + ATP^{4-} + H^+ \quad (11)$$

Gene knockouts and enzymatic knockouts have shown both enzymes to be essential in the production of acetate[103].

In low extracellular pH, or high acetic acid concentrations, further production of acetate can lead to the destruction of the proton gradients and lead to a loss of viability of the cell as membranes dissociate with further increase of H^+ ions [40]. The conversion of acetate into ethanol using the AOR enzyme previously mentioned increases the pH. The production of acetate is more energetically favourable for the cell as its generation leads to the production of ATP, however, the production of ethanol can in some conditions be more favourable to the cell as it reduces the capacity of the cell to acidify itself to death. Production of too much ethanol can also damage the cell, as exemplified in the brewing industry, as ethanol interferes with membrane fluidity and can disturb membrane associated tasks such as proton motive gradients, and membrane transport proteins [97, 109]. Acetate can be reassimilated into Acetyl-CoA by CoA transferase [102, 110, 111]. And the recycled Acetyl-CoA can be converted into ethanol or acetate again.

1.4.7.3 2,3-Butanediol Formation

2,3-butanediol is a platform chemical which can be used in products from plasticisers to medicine and jet fuel to hand cream. As shown in Figure 4, 2,3-Butanediol and lactate are produced by acetogens from Acetyl-CoA by a series of enzyme catalysed reactions downstream of pyruvate production. Pyruvate can be turned into lactate, formate or acetolactate. Acetolactate can then either be used to generate some essential amino acids or be converted by AldC into acetoin and then by 2,3-butanediol by 2,3-butanediol dehydrogenase. Reports of 2mM of 2,3-butanediol from *C. autoethanogenum* and *C. ljungdahlii* have been made

when the organisms are growing on syngas [95] [112]. A small amount of lactate is also produced by *C. autoethanogenum* and *ljungdahlii* and catalysed by Ldh (lactate dehydrogenase) [95, 110].

1.4.8 Alternative carbon source metabolism

Although industrially speaking, (and to garner the benefits of gas fermentation over sugar fermentation) metabolism of CO is more important, it is also essential to note that if possible, *C. autoethanogenum* is capable of growth using sugars. Fructose can be converted into pyruvate by glycolysis using the Embden-Mayerhof-Parnas (EMP) pathway and xylose can be converted into pyruvate via the pentose-phosphate pathway also known as PPP. The pathways also generate CO_2, NAD(P)H and ATP. The pyruvate generated from the PPP or EMP can then be converted into a range of downstream products by PFOR, whilst producing reduced ferredoxin, Acetyl-CoA and CO_2. The reduced Ferredoxin and CO_2, alongside the NAD(P)H and ATP (generated from the pathway) can be recycled to be used in the Wood-Ljungdahl pathway to create an additional Acetyl-CoA which can be converted to acetate to produce more ATP[113]. In growth using Carbon monoxide as the sole carbon source, many of the metabolic intermediates produced by the PPP or EMP are required by the cell, despite the lack of sugars present. These intermediates can be generated by gluconeogenesis, whereby pyruvate is converted into phosphoenol-pyruvate and then converted into other compounds. Phosphoenol-pyruvate is most likely formed though oxaloacetate[114].

1.5 Genetic Tools

The generation of industrial products by gas fermentation can be very profitable and utilising *C. autoethanogenum* and *C. ljungdahlii* to produce them can lead to a viable alternative to generating these products from non-renewable sources. However, it is essential to optimise the process in order to make the method as competitive as possible with the production of chemicals by traditional routes. Since the dawn of the "genetic age" and the reduction in cost of sequencing; the genomes of many solventogenic species of Clostridia have been ascertained [84, 96, 115-118]. Despite this, the generation of stable recombineered *Clostridium autoethanogenum* and *ljungdahlii* has remained difficult, but recent development of tools has alleviated some of this struggle [84, 96, 115-118]. Synthetic manipulation of the genome to introduce desirable traits from other organisms could lead to an increasingly competitive route to industrial chemical production by these bacteria. Desirable traits could include high product yield and selectivity, stable integration of genes, increased viability of cells and robustness, increased product tolerance and tolerance to contaminants, high biomass production and lower requirement for chemicals within substrates.

1.5.1 Genome annotation

The work shown in this thesis was funded as part of the wider "GASCHEM" grant, which set out to "Optimise industrial gas fermentation using *C. autoethanogenum* and to expand the product profile through synthetic biology." In order to carry out the brief of the GASCHEM project it was essential that a detailed knowledge of the genome was gleaned, as the first draft annotated genome announcement came just two months before the beginning of the project[119] and was composed of 100 contigs [119]. The sequencing of the

genome was complex due in part to the low GC content of the genome and the frequent repeated sections[55], although this was aided in part by the availability of a fully closed genome of the close cousin, *C. ljungdahlii [77]* . A closed genome sequence for *C. autoethanogenum* was published in 2014, however, the genome contained many inconsistencies such as frame shifts and stops in the middle of essential genes when compared to the *C. ljungdahlii* sequence. Due to the nature of the available announcements, it was decided that the GASCHEM group would produce its own annotated genome for publication. Illumina MiSeq sequenced a direct stock of *C. autoethanogenum* from the DSMZ. The incongruities between our sequences and other genomes were gathered, and 10% of these were sequenced by Sanger sequencing and in all instances the Sanger sequencing results agreed with our data. The size of the GASCHEM group led to the ability and workforce to make a manual annotation of the genome possible. This reduced the propagation of mistakes in previously sequenced genomes which automated annotation can lead to, as an issue with many bacterial genome sequences is the annotation of genes is often carried out by full automation, which leads to propagation of mistakes in previously sequenced genomes. Each person manually curated 336 genes, removing 482 "hypothetical proteins" and assigning them a function, and 182 supposed annotations were reduced to "hypothetical proteins". Another member of the team double checked each elevation or reduction of function [120]

The genome consists of just under 4.4 MB, 84.7% of which is comprised of 4039 genes. The GC content is just under a third of the total bases. The *C. ljungdahlii* genome is around 4.6MB and contains 4198 predicted genes[77]. Despite the size difference, *C. autoethanogenum*'s genome contains genes for mannose

metabolism and additional genes for aromatic compound degradation. In addition, *C. autoethanogenum* also contains a gene which encodes a Fe/Fe hydrogenase which is paired in an operon with NuoF-like oxidoreductases. This cluster has been shown to be highly expressed during CO2/H2 growth, and could be responsible for an improvement in growth on syngas.

1.5.2 Genetic manipulation of *Clostridium autoethanogenum* and *C. ljungdahlii*

Despite published methods for mutagenesis of the species [121-123], *Clostridium autoethanogenum* remains recalcitrant to recombineering. One of the major challenges of engineering the species is the struggle to insert DNA from other species into the organism. Two methods are reported as possible: conjugation and electroporation. Many clostridial species are capable of both; conjugation is used predominantly in species such as *C. difficile* and *C. sporogenes* and electroporation is used in *C. celluluyticum* and *C. perfingens. C. ljungdahlii* is capable of having DNA introduced by either method [84, 124-133]. The genome annotation has provided insight into a further difference between *C. autoethanogenum* and *C. ljungdahlii* and this lies in the reason *C. ljungdahlii* is much more genetically tractable than *C. autoethanogenum.* Whereas *C. ljungdahlii* can be transformed by either common method, *C. autoethanogenum* can only be transformed by conjugation[134]. The main culprit for *C. autoethanogenum's* recalcitrance to transformation by electroporation may be the presence of active methylation- restriction systems [135]. *C. autoethanogenum* possesses four type IV restriction systems and a native CRISPR system, neither of which are present in *C. ljungdahlii.* Due to the reduction of these defence

systems, *C. ljungdahlii*'s genome is littered with prophage integrations[136], accounting for a large part of the discrepancy in genome size between the two species. Although *C. autoethanogenum* is less prone to phage integration due to its excellent native defence systems it does make gene editing difficult as exogenous DNA is also subject to degradation by the complex restriction systems native to *C. autoethanogenum*. The restriction systems will attack modified DNA and have low sequence specificity. This results in the attack and destruction of DNA inserted from organisms such as *E. coli*, which may have been used as a cloning strain for the inserted DNA and will have previously methylated the DNA.

1.5.3 Plasmid transfer in *C. autoethanogenum*

Due to the aforementioned recalcitrance to transformation in *C. autoethanogenum*, electroporation (of electrocompetent cells) is extremely inefficient and results in very low numbers of successful transformants. In order to mitigate this effect, conjugative *E. coli* cells are used to clone plasmids and for DNA transfer. The *E. coli* strain; CA434, contains an R702 plasmid with conjugative capacity and is commonly used in clostridia [137-139]. Unfortunately, this strain is a Dcm+ strain (DNA cytosine methyl transferases). In Dcm+ strains, the methyl transferases methylates cytosines which can be attacked and degraded by the type IV restriction systems previously mentioned. During the course of his PhD, Craig Woods developed a strain of Dcm- NEBexpress cells containing the R702 plasmid (thus enabling conjugation to occur). The resulting strain is known as "Sexpress" and led to much greater transformation efficiencies.

1.5.4 pMTL system

The pMTL80000 series [138], is a modular shuttle plasmid system for the clostridial genus. The easily manipulated modular system consists of four elements, arranged in the same order and bound by four 8bp type II restriction sites- SbfI, AscI, FseI and PmeI. The standard arrangement (as shown in Figure 6) consists of a Gram positive replicon for amplification in Clostridia (0-spacer, 2-pBP1,3-pCB102, 4-pCD6, 5-pIM13), an antibiotic resistance gene (1-CatP, 2-ermB 3-aad9 4-tetA), a gram negative replicon for amplification in E. coli (1-p15a, 2-p15a+tra, 4- ColE1, 5-ColE1+tra) the "tra" (origin of transfer) element of which allows for conjugation to occur) and an application specific module which is upstream of the insertion site for genes of interest with promotors and Multiple Cloning Sites (0- Spacer, 1-just MCS site, 2-pTHL+MCS, 3-pFDX+MCS, 4-CatP reporter). The gram positive replicons 2 and 3 were the most stable in *C. autoethanogenum* and *C. ljungdahlii* (Liew, University of Nottingham, unpublished). The Thiolase promotor (pTHL) was a very strong promotor in the species but ferredoxin (pFDX) was also highly effective (B. Pander, University of Nottingham, Unpublished). As many of the plasmids created in this project will eventually be conjugated into *Clostridium autoethanogenum* the gram negative replicons 2 and 3 were preferentially used. Plasmid names, or plasmid backbone names, are determined by the combination of modules preceded by "pMTL8" followed by the sequence of modules as shown above.

Restriction digest of the pMTL backbone with AscI and SacII breaks open the backbone after the application specific module for insertion of genes of interest

into the plasmid but alternative combinations of restriction enzymes in the same location will also function.

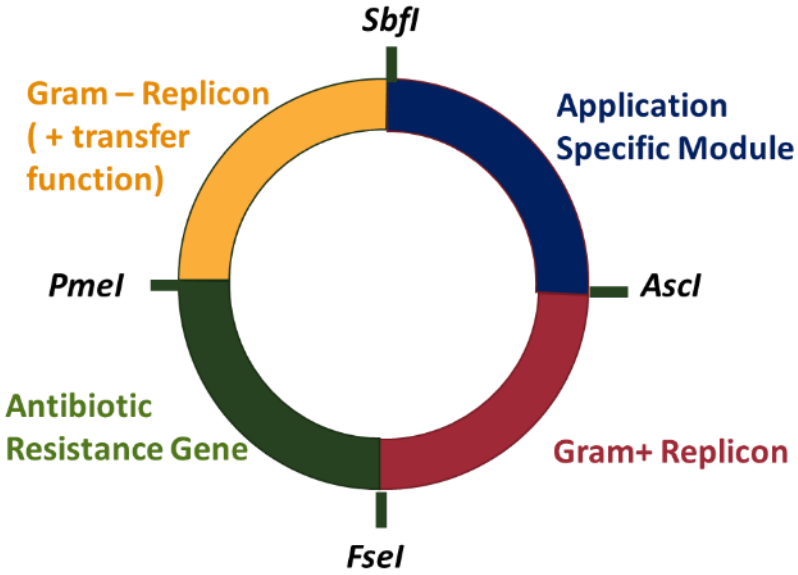


Figure 6 pMTL modular plasmid structure.

Novel DNA can be inserted into an organism by the transformation of the species with replicative plasmids to confer genes from one species to another. However, these plasmids are unstable and if not selected for can be easily lost due to the cost to the cell of their replication. Selection is generally by antibiotic resistance in order to be maintained.

1.5.5 Homologous Recombination and Allelic Coupled Exchange

The ability to manipulate the genome effectively means that we can study the effects through reverse genetics. Much work has been done both in the SBRC

(University of Nottingham) and other groups, to develop tools in *C. autoethanogenum* which allow for the insertion and deletion of specific genes allowing us to eliminate and insert novel pathways into the species [140]. Perhaps the most famous of these is the ClosTron system which allows the specific knockout of single genes[140]. Early knockout mutants were generated by single crossover events by homologous recombination with replication defective plasmids [141-147]. However, these mutants were segregationally unstable and the risk of the crossover reoccurring in the duplicated regions of the plasmid and return of the genotype to the wild type was high.

Homologous recombination can be spontaneous and the larger the homologous sequence, the greater the likelihood of recombination. This can result in accidental single crossovers in individual cell lines and in rare cases can lead to the full integration of the plasmid. If undirected this can be costly and undesirable. Although single crossovers of plasmids are generally undesirable, this propensity can be exploited to produce a stable integration when a plasmid contains two separate homology arms and two separate homologous recombination events can occur, one at each homology arm. Due to the exceedingly low probability of a double crossover event happening spontaneously, techniques have been developed to encourage this occurrence in order to generate a stable double crossover integration of DNA. A stepwise methodology in which a single crossover integrant is generated and isolated, followed by a counter selection method to select for colonies with a double crossover (as shown in Figure 7).

In 2012, Heap et al published a method for double crossover selection without the mandated inclusion of an antibiotic resistance gene [139] called “allelic

coupled exchange". Although the work was carried out in *C. acetobutylicum* it has been proven to work in all clostridial species it has been attempted in so far. As demonstrated in Figure 7, the method involved two homology arms; a long one and a short one, the long homology arm contained a normal sequence for a functional counter selectable gene, whereas the short homology arm carried the sequence for a truncation of the selectable gene in order to inactivate it. Initially, a selectable single crossover integration would occur at the longer more dominant homology arm (length corresponds to probability of crossover). Once this was selected for, a double crossover event at the short homology arm could be selected for through the counter selection of a functional gene product at the insertion site. This allowed for site directed insertion of foreign DNA into the chromosome. The short homology arm led to the truncation of a counter selectable gene which allowed for the selection of double crossover mutants. This can be repaired by a second plasmid containing an enlarged short homology arm with the correct full gene sequence to "repair" the deleted selectable gene.

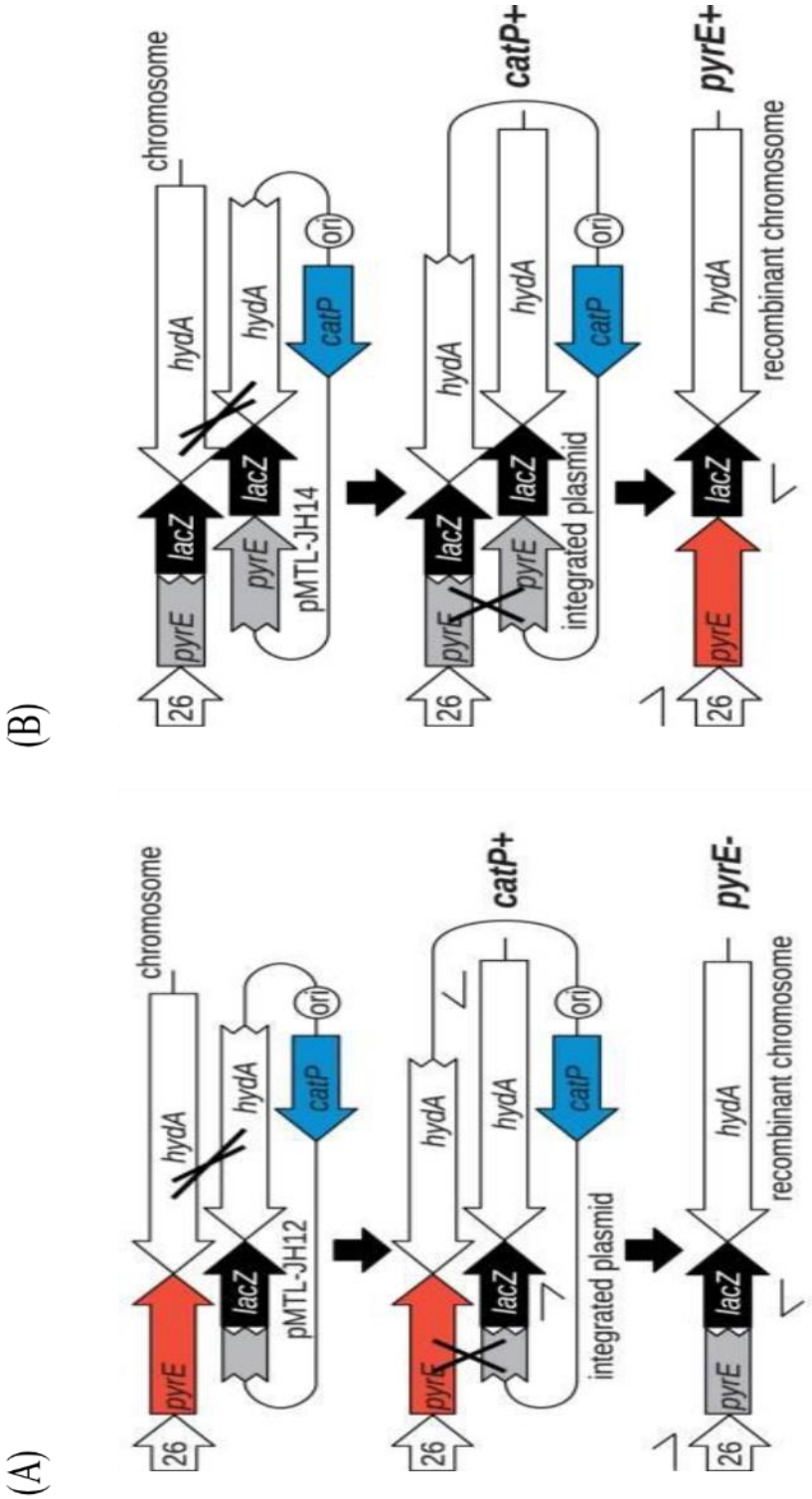


Figure 7 exemplification of the ACE selection system using pyrE as a selectable marker.
The lacZ gene is integrated into the genome at the pyrE locus through Allelic coupled exchange. In the first stage transformants containing the plasmid can be selected by thiamphenicol resistance conveyed by the catP gene. The initial crossover occurs at the large homology arm. The second recombination event occurs at the short homology arm and is isolated by the exposure to 5-FOA which will kill any cells with a functional pyrE gene. Repair of the truncated pyrE can occur by the transformation of the strain with a complementation plasmid which carries a longer short homology arm with the 5' terminal of the gene which repairs the truncation to the wild type. Single crossover strains can again be selected and successful double crossover and repair of the truncated gene can be selected for by FOA toxicity and uracil prototrophy. Figure sampled from Heap et al, 2012[139].

Currently the main selection method for In Frame Deletion/ Allelic Coupled Exchange (IFD/ACE) is selected for by deleting and replacing the gene *pyrE*. The intention in IFD is to specifically cut out a gene cleanly so there is no downstream effect except one seen because of the interactions of that gene. It can be used for Directed mutagenesis for multiple genes using two main components – SOEing PCR to get rid of the gene and *pyrE* selection to make sure it works.

SOEing (Splicing by Overlap Extension) PCR is a PCR homology based technique which is used to join the two regions flanking a gene that you want to remove, whilst excising the genetic material in between the flanking primers. The SOEing primers consist of regions of homology with each other, during PCR these are joined and "cut" the gene of interest out. This is then ligated into a plasmid with a selectable marker. This is described in detail in the methods section 2.6.9.

Selectable markers for successful mutagenesis are removable in the ACE/ IFD system, meaning they can undergo more than one round of mutagenesis[148]. As described in the seminal paper by Ng[149], a *pyrE* deficient strain is developed and then transformed back with a functional heterologous *pyrE* (*pyrE* correct) that acts as a selectable marker[148]. They are heterologous to ensure no site specific recombination with the disrupted native gene. This selection works on two levels: because *pyrE* is responsible for uracil synthesis, strains without a functional *pyrE* gene are not able to grow on plates without added uracil. The gene *pyrE* is also necessary for the metabolism of 5-fluoroorotic acid (5-FOA) that results in a toxic product that kills the organism[148]. If *pyrE* has been

affected, (Δ *pyrE*/ deficient in *pyrE*) then the organism cannot survive on plates without uracil because it is an auxotroph for uracil, but can grow with FOA because no toxic product is formed. If *pyrE* has been corrected it can grow on plates without uracil because it can make its own, but cannot grow on plates with 5- FOA because it makes a toxic product[148]. The great triumph of the ACE and *pyrE* selection system is that you can get as close to the wild type as possible (minus the genes you have removed), and the rest of the genome returned to the wild type by the end of the system so the only differences in the strains growth, viability or metabolite production is due to having that particular gene knocked out.

The ability to delete or insert genes using this method will allow for the specific alteration of the genome of *C. autoethanogenum* or *C. ljungdahlii* in order to insert or delete parts of pathways which could lead to the optimisation of the organism and convey industrially desirable characteristics.

1.6 Aims

1. To determine which of the currently added B-vitamins in the media are true vitamins for the species, and determine which are not actually required for growth and viability of the organism.
2. To try to introduce the missing genes from closely related species via plasmid and through transformation of the genome to make strains which can biosynthesise missing vitamins in order to generate autotrophic strains.
3. The assessment of the utility of the generated strains as surrogates for the wild type organism, through comparative assessment of physiological characteristics, growth, viability and product formation
4. To determine the effects of pantothenate (and therefore, CoA and Acetyl-CoA) limitation on growth, gas output and economic product formation in industrial conditions in a continuous stirred reaction tank reactor.

2 Materials and Methods

2.1 Bacterial Strains and Plasmids

2.1.1 Bacterial Strains used in this Study

Strain	Description/ Genotype	Source/ reference
Escherichia coli Top10	Plasmid storage strain	Invitrogen ltd.
Escherichia coli Ca434	Conjugative transfer strain	M. Young UCW, Aberystwyth, UK (Purdy et al.,2002)
E. coli Sexpress	NEB Express with RO210	C. Woods
Clostridium ljungdahlii	Wild type isolate	DSM 13528T DSMZ, Braunschweig, Germany
Clostridium acetobutylicum	Wild type isolate	ATCC 824, N.P.Minton, University of Nottingham, UK
Desulfotmaculum nigrificans	Wild type isolate	DSM 14008 DSMZ, Braunschweig Germany
Clostridium autoethanogenum ΔpyrE	*pyrE* in frame deletion by ACE	This study
Clostridium ljungdahlii ΔpyrE	*pyrE* in frame deletion by ACE	A. Henstra, University of Nottingham
C. autoethanogenum::pFJAL3	Contains the pMTL_FJAL3 plasmid	This study
*C. ljungdahlii****::pFJAL3***	Contains the pMTL_FJAL3 plasmid	This study
C. autoethanogenum::pFJAL4	Contains the pMTL_FJAL4 plasmid	This study
*C. ljungdahlii****::pFJAL4***	Contains the pMTL_FJAL4 plasmid	This study
C. autoethanogenum::pFJAL5	Contains the pMTL_FJAL5 plasmid	This study
*C. ljungdahlii****::pFJAL5***	Contains the pMTL_FJAL5 plasmid	This study
C. autoethanogenum::pFJAL6	Contains the pFJAL6 plasmid	This study
*C. ljungdahlii****::pFJAL6***	Contains the pMTL_FJAL6 plasmid	This study
C. autoethanogenum::pFJAL7	Contains the pMTL_FJAL7 plasmid	This study
*C. ljungdahlii****::pFJAL7***	Contains the pMTL_FJAL7 plasmid	This study
C. autoethanogenum::pFJAL8	Contains the pMTL_FJAL8 plasmid	This study
*C. ljungdahlii****::pFJAL8***	Contains the pMTL_FJAL8 plasmid	This study

C. autoethanogenum::pFJAL4+pFJAL6	Contains the plasmids: pMTL_FJAL4 and pMTL_FJAL6	This study
*C. autoethanogenum****::pFJAL5+pFJAL7***	Contains the plasmids: pMTL_FJAL5 and pMTL_FJAL7	This study
C. ljungdahlii::pFJAL4+pFJAL6	Contains the plasmids: pMTL_FJAL4 and pMTL_FJAL6	This Study
*C. ljungdahlii****::pFJAL5+pFJAL7***	Contains the plasmids: pMTL_FJAL5 and pMTL_7	This study
+pantothenate (C. autoethanogenum)	Repaired *ΔpyrE* strain with *panBCD* genes inserted at the *pyrE* locus under the control of a thiolase promotor	This study
+pantothenate (C. ljungdahlii)	Repaired *ΔpyrE* strain with *panBCD* genes inserted at the *pyrE* locus under the control of a thiolase promotor	This study
Weak pantothenate	Repaired *ΔpyrE* strain with *panBCD* genes inserted at the *pyrE* locus under the control of a weak (sac_fdx)promotor	This study
*C. autoethanogenum****::pFJAL20***	Contains the pMTL_FJAL20 plasmid	
*C. autoethanogenum****::pFJAL21+pFJAL22***	Contains the plasmids: pMTL_FJAL21 and pMTL_FJAL22	
+biotin	Repaired *ΔpyrE* with the *bioBDFHCA* genes at the *pyrE* locus	
+biotin+pantothenate	Contains the plasmids pMTL_FJAL3 and pMTL_FJAL24	

Table 1 Strains used in this project

2.1.2 Plasmids used in this study

Plasmid Name	**Function**	**Source of Plasmid**
R702	Conjugation plasmid in Ca434	M. Young, Aberystwyth
pAnne12	Plasmid deletes part of the gene involved in the production of *pyrE*. When deleted the strain becomes a uracil auxotroph and tolerant to 5-FOA	Donated by Anne Henstra
***pyrE* Knockout Vector**	Used to knock-out a gene already present in the organism. To use the vector the long and short homology arms are removed and SOEing PCR section is inserted between SacII and AscI.	Donated by Anne Henstra
pMTL_CH20	Can bring in a novel gene or a gene with an overexpression promotor into the genome of a *ΔpyrE C. autoethanogenum* strain.	Donated by C. Humphries
pMTL_CH23	Can bring in a novel gene or a gene with an overexpression promotor into the genome of a *ΔpyrE C. autoethanogenum* strain. Also contains a P_{thl} promotor ahead of the site for inserting payload genes	Donated by C. Humphries

pMTL_82151	Modular plasmid containing pBP1, ColE1+*tra*, CmR/TmR, MCS	Heap *et al*., 2009
pMTL_83151	Modular plasmid containing pCB102, ColE1+*tra*, CmR/TmR, MCS	Heap *et al*., 2009
pMTL_82252	Modular plasmid containing pBP1, ColE1+*tra*,ermR, pTHL+MCS	Heap et al., 2009
pMTL_82353	Modular plasmid containing pBP1, ColE1+*tra*,aadR, pfdx+MCS	Heap et al,2009
pMTL_83153	Modular plasmid containing pCB102, ColE1+*tra*,CmR/TmR, pfdx+MCS	Heap et al, 2009
pMTL_83152	Modular plasmid containing pCB102, ColE1+*tra*,CmR/TmR, pTHL+MCS	Heap et al, 2009
pMTL_82251	Modular plasmid containing pBP1, ColE1+*tra*,ermR,MCS	Heap et al, 2009
p*_panBCD*_82151	Modular plasmid 82151 containing the *panBCD* genes	Donated by A. Henstra
pMTL_FJAL3	Modular plasmid 82152 containing the genes *panBCD* under the control of a thiolase promotor	This project
pMTL_FJAL4	Modular plasmid 83153 containing the genes *panCD* under the control of a fdx promotor	This project
pMTL_FJAL5	Modular plasmid 83153 containing the genes *panBC* under the control of an *fdx* promotor	This study
pMTL_FJAL6	Modular plasmid 82252 containing the genes *panB* under the control of an *thl* promotor	This study
pMTL_FJAL7	Modular plasmid 82252 containing the genes *panC* under the control of an *thl* promotor	This study
pMTL_FJAL8	Modular plasmid 82252 containing the genes *panD* under the control of an *thl* promotor	This study
pMTL_FJAL10	CH23 plasmid containing the genes *panBCD*. Used to bring *PanBCD* genes under the control of a thiolase promotor into the genome and restore *ΔpyrE*.	This study
pMTL_FJAL11	CH20 plasmid containing the genes *panBCD* under the control of a weak promotor – *sac_fdx*. Used to bring in the genes and restore *ΔpyrE*.	This study
pMTL_FJAL20	Modular plasmid 82252 containing the genes bioYDA under the control of a P_{thl} promotor	This study
pMTL_FJAL21	Modular plasmid 82252 containing the genes *bioBDF* under the control of an *thl* promotor	This study
pMTL_FJAL22	Modular plasmid 83153 containing the genes *bioHCA* under the control of an *fdx* promotor	This study
pMTL_FJAL23	Ch23 plasmid containing a cassette with the *bioHCA* genes under the control of a fdx promotor and *BioBDF* genes under the control of an *thiolase* promotor designed to insert the genes into the genome of *C. autoethanogenum* and fix *ΔpyrE*	This study
pMTL_FJAL24	Modular plasmid 83251 containing the genes *bioHCA* under the control of an *fdx* promotor and *bioBDF* under the control of a thiolase promotor.	This study
Intermediate pMTL_FJAL23	Modular plasmid 83153 containing the genes *bioHCA* under the control of an *fdx* promotor	This study

Table 2 Plasmids used in this study

2.2 Oligonucleotide primers

2.2.1 Oligonucleotide primers

Oligonucleotide	Sequence	Function
***PanBCD*1FWD**	tatagcggccgcatgaaaaataccactgaaa	To amplify *panBCD* from C. acetobutylicum
***PanBCD*1REV**	tatagctagcttagccaattgtcc	To Amplify *panBCD* from C. acetobutylicum
PanCD1FWD	tataatcgatatggaaattttgcattcc	To amplify panCD from C. acetobutylicum
PanCD1REV	tatagctagc ttagccaattgtcc	To amplify panCD from C. acetobutylicum
PanBC1FWD	tataatcgatatgaaaaataccactgaaac	To amplify panBC from C. acetobutylicum
PanBC1REV	tatagctagcttatatattccaagtgaaattatctataagg	To amplify panBC from C. acetobutylicum
PanB1REV	tatagctagcttatttggtaatttcgtctataatg	To amplify panB from C. acetobutylicum
PanB1FWD	tatagcggccgcatgaaaaataccactgaaac	To amplify panB from C. acetobutylicum
PanD1FWD	tatagcggccgcatgcatttaaatatgttaaaatctaaa	To amplify *panD* from C. *acetobutylicum*
PanD1REV	tatagctagc ttagccaattgtcc	To amplify *panD* from C. *acetobutylicum*
PanC1FWD	tatagcggccgcatggaaattttgcattcc	To amplify *panC* from C. *acetobutylicum*
PanC1REV	tatagctagcttatatattccaagtgaaattatctataagg	To amplify *panC* from C. *acetobutylicum*
ACE*pyrE*FSP	gagcttatgcaattcaagtaggtactgcaaac	To screen for a double crossover event to ensure *pyrE* is truncated
CMH1	gagcaaggcaagacc	Colony PCR primer
M13 FSP	ccccccgcggcagcccaatattcc	To screen for a double crossover event to ensure *pyrE* is truncated
M13R	caggaaacagctatgacc	
ACE*pyrE*RSP	catcaaagctatactattttccgtatttacatttggg	Primer binds further downstream to isolate the *pyrE* deletion region for sequencing
ACE*pyrE*-F	agttggaataggtggaatagtaagc	Primer binds 200bp upstream of short homology arm integration site
ACE*pyrE*-R	cactatattcactgatactatttaaaaatacttctcc	Primer binds 200bp down stream of long homology arm integration site
***PanBCD*2FWD**	tataatcgatatgaaaaataccactgaaac	Amplification of *panBCD* genes from C. acetobutylicum for use in pFJAL10 and 11
***PanBCD*2REV**	tatagctagc ttagccaattgtcc	Amplification of *panBCD* genes from C. *acetobutylicum* for use in pFLAJ10 and 11

BioYDAF1	tatacatatgattatggtttctatttttgc	Amplification of *bioYDA* genes from *C. acetobutylicum*
BioYDAR1	tatacctagcttattcaagatactgctctatactttc	Amplification of *bioYDA* genes from *C. acetobutylicum*
bioBDFF1	tatacatatgagcaatattttgaaacatatcg	Amplification of *bioBDF* genes from *C. acetobutylicum*
bioBDFR1	tatagctagcttaaatcaatcccagttc	Amplification of *bioBDF* genes from *C. acetobutylicum*
bioHCAF1	tatacatatgttggaaaaacagagattaatcc	Amplification of *bioHCA* genes from *C. acetobutylicum*
bioHCAR1	tatagctagcttaatctattccaaaatagttattaattgc	Amplification of *bioHCA* genes from *C. acetobutylicum*
CH23:BioBDF	atgaaatagattgaaatgagcaatattttgaaacatatc	Gibson assembly primer 1 for pFJAL23
fdxBioHCA:BioBDF	gctactacacttaaatcaatcccagttcttttc	Gibson assembly primer 2 for pFJAL23
BioBDF:fdxBioHCA	ggattgatttaagtgtagtagcctgtgaaataag	Gibson assembly primer 3 for pFJAL23
CH23:fdxBioHCA	ttgatttttcgacgctagcttaatctattccaaaatagttattaattgc	Gibson assembly primer 4 for pFJAL23

Table 3 List of Primers used in this Study

2.3 Chemicals and suppliers

All molecular biology enzymes were ordered from New England Biolabs, (Ipswich, MA, USA), as well as the Ladders (2-log quickload ladder (0.1-10kb)), loading dyes (arrived as part of the 2-log ladder set) and DNTPs. Oligonucleotides and primers were ordered from Sigma-Aldrich Company Ltd (Dorset, England). The polymerases used were either Dream Taq (from Sigma-Aldrich) Q5 Polymerase or Phusion high-fidelity polymerase (from New England Biolabs). Plasmid Miniprep kits were from Sigma-Aldrich as were the Genomic DNA extraction kits. Gel extraction kits were either from Sigma-Aldrich, Invitrogen (Paisley, UK.) or Qiagen (Manchester, UK).5-Fluoroindole and 5-Fluoroorotic acid were from Sigma-Aldrich. All chemicals were from Sigma-Aldrich Company Ltd (Dorset, England).

2.4 Bioinformatics tools.

2.4.1 GenDB

In 2014, Brown *et al.* announced the release of a complete annotated genome of *C. autoethanogenum*. However, due to the genetic differences in commercial strain used by Brown et al to the first isolated native strain by Abrini *et al.* [18] it was considered prudent to reannotate the wild type strain used by the GASCHEM project, as it was believed to be closer to the wild type Abrini strain. In order to facilitate this, the programme GenDB was used. The work to annotate the genome, was split a group of researchers within the GASCHEM project and a variety of online databases of proteins, enzymes and genes were used. The main programmes and databases we used were NCBI Blast[150], KEGG[151], PFAM[152] and UniProt[153], which we used to compare the sequences from our wild type strain to previously published strains in order to elucidate gene functions, reactions and enzymatic functions they were potentially involved in, any previously allocated gene names and homologies with other bacteria. The results of this database mining was added to the GenDB file and the gene marked as annotated until the complete genome had been annotated. If no information could be found about the specific gene it would be labelled as 'incomplete annotation' but as much information as possible was added.

This work resulted in the paper by Humphreys *et al*. [1], "Whole genome sequence and manual annotation of *Clostridium autoethanogenum*, an industrially relevant bacterium".

2.4.2 DNA Sequence Analysis

Genomic DNA sequence analysis was conducted primarily using the NCBI Graphic Reporting Software. Basic Local Alignment Searches (BLASTn) for nucleotide comparisons against a database of nucleotides or proteins (BLASTp) used the algorithms provided by NCBI[150] or the UNIPROT Website[153]. Searches for sequences of nucleotides were conducted primarily through the UNIPROT search tools and biosynthetic pathways were searched for primarily using KEGG[151]. EMBOSS Needle[154] was used for DNA sequence alignment analysis. This function was provided by the European Bioinformatics Institute.

2.4.3 Protein Structure Analysis

The sequence database Pfamseq predicted functional domains of protein sequences, accessed and calculated by the website pfam.xfam.org[152] provided by the Wellcome trust Sanger Institute.

2.4.4 Plasmid Design and visualisation.

Plasmid maps were designed and visualised by three programmes, Ugene, GENtle, and Benchling.

2.4.5 Oligonucleotide design

Oligonucleotide primers were designed at the start of the project using Ugene software and checked for predicted primer-dimers, the absence of predicted secondary structures, melting point calculations and GC% content using Sigma Aldrich's "OligoEvaluator™" oligonucleotide calculator programme. Later in the study, the programme Benchling became the primary source for oligonucleotide design. Primers were still checked for secondary structure,

predicted primer-dimers and other characteristics using the dedicated Sigma Aldrich programme. Annealing temperatures were calculated by the manufacturer using the equation:

$$\mathbf{Tm}\ (°\mathbf{C}) = \mathbf{81.5} + \mathbf{0.41}(\%\mathbf{GC}) - (\mathbf{675/N})$$

Equation 1 how to calculate annealing temp for primers.
Where %GC refers to the percentage of G and C bases in the primer and N is the number of nucleotides in the primer.

2.4.6 Data analysis and presentation

Statistical analysis was performed using the programmes Microsoft Excel for spreadsheet analysis and data entry. Graphical representation of results and error bar calculations were provided by the programme GraphPad Prism version 6, or Microsoft Excel.

2.5 General Microbiological techniques

2.5.1 Growth Media

Bacteria were grown in liquid or solid media at 37°C. General Media used in the growth of bacteria was made by the recipes outlined below. Solid media was obtained by the addition of 1.5% w/v of number 1 Bacteriological agar powder. All media was autoclaved for 20 minutes at 120°C. All recipes were made using RO water.

2.5.1.1 Luria Broth

E. coli cells were grown over night in either liquid LB medium or on agar plates at 37 °C. If necessary, supplements listed in Table 15 were added.

Luria-Bertaini Media (LB)	In 1 Litre
Tryptone	10g
Yeast extract	5g
Sodium Chloride	10g

Table 4 Luria broth recipe

The pH of this media is naturally around 7-7.5.

2.5.1.2 Yeast Tryptone Fructose Media

To cultivate *C. autoethanogenum* in a rich medium Yeast Tryptone Fructose media or YTF media was used. YTF was used only in anaerobic cabinets or occasionally as a control in serum bottles. In order to make the YTF anaerobic either plates or bottles of media were left in the cabinets for a minimum of four hours or until the rezasaurin in the media had turned from pink (indicating oxygen presence) to clear (so plates looked yellow). The pH was set to 5.8 using HCl.

The components of the macronutrient solution were added in order of appearance. Each component was completely dissolved before the next one was added.

Yeast-Tryptone-Fructose Media	Per litre of Media
Yeast Extract	10g
Fructose	10g
Tryptone	16g
NaCl	0.2g
Vitamin solution (as seen in Table 9)	10mL
Trace Metal Solution (As seen in table 7)	10ml
RO Water	970ml

Table 5 Yeast Tryptone Fructose Media (YTF)

2.5.1.3 PetC-MES

Anaerobic PetC-MES media was conducted according through Breznak and Costilow [78] in Hungate-tubes or serum bottles. An appropriate volume of dH2O was placed in the anaerobic tent 48 hours before the preparation of the

media in order to dispel all of the oxygen. Tubes containing the required volumes of stock solutions and chemicals were placed in the anaerobic tent 24 hours before the media was made. The required serum bottles, caps, and butyl rubber stoppers were placed into the anaerobic tent two hours before use, along with an electric pipette and graduated 50ml stripettes. All the components listed below were added to the deoxygenated water and mixed thoroughly before aliquots being removed for pH testing and the pH adjusted with 3M NaOH until the pH was approximately 5.8. 50ml was then aliquoted into each serum bottle, the bottles capped and autoclaved for 20 minutes at 121ºC.

Fructose and further media supplements were added after autoclaving from separate anaerobe and sterile stock solutions. Cysteine or sodium-sulfide was added before using the medium to act as reducing agent and additional sulphur source. Although in some cases none of the mentioned reducing agents were added into the medium.

STOCK SOLUTION	**CONCENTRATION**	**VOL FOR 1L**
NH_4Cl	200g/L	5mls
KCl	100g/L	1ml
$MgSO_4.7H2O$	200g/L	1ml
KH_2PO_4	200g/L	1ml
$CaCl_2$	20g/L	1ml
Nitriolotriacetic Acid	50g/L	1ml
Trace metal solution (as seen in Table 7)	100X	10mls
B vitamin solution (as seen in table 9)	100X	10mls
Rezasurin	2g/L	0.5ml
3M NaOH	3M	~10mls-to adjust to pH 5.8

Table 6 PetC- MES Recipe

2.5.1.4 Standard defined minimal media +/- Uracil

Used to determine whether there is an autotrophy or auxotrophy for uracil during the *pyrE* selection system. In order to make the media solid 15g of Oxoid agar

number 1 per litre could be added. The pH was set to 5.8-6.0 using 3M HCL and 3M NaOH.

Component	In 1 Litre of media
MES	5g
D-fructose (40 mM)	7.2g
Sodium Formate	1g
5M NaOH	2ml
1000x acidic trace solution	1ml
1000x basic trace solution	1ml
Vitamin solution (Table 9)	1ml
50x macronutrient solution	20ml
Vitamin free Casamino acids	10g

Table 7 SDMM +/ - uracil

2.5.1.5 Initial Bioreactor Media

Media component	Amount per 1.0 L of media	Amount per 1.5 L of media
$MgCl_2.6H_2O$	0.5g	0.75g
$CaCl_2.2H_2O$	0.37g	0.555g
KCl	0.15g	0.225g
NaCl	0.12g	0.18g
85% H_3PO_4	0.38ml	0.57mls
NH_4Cl	1.0g	1.5g
Metal mix 1	1ml	1.5mls
Metal mix 2	0.2ml	0.3mls
Tungsten solution	0.2ml	0.3mls
Rezasurin (2g/L)	0.25ml	0.375mls

Table 8 Initial media recipe for bioreactor. Source: Louise Sewell

2.5.1.6 Standard Bioreactor Media

MEDIA COMPONENT	In 20L
$NaH_2PO_4.2H_2O$	17.32g
$MgCl_2.6H_2O$	10g
$CaCl_2.2H_2O$	7.4g
KCl	3g
NaCl	2.4g
NH_4Cl	20g

Metal mix 1	20mls
Metal mix 2	4mls
Tungsten solution	4mls
Rezasurin (1g/L solution)	10mls (or 10mg)
B-Vitamin solution	200mls

Table 9 Standard Bioreactor media recipe

2.5.2 Stock solutions

2.5.2.1 B vitamin Solution

Stock component	**Amount per L of stock**
Thiamine HCl	50mg
Riboflavin	50mg
Nicotinic Acid	50mg
Pantothenic Acid	50mg
Pyridoxine HCl	10mg
Biotin	20mg
Folic Acid	20mg
4-Aminobenzoic Acid	50mg
Cyanocobalamin	50mg
Lipoic Acid	50mg
Distilled Water	To 1L

Table 10 B vitamin stock solution

2.5.2.2 Trace Metal Solution

Trace Metal Solution	**In 1L of stock**
Nitrotriacetic Acid	2g
$MnSO_4.H_2O$	1g
$Fe(SO_4)_2(NH_4)2.6H_2O$	.8g
$CoCl_2.6H_2O$	.2g
$ZnSO_4.7H_2O$	.02g
$CuCl_2.2H_2O$	.02g
$NaMoO_4.2H_2O$	.02g
Na_2SeO_3	.02g
$NiCl_2.6H_2O$	.02g
$Na_2WO_4.2H_2O$	.02g

Table 11 trace metal solution

2.5.2.3 Macronutrient Solution

Component	g/L	g/500mL
	(1x)	(50x)
NH_4Cl	1	25
NaCl	0.8	20
KCl	0.1	2.5
KH_2PO_4	0.1	2.5
$MgSO_4.7H_2O$	0.2	5
$CaCl_2.2H_2O$	0.04	1

Table 12macronutrient solution

2.5.2.4 Acidic Trace Element Solution

1000x Acidic trace element solution (per L)	Per Litre Media
HCl	50 mM
H_3BO_3	100 mg
$MnCl_2.4H_2O$	230 mg
$FeCl_2.4H_2O$	780 mg
$CoCl_2.6H_2O$	103 mg
$NiCl_2.6H_2O$	602 mg
$ZnCl_2$	78 mg
$CuSO_4.5H_2O$	50 mg
$AlK(SO_4)_2.12H_2O$	50 mg

Table 13 Acid Trace Element solution

2.5.2.5 Base Trace Element Solution

1000x Basic trace element solution	Per Litre
NaOH	10 mM
Na_2SeO_3	58 mg
Na_2WO_4	53 mg
$Na_2MoO_4.2H_2O$	52 mg

Table 14Base trace element solution

2.5.2.6 Supplements

All media supplements were made in accordance with manufacturer's instructions and stored for the recommended length of time at the recommended temperatures. Supplements used in this study are outlined below. All additions to the media post- autoclave were syringe filter-sterilized using a Minisart filter with

membrane pores no larger than 0.2µm under a Pasteur hood created by a Bunsen burner.

Supplement	Solvent	Stock conc. mg/ml	Working conc. *E. coli* µg/ml	Working conc.. Clostridia sp. µg/ml
Ampicillin (amp)	water	50	100	-
Chloramphenicol (Cm)	100% EtOH	25	25	-
Thiamphenicol (Tm)	50% EtOH	15	-	15
Erythromycin (Erm)	100% EtOH	50	500	10
Kanamycin (Kan)	water	50	50	-
D-cycloserine (DC)	water	50	-	250
5-fluoroorotic Acid (5-FOA)	DMSO	150	-	4000
Uracil	water	1	-	20

Table 15 supplement table

2.5.3 Individual Vitamin stocks

In order to make up specific vitamin concentrations and compositions, sealed sterile serum bottles containing filter sterilised concentrated amounts of the individual vitamins were made. The stock concentrations were made using the amounts of each vitamin from 2.5.2.1 but in only 100ml of liquid. In the cases of 4-Aminobenzoic acid and lipoic acid, 50mg was added to a bottle containing approximately 10mls of dH2O, this was then titrated with 3M NaOH until dissolved while mixing regularly and then made up to 100 ml. In the case of riboflavin the vitamin was dissolved in 100ml of approximately 50ºC of dH_2O. In cases where specific vitamin mixes were being tested, these were added post autoclaving of the media due to the controversy about whether certain vitamins are heat labile and at what temperature. All vitamin bottles were protected from

light degradation and heat damage by wrapping them in aluminium foil and keeping them at 5ºC.

2.5.4 Aseptic technique

In order to sterilise solutions and bottles or other essential apparatuses such as Eppendorfs or pipette tips, components were autoclaved for at least 20 minutes at 121ºC. Benches or other worktops, and tops of bottles during sampling were sterilised with 70 % ethanol, and dried. Filters were used to sterilise heat labile components which needed to be added to media after autoclaving. Consumables such as spreaders or loops, petri dishes or falcon tubes were bought sterile and discarded after use.

2.5.5 Growth Conditions

Bacteria were grown in liquid or solid (1% agar) media at 37 °C. Aerobic organisms were cultured in liquid media in a rotating 37 ºC incubator at 225 rpm and in solid media in a 37 or 30 ºC standing incubator. Anaerobic strains were cultured in PetC-MES (American Type Culture Collection (ATCC) medium 1754) or YTF in anaerobic cabinets or in sealed serum bottles and Hungate tubes, with an atmosphere of 80% Nitrogen, 10% Carbon dioxide and 10% hydrogen. If the strains were using “Gas” as their carbon source then post inoculation the serum bottles containing anaerobic mixture were overpressured using a bar of carbon monoxide added by a filtered needle using a gas exchange. Growth experiments were carried out in PetC-MES in anaerobic conditions, with vitamin concentrations or contents modified according to experiment. Appropriate antibiotics were used for *C. ljungdahlii* and *autoethanogenum* (250ug/ml of erythromycin, 20 µg/mL thiamphenicol) and E. coli (, 250 µg/mL erythromycin, 30 µg/mL chloramphenicol, 50 µg/mL spectinomycin). Before inoculation

medium was reduced with 2% v/v L-cysteine solution and 0.2% v/v Ti-NTA solution. Different vitamin tests were carried out by modifying the original media recipe by leaving out the relevant vitamins. The Casamino acids were vitamin free. The B-vitamin stocks were filter sterilised and added after autoclaving. Separate stock solutions were made not containing one of the vitamins. 1 ml of these was added per litre of media.

2.5.5.1 Gas Adaptation

Transformed *C. autoethanogenum* and *C. ljungdahlii* strains were often not able to grow abundantly on carbon monoxide alone initially and so a weaning process was implemented called gas adaptation. Transformed cultures were grown in 5ml cultures containing fructose and then inoculated into 50 ml serum bottles containing 25% of the standard fructose concentration and 1bar of CO. If cultures successfully grew in these conditions a subculture was made into a serum bottle containing just 1 bar of CO, and if growth was successful into a final culture in conditions as outlined above. If the cultures were being tested for vitamin prototrophy they were not exposed to the tested vitamin in the second bottle but they were in the first in order to try to gently expose them to new conditions rather than risk the non-viability of the culture because it was expected to grow on both a new substrate and without a vitamin immediately. This step was often fraught with losses, and extremely time consuming.

2.5.6 Strain storage and revival

Clostridial strains were stored by inoculating 4ml of overnight cultures and then pipetting 200µl of this culture into Microbank^{Tm} Long Term Bacterial storage system tubes by Pro-lab Diagnostics, or by adding glycerol to make a 10%

solution and storing in the -80 ºC freezer or the culture collection. *E coli* strains containing plasmid DNA were stored in top10 cells in the same manner.

2.5.7 Preparation of competent cells.

2.5.7.1 Electrocompetent *E. coli* Cells

Electrocompetent cells were prepared as described in[155]. *E. coli* cells previously transformed with the top10 plasmid or the CA434 plasmid were removed from the culture collection and grown over night on LB plates (all media contained kanamycin if CA434) and incubated overnight in a 37 ºC incubator. The following evening a colony was picked from this plate and used to inoculate a 10ml culture of LB which was grown overnight in 37 ºC shaking incubator. 500µl of this culture was used to inoculate 100ml of unsupplemented LB broth and grown until the culture reached an OD (600) 0.5-0.6 at 600nm. The following stages all took place in an ice bath. The 100 ml culture was split between three 50ml falcon tubes and centrifuged at 4000x G for 10 minutes in a cooling incubator set to 4 ºC in order to harvest the cells. The pellet was washed gently in 35ml of sterile ice cold dH2O and centrifuged in the same conditions and the supernatant removed. This step was repeated and then cells were finally resuspended in 500µl of sterile 10% glycerol before being pooled and separated into 50µl aliquots and stored in the -80 ºC freezer.

2.5.7.2 Chemically competent E.*coli* Cells

Chemically competent cells were prepared as described in[155]. *E.* coli cells previously transformed with the Top10 plasmid, the CA434 plasmid, or Sexpress strains were removed from the culture collection and grown over night on LB

plates and incubated overnight in a 37ºC incubator. A colony was picked from this plate and inoculated into 5ml of LB containing an appropriate antibiotic and grown for 14 hours. One ml of this resultant growth was inoculated into 100ml of LB medium in a sterile conical flask and agitated in a 37ºC incubator shaking at 225rpm until the $OD_{(600)}$ reached 0.4-0.6. This was then decanted into 50ml falcons and kept on ice for 30 minutes. The falcon tubes were then centrifuged in a cooling Eppendorf™ Centrifuge (5430R) at 4ºC for 10 minutes at a low speed (3000 x g). The resulting supernatant was discarded and ice cold $CaCl_2$ buffer (100mM $CaCl_2$. 20% glycerol) was used to resuspend the pellet this was then incubated on ice for 15 minutes, and then the centrifugation process was repeated and the supernatant discarded. The pellet was then resuspended in 4ml of the buffer and chilled for one hour, before being aliquoted into 50µl samples and stored in the -80ºC freezer.

2.5.7.3 Electrocompetent *C. ljungdahlii* Cells

To produce electrocompetent *C. ljungdahlii* cells, 300 ml of YTF was inoculated with 30ml of early stage exponentially growing cells and incubated in the 37ºC anaerobic workstation overnight. When the OD had reached 0.4-0.5, the culture was transferred in an anaerobic gas canister to the coy tent, and cells were harvested by centrifugation at 4226 x g for 5 minutes at room temperature, and supernatant discarded. The cell pellet was washed using 30ml of SMP buffer (1mM magnesium chloride, 7mM phosphate and 270mM sucrose) which had been prepared the day before and left to degas overnight. The centrifugation step was repeated and then the pellet wash washed twice in a further 30ml of SMP. After the discarding of the supernatant the cell pellet was finally resuspended in 2ml of SMP and 10% (v/v) of DMSO. The competent cells were aliquoted into

100µl samples in 2ml eppendorfs and flash frozen in liquid nitrogen and stored in the -80ºC freezer.

2.5.8 Transformation

2.5.8.1 Electroporation of *E. coli*

Previously prepared electrically competent *E. coli* strains Ca434 and Top10 were transformed by incubation of 50µl of competent cells with 5µl of plasmid prepared from a Sigma Aldrich plasmid preparation kit for 30 minutes on ice. The mixture was transferred to an electroporation cuvette (BioRad 0.2cm) and electroporation occurred by an electropulse shocking in a Biopulser (BioRad Gene Pulsar) at setting Eco2 (2.5 kV, 200Ω and 25µF capacitance). After two minutes to recover on ice, the electroporation cuvettes had 250µl of SOC media added to them, the cells and media were transferred to a labelled Eppendorf tube and they were left to recover for two hours in a 37 ºC shaking incubator. Successful transformants were screened for on LB agar plates with working concentration of 25 µg/ml of chloramphenicol or 500 µg/ml of erythromycin as required.

2.5.8.2 Chemical transformation

Chemically competent *E. coli* Ca434 and Top 10 were made and transformed by heat shock at 42°C for 45 seconds as outlined in [155]. 5µl of DNA were incubated for 30 minutes with 50 µl of competent cells. Cells were then heat shocked and returned to ice for two minutes, after which 500 µl of SOC media (supplied by Sigma Aldrich) was added to the cells and then incubated in a 37ºC shaking incubator for 2 hours. Transformants were selected for on LB agar plates with an appropriate antibiotic.

2.5.8.3 Conjugation

C. autoethanogenum was transformed by conjugation with *E. coli* Sexpress (developed by C. Woods, Nottingham) which contains a conjugation plasmid R702 inside NEB Express cells. The plasmid of interest was transformed by electroporation or chemical transformation into competent Sexpress cells. Successful conjugants (screened for by antibiotic resistance) were inoculated overnight into sterile falcon tubes containing 5 ml of LB supplemented with 100 µg/ml of spectinomycin and a relevant antibiotic. When the optical density had reached 0.8, 1.5 ml aliquots of the overnight culture were washed in 500 µl of PBS aerobically. The pellet was transferred to an anaerobic cabinet and suspended in 200µl of a wild type *C. autoethanogenum* culture (in exponential phase grown to an OD of approximately 0.2-0.4), then the whole suspension was plated onto non-selective YTF mating plates. After 24 hours the resulting growth was suspended in 500µl of PBS and then plated onto YTF selective plates containing a relevant selective antibiotic, and 10µg/ml of trimethoprim.

2.5.8.4 Transformation of Electrocompetent *C. ljungdahlii*.

100µl of electrocompetent *C. ljungdahlii* cells were thawed on ice with 5 µl of the transformation plasmid for 30 minutes. The cells were then transferred to a chilled electroporation cuvette with a 2mm electrode gap. The cuvettes were then electrocuted at 1kV from a 50µF capacitor using the Gene Pulse Xcell electroporation machine (Bio Rad laboratories). The shocked cells were suspended in 10ml of YTF and left to recover for 20 hours before plating onto selective media for transformants.

2.5.8.5 Plasmid stability assay

In order to ascertain the stability of transformed plasmids in *C. autoethanogenum*, transformed by either electroporation or by conjugation, a single colony from a selection plate was inoculated into 4ml of YTF (with appropriate antibiotic selection supplementation) in triplicate and incubated anaerobically overnight. 1ml of this overnight culture was transferred to a 2ml Eppendorf and centrifuged at 8000 x g at room temperature for 2 minutes. The supernatant was then removed and discarded and the pellet resuspended in 1ml of anaerobic PBS and centrifugation process repeated, and washed with a further 1ml of anaerobic PBS and finally resuspended in 1ml of anaerobic PBS. 100µl of the resuspended cells were used to inoculate two 5ml sample tubes of YTF in the anaerobic cabinet, one containing antibiotic supplementation to act as a selection pressure for the plasmid and one without. These tubes were left to grow for 24 hours. Every 24 hours, 1ml of the sample was removed, washed in the aforementioned manner and serial dilutions of the sample were made to $10^{-6.}$ 20µl of each of the dilutions were plated onto plates with and without antibiotic supplementation. Colonies were enumerated after 24 hours.

2.6 Development of the *ΔpyrE* strain

2.6.1 *pyrE* selection.

The *pyrE* selectable marker system is a powerful system for introducing genes and for deleting them. As described in the seminal paper by Ng[149], a *pyrE* deficient strain is developed and then transformed back with a functional *pyrE* heterologous (*pyrE* correct) that acts as a selectable marker.[148]

To use the *pyrE* selection system the plasmids involved (*ΔpyrE*, knockout vector, complementation vector and correction vector) had to be created. As mentioned some of these plasmids were donated. Plasmids were taken from -80ºC stock and electroporated into electrically competent *E. coli* Ca434 cells. After electroporation they were plated onto LB plates containing chloramphenicol. Colonies which had taken up the plasmid, and therefore, the *CatP* gene (which makes a cell chloramphenicol resistant) survived and were then used to inoculate an overnight culture of 5ml of LB+ Chloramphenicol, and the plasmids extracted using a Miniprep kit. Once the *ΔpyrE* plasmids were extracted they were conjugated into *C. autoethanogenum.* The conjugations were then tested on selective plates with trimethoprim and 7.5µg/ml Thiamphenicol. Colonies able to survive this selection were purified, and plated onto YTF agar plates containing 5-Fluoroorotic acid. Colonies which survived the Fluoroorotic acid test were then tested for auxotrophy on plates which contained minimal media with and without uracil (as outlined in 2.6). The auxotrophs (those that could not survive without uracil) glycerol stocks taken and genomic DNA extracted. This was tested using a PCR amplification of the section between the M13 primers. Those which had 600 base pairs between them were sent for whole genome sequencing.

2.6.2 Complementing a *ΔpyrE* strain and bringing in a payload

To use the *ΔpyrE* Selection System to bring in a payload of genes, plasmids were designed using the CH20 (no promotor) or the CH23 (*thl* promotor) which contained the appropriate homology arms. *LacZα* (the placeholder gene) was removed and the required genes inserted into its place. These plasmids were then conjugated into the *pyrE* deficient strains of *C. autoethanogenum* and successful

conjugants were plated onto 25ml YTF agar plates with and without a working concentration of 1000µg/ml of 5-fluoroorotic acid. Colonies not able to survive the Fluoroorotic acid test were tested for uracil autotrophy by plating a sample of the same colony onto 25ml agar plates containing a minimal media with 20µg/ml^{-1} uracil and 25ml agar plates containing no uracil. Successful complements should have been able to survive without uracil but not when fluoroorotic acid was present.

2.7 Microscopy.

2.7.1 Phase contrast microscopy.

Samples were often viewed under a phase contrast microscope in order to confirm at least phenotypically they were the correct shape, and reasonably healthy with lots of living cells (short, clearly outlined, densely filled rod shaped bacteria with lots of dividing cells as opposed to very long fainter cells with rough edges, indicating stress.) and cultures were not contaminated. A Nikon H5505 microscope was used for this purpose.

2.7.2 Transmission Electron Microscopy

For better quality images of wild type *C. autoethanogenum* a project was undertaken with the help of Dr. Denice McLean from the Advanced Microscopy unit in the QMC. High quality samples of cells growing in exponential culture were harvested at 1000rmp. The supernatant was aspirated and discarded and a fixing agent (2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer) was used to resuspend the pellet and incubated for 30- 45 minutes. The suspension was recentrifuged, and the excess fixative agent (that which was not in the pellet) was removed and a solution of 0.1 M cacodylate/phosphate buffer

was used to resuspend the pellet. The pellet was washed in the buffer and centrifuged a further three times and then the pellet was dissolved in 1 % aqueous osmium tetroxide and centrifuged again. The pellet then underwent four more washing steps in 0.1 M cacodylate/phosphate buffer and then washed for a further 5 minutes in distilled water. The pellet was then gradually dehydrated in different concentrations of ethanol (twice for 15 min 50 % ethanol, twice for 15 min 70 % ethanol, twice for15 min 90 % ethanol, three times for 20 min 100% ethanol, and finally twice for 15 min 100 % propylene oxide (propox)). Resin was then added to the dehydrated pellet. The resin was composed of: 25 ml araldite CY212 resin, 15 ml agar 100 resin and 55 ml DDSA, 2 ml dibutyl phthalate and 1.5 ml DMP 30 added. The pellet was continually stirred during this resination process, and a colour change from a brick red to a bright orange occurred. The pellet was then left to polymerise for 48hours at 60°C. There were three stages to the infiltration of the resin into the fixed, washed pellet. Four hours in a 1:3 resin to propox mix, then overnight in a one to one ratio of resin to propox and finally three round of 2.5 hours in pure resin. The samples were then incubated in an oven for 48 hours at 60 °C. When they were dry, slices of the pellet were cut and mounted on slides. For the imaging, uranyl acetate was used as a negative stain. A Tecani Biotwin Transmission Electron Microscope using a voltage of 100Kv and a digital camera was used in order to take the images. In all stages we were carefully coached by Denise McLean.

2.8 General Molecular Techniques.

2.8.1 Preparation of Genomic DNA from Clostridial species

In order to obtain genomic DNA from clostridial species the Sigma Aaldrich GenElute™ Bacterial Genomic DNA Kit was used. 1.5ml of exponential culture of the desired strain was removed decanted into an Eppendorf and centrifuged for 3 minutes at 12000x g. The supernatant was removed and 200µl of lysis buffer (10mg/ml lysozyme in PBS) was used to resuspend the pellet of cells. Samples were then gently shaken and incubated at 37 ºC for about half an hour. Then 20µl of proteinase K solution and 200µl of lysis solution C were added, followed by votexing and incubation at 55ºC for 10 minutes. GenElute Miniprep Binding columns were then prepared and the elution discarded, in order to maximise DNA binding to the column. 200µl of ethanol (95-100%) was added to the lysate and vortexed, and the solution transferred to the binding column and centrifuged at 6500Xg for 1 minute, before discarding the collection tube and moving the binding column to a fresh collection tube. The column was then washed twice in 500µl of wash solution and eluate discarded. The column was then dried by centrifugation at 6500xg to remove any residual ethanol. 200µl of nuclease free water was then used to elute the DNA from the binding column at 65000xg after incubation of the sample for 15 minutes at room temperature. The gDNA was stored at -20ºC.

2.8.2 Plasmid DNA from *E. coli*

Plasmid DNA was removed from cultures of *E. coli* by inoculating 5ml of LB media with an appropriate selection antibiotic and growing overnight and then using the QIAprep Miniprep kit, the Sigma Aldrich Miniprep kit or the Monarch

NEB Miniprep kit according to the manufacturer's instructions. Storage was in nuclease free water in -20 ºC freezer or by transformation into Top10 *E. coli* strains.

2.8.3 Restriction Enzyme Digests

New England Biolabs supplied all restriction enzymes and appropriate buffers, and all digestions were carried out as per the appropriate instructions. In almost all instances the digestion mix was made of 1µg of DNA, 1µl of restriction enzymes and 5µl of reaction buffer – using which ever had the highest combined efficiency for all enzymes being used and making the total reaction up to 20µl with nuclease free water

2.8.4 Dephosphorylation of linear DNA

Vectors were dephosphorylated in order to prevent religation of lineralised plasmids. Antarctic phosphatase (NEB) was used for the Dephosphorylation according to the instructions supplied by NEB. This included 1µg of vector DNA, .5µl of Antarctic phosphatase and 1x Antarctic phosphatase buffer and then the reaction volume was made up to 20µl with nuclease free water. The reaction was then incubated at 37 ºC for 15 minutes and the enzymes inactivated for 5 minutes at 65 ºC.

2.8.5 Plasmid DNA Ligation

In order to ligate plasmids, the Rapid T4 ligation pack from Promega was generally used. Lineralised DNA fragments were mixed in a ratio of 2:1 vector to insert and combined with 1µl of T4 DNA ligase and 1X T4 ligation buffer and the total volume of the reaction was made up to 20µl using nuclease free water. Ligations were generally carried out at room temperature for 30 minutes, but in

recalcitrant plasmids could be carried out at a temperature gradient over night by incubating in thawing ice (-10ºC to 10 ºC) plasmids were then transformed into competent cells in a manner written in these methods previously.

2.8.6 Hi-Fi or Gibson's Assembly

Hi-Fi assembly is an efficient method for the combination of two or more fragments into a vector. The NEBuilder® Tool was used to design primers and calculate optimal annealing temperatures for Hi-Fi Assembly. NEBuilder® HiFi DNA Assembly Master Mix was used supplied by New England Biolabs. Fragments amplified by specific primers were combined in an Eppendorf on ice, in a ratio of 2:1 insert: vector and combined with 10µl of NEBuilder® HiFi DNA Assembly Master Mix and the reaction volume made up to 20µl and incubated for 30 minutes. They were then incubated in the thermocycler at 50ºC for 60 minutes and then transformed into competent cells.

2.8.7 Agarose Gel Electrophoresis

TAE buffer was made using 40mM Tris, 1mM EDTA and 0.1% glacial acetic acid. To make agarose gels, TAE buffer was used to dilute a 1% (w/v) concentration of agarose powder. This was then boiled in a microwave and left to cool to 55 ºC in a 55 ºC water bath. The gel mould and comb was set up and 1µg/ml of sybrsafe was added to the agarose gel and the cooled agarose gel was poured into the mould. When the gel was set the mould and comb was removed and the gel placed in an electrophoresis tank filled with TAE. DNA (in plasmid or fragment form) and NEB loading buffer (6X concentration) were mixed

thoroughly and then loaded into the gel. Electrophoresis separated the bands at 100V for 45 minutes.

2.8.8 DNA Extraction from Agarose gel

Gels were visualised under blue light or in the Licor™ machine. Once images were taken, if the gel was successful and the band needed to be excised a scalpel would be used to trim as much of the excess gel as possible from the useful band. The DNA would then be extracted using either a Zymoclean® Gel DNA Recovery Kit or Monarch® DNA Gel Extraction Kit (supplied by New England Biolabs (UK), as per the manufacturer's instructions. DNA would be eluted in 10µl of nuclease free water.

2.8.9 Polymerisation Chain Reaction

Polymerase Chain Reaction (PCR) was used to amplify specific regions of DNA. As outlined in section 2.4.4 and 2.4.5, primers were designed using the bioinformatics programmes: Benchling, Ugene or Gentle, and annealing temperatures calculated by the Sigma Aldrich oligo analysis tool. Primers were synthesised by Sigma Aldrich, and the desiccated primers were rehydrated to a stock concentration of 100pm.μl^{-1} using nuclease free water, and then diluted further to a working concentration of 1 in 10. PCR reactions contained 100-200µg of DNA as a template, 1 µl each of forward and reverse primers, 1 µl of DNTP mix .5µl of Phusion or Q5 polymerase if high fidelity was required (for ongoing reactions) or DreamTaq if lower fidelity was acceptable (for screening primers), 5µl of an appropriate buffer made up with nuclease free water to a final reaction volume of 25µl. Thermocycling occurred in either a TProfessional Trio Thermocycler by Biometra if there was high confidence in the annealing temperature or if a graduated temperature scale was required then a SensoQuest

Labcycler by Geneflow was used. with the calculated annealing temperatures as mentioned before and an elongation time of 30 seconds per 1 KB of DNA to be copied. PCR reaction programmes were adapted according to guidance by the manufacturer of the polymerase for the appropriate denaturation, annealing, elongation and final elongation times, temperatures, and number of cycles of the programme to be carried out, a typical example of a PCR programme is below. PCR products were stored short term at 4 ºC or long term at -20 ºC.

Cycle Step	Temperature (ºC)	Duration	Program Cycle
1. Initial Denaturation	95	2 min	
2. Denaturation	95	45 sec	
3. Annealing	55*	45 sec	
4. Elongation	72	2 min**	Return to step 2. (35x)
5. Final Elongation	72	5 min	
6. Hold	4	-	

Table 16 standard PCR programme for amplification of DNA fragments.
** annealing temperature dependent on melting temperature of the primer in each reaction. **elongation time dependent of product length ~30 seconds per 1KB*

2.6.9 SOEing Polymerase Chain Reaction

Knock out clusters were generated by SOEing PCR in order to generate two regions of homology to the genome at the desired sites, flanked by restrictions sites. Overlapping sections of homology were designed by visual analysis, and the procedure is outlined in Figure 8. Primers A and D contain the restriction sites X in the 3' terminal and Y in the 5' terminal respectively. B and C were designed with over lapping homology arms for each other of 10 nucleotides each (in their 5' and 3' terminals respectively). In the first round of PCR the two DNA fragments are generated and in the second round of PCR the fragments are combined as the 20bp overlap causes the two fragments to anneal together, and the final recombinant product is generated when the primers A and D anneal to the terminals and DNA polymerase fills in the gaps.

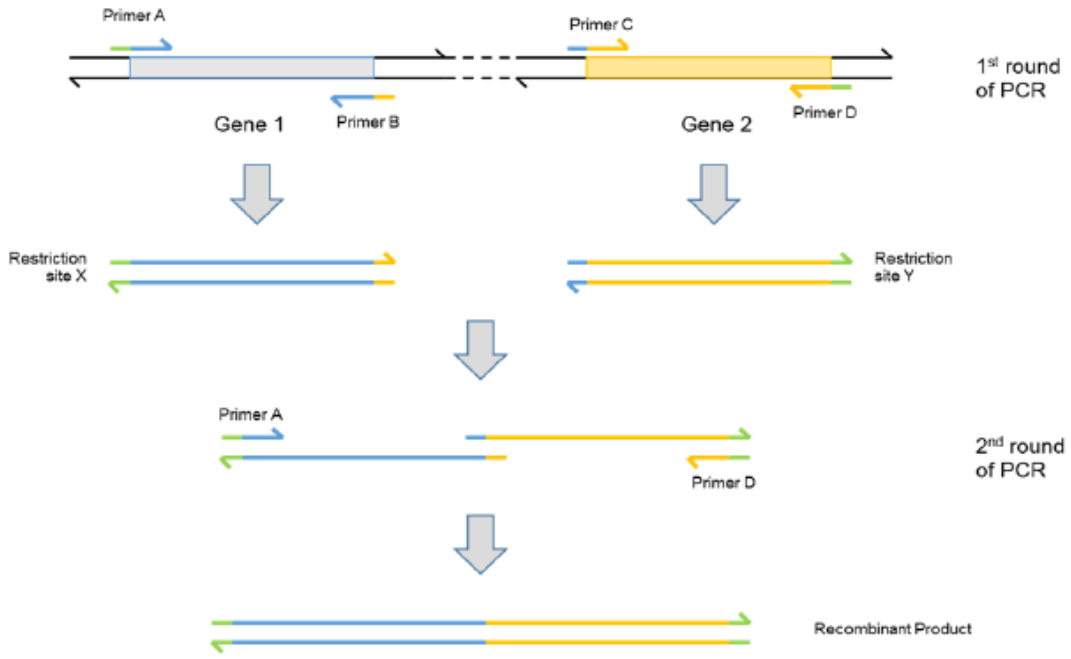


Figure 8 A representation of SOEing PCR.
image sampled from Dr. C. Humphreys (University of Nottingham) Primers A and D have restriction sites X and Y respectively integrated into their design and B and C have a 10bp homologous overlap.

2.8.9.1 Colony PCR

In order to perform Colony PCR for a rapid confirmation of mutants, transformed colonies were first restreaked and then a new colony was picked and resuspended in 20µl of dH2O in a PCR tube and then heated in the thermocycler at 99 ºC in order to be denatured and used as a DNA template for PCR. 3µl of this denatured bacteria was used as the template along with 10µl of DreamTaq mastermix buffer, 1µl of each primer and 10µl of water. The thermocycling programme was set up as per the DreamTaq instructions.

2.8.10 DNA Fragment Sequencing

Sequencing of plasmids and fragments generated by PCR were sequenced using Source Bioscience – Life Sciences UK, with primers supplied with samples.

2.9 Characterisation Methods

2.9.1 Plate viability tests

A series of viability tests were made on agar plates, to test whether *Clostridium autoethanogenum* could grow with and without each vitamin. In order to produce an inoculum without each vitamin, initial cultures of the wild type *autoethanogenum* were grown until they reached the exponential phase and then serially diluted by diluting 1ml :10ml culture: liquid minimal media (-vitamins) 3 times then plating 40µl onto plates with different conditions. Experiments were performed in biological and technical triplicate. A, YTF plates, B minimal media plates with B vitamins (+1 negative control, and C. minimal media plates with one vitamin excluded but containing all other vitamins. (+1 negative control)

2.9.2 Serial dilution tests

Requirement for pantothenate by *C. autoethanogenum* and *ljungdahlii* was studied in a serial dilution experiment with vitamin free Casamino acids containing medium amended with complex vitamins solutions that lacked pantothenate. This was carried out at 37ºC in 25ml Hungate tubes, containing 25 ml of media The first tube was inoculated with 200µl of an culture in exponential phase and contained a normal amount of the tested vitamin present in the media. Cultures were serially diluted over 5 test tubes with one dilution every 48 hours with 1% inoculum volume, covering dilution up to 10^{-10}. These tubes contained all other vitamins but lacked any of the tested vitamin, save what was added

during the serial dilution. Growth was indicated by an increase in OD, monitored after several days, and confirmed by phase contrast microscopy.

2.9.3 Growth Experiments

Growth experiments for *C. autoethanogenum* and *C. ljungdahlii* were carried out in a volume of 50ml media using 150ml serum bottles with butyl rubber stoppers. All cultures were inoculated with 500µl inoculant made by serial dilution (as shown above) with a pH of 5.8-6.0, and also contained D-cycloserine and cysteine. Time points were taken at intervals until 120 hours of growth had occurred. At every time point samples were taken to analyse OD, and to test the pH to make sure it remained constant. All growth experiments were performed in biological and technical triplicate, and used the *Clostridium autoethanogenum* or *C. ljungdahlii* wild type in YTF media as the control. Either 10g/L fructose or one bar of carbon monoxide was used as the carbon source.

2.10 Measurement Techniques

2.10.1 Optical Density

Optical Density was measured using a Spectrophotometer (Jenway 7305). Measurements were always taken by the same machine and always at 600nm. An appropriate blank – either sterile water or sterile media was always used and measurements were always carried out in triplicate and averaged. If the result was a reading over .800 a one in ten dilution, of 100µl of sample in 900µl of the blank liquid (whether this was water or sterile media), and then the resulting reading multiplied by 10 in order to receive a more accurate reading.

2.10.2 HPLC

The mM yields of industrially relevant chemicals produced by the bacteria when growing in media with different conditions were ascertained by using a Thermo Scientific Dionex UltiMate 3000 HPLC system with a refractive index (RI) detector and diode array detector (DAD) at 210nm using a Rezex ROA-Organic Acid H+ (8%) (150mm x 4.6mm x 8µm) column from Phenomenex operated at 35ºC. Mildly acidified water (0.005M H_2SO_4) was used as a mobile phase with the flow rate of 0.5 millilitres per minute. To remove cell debris, samples were centrifuged at 20,000 X g for 5 minutes, and the supernatant was filtered with 0.22µm regenerated cellulose syringe filters. The supernatant was diluted 1:1 with mobile phase containing 50mM valeric acid as an internal standard and pipetted into a glass HPLC vial containing an insert. Twenty microliters of the supernatant was then injected into the HPLC for analysis. Products measured were ethanol, acetate, 2,3-butanediol, lactate and formate. These were measured in order to see whether changes to the product profile occurred when a key vitamin was left out of the media.

3 Chapter Three: Elucidation of Vitamin Requirements for Two Gas Fermenting Clostridia Species.

3.1 Introduction

Clostridium autoethanogenum and *Clostridium ljungdahlii* are acetogens that can readily produce acetate, ethanol, formate and lactate from Acetyl-CoA as well as a range of other useful chemicals such as 2,3-butanediol[50]. Microbial production of these resources offers a useful alternative to current industrial production methods[156]. Further developments in the process and in the organism will provide a bright new future for the production of these useful fractions by *C. autoethanogenum* and *C. ljungdahlii*.

One way to increase the economic viability of this process is to reduce the feedstock and input costs, as this has a high effect on the eventual cost of the chemicals which can be produced by this process. One of the facets of the process where input costs can be reduced is in the media contents. It is important to characterise the essential vitamins required for growth, as if unnecessary supplementation is added, these can greatly increase the input costs. Like other Clostridia, *C. ljungdahlii* and *C. autoethanogenum* are fastidious and are generally grown in undefined medium containing yeast extract and a full complement of B vitamins[13]. In this chapter, we set out to establish the "true" vitamins actually required for growth of *C. ljungdahlii* and *C. autoethanogenum*. Current media protocols generally require the addition of ten B-vitamins – biotin, folic acid, pyrodoxide hydrochloride, thiamine, riboflavin, nicotinic acid, pantothenate, cyanocobalamin, benzoic acid and thioctic or lipoic acid, but this is a standard complement used for many bacterial species and not all of them may be required.

3.1.1 Thiamine

Vitamin B1, also known as thiamine, is an essential cofactor for most species. In its active form - thiamine diphosphate (ThDP) or Thiamine pyrophosphate (TPP) it is used as a co-enzyme in the catabolism of sugars and amino acids. The biosynthesis of thiamine in bacteria is well-documented. Many of the different genes have been extensively characterised and over expressed[16] and although there is some degree of variation of the route to the components, the biosynthesis involves the creation of a thiazole component for which the genes *thiFSGO*, *thiL*, and *dxs* are commonly involved (in firmicutes) to produce 4-methyl-5-β-hydroxyethyl thiazole phosphate (THZ-P), and a pyrimidine component which require the gene *thiC* to produce 4-amino-5-hydroxymethyl-2-methyl-pyrimidine pyrophosphate (or HMP-PP) separately. These moieties are then combined to form ThMP (thiamine monophosphate) by thiamine-phosphate synthase which is encoded for by *thiE*. In many species of gram positive bacteria, the ThMP is hydrolysed to thiamine, which can be converted into its active form ThDP by thiamine diphosphokinase(*thiL*) [16]. There are four genes associated with thiamine kinases and these are *thiDML* and *pdxK*[16].

3.1.2 Riboflavin

Riboflavin, and its active forms - flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) act as cofactors for reactions which are involved in the electron transport chain. Riboflavin (and its derivatives) are also involved in flavoprotein enzyme reactions, the oxidation of pyruvate, and can be involved in the reduction of tryptophan to niacin (B3). There are two main sections of the pathway –the pentose-phosphate pathways in which ribulose-5 –phosphate is converted by the enzyme 3,4-DHBP synthase (*ribA* and *ribB*) into 3,4-dihydroxy-

2-butanone-4-phosphate. The other phase of biosynthesis is the purine biosynthesis pathway in which a cascade of enzymes (encoded by: *ribA, ribG, ribD*) convert Glyceraldehyde-3-phosphate into 5-amino-6-(5'-phosphoribosylamino) uracil. 6,7-dimethyl-8-ribityllumazine synthase (*ribH)* is then involved in the conjoining of these two pathways and their products into 6,7-dimethyl-8-ribityllumazine which is converted into riboflavin by Riboflavin biosynthesis protein RibBA (*ribBA).*

3.1.3 Niacin

Nicotinic acid, also known as niacin, acts as a precursors to the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which aid many dehydrogenase reactions[157]. Generation of NAD and NADP are essential for a functioning metabolism in all organisms[158]. These NADs act as either electron donors or acceptors in oxidation-reduction reactions within cells. They are also used as substrates in a multitude of other classes of non-redox reactions, for example, acting as a dehydrating agent in DNA repair for the ligase enzyme[158]. There is some variation on the metabolic pathway for biosynthesis of NAD+ in bacteria. In the *Clostridium* genus, the pathway for the generation of these two useful coenzymes is produced by a cyclical production of niacin which can be converted to NAD and then broken down to form niacin again. The process starts with the production of nicotinic acid mononucleotide (NAMN) which is produced from a tri-step de novo pathway from aspartic acid containing the genes *nadB, nadA and nadC (*L-aspartate oxidase; quinolinate synthase; *and* quinolinic acid phosphoribosyltransferase respectively)*[159]*. Nicotinic acid mononucleotide adenylyltransferase (*nadD*) then converts NAMN into nicotinic acid adenine

dinucleotide, which is in turn converted via NAD synthetase (*nadE*) into NAD which can either be used, converted into NADP via NAD kinase (*nadK)* or recycled into niacin. To convert NADP to niacin, NAD is broken down partially by DNA ligase into nicotinamide mononucleotide, which is converted by Nicotinamide phosphoribosyltransferase (*nadV)* into nicotinamide, which nicotinamide deamidase (*pncA)* will convert into niacin. Niacin can then be converted to NAMN by nicotinic acid phosphoribosyltransferase (*pncB)* and the process can continue without input from the de novo pathway[159].

3.1.4 Pantothenate

Pantothenate is intrinsic in the production of CoEnzyme A which in turn is essential for the donation of an acyl group in the production of Acetyl-CoA, which acts as the energy currency of the cell. It is also used in the synthesis and metabolism of fats, carbohydrates and proteins. For the biosynthesis of pantothenate, pyruvate is converted into acetolactate via Acetolactate synthase. Ketol-acid reductoisomerase (*ilvC*) converts the acetolactate into dihydroxyisovalerate which in turn is made into ketoisovalerate by Dihydroxy-acid dehydratase (*ilvD*). Ketoisovalerate is the platform chemical for a range of different products in the cell, but in the generation of pantothenate, the ketoisovalerate is converted to ketopantoate by 3-methyl-2-oxobutanoate hydroxymethyltransferase (*panB*), *ilvC* then converts this into pantoate. Simultaneously, L-Aspartate is converted into β-alanine by Aspartate 1-decarboxylase (*panD*). Pantothenate synthetase (*panC*) then converts the β-alanine and pantoate into D-pantothenate, which via further conversion become Coenzyme A.

3.1.5 Pyridoxine

Pyridoxine is an unusual vitamin in that the pathway is not highly conserved amongst organisms and there are an abundance of different pathways and routes to its biosynthesis. Within the cell pyridoxine acts as a cofactor in its active form – pyridoxal 5'-phosphate, also known as PLP[86]. PLP is a cofactor for enzymes involved in reactions for amino acids, glucose and lipid metabolism. In the majority of Clostridial species, including the two involved in this study, ribulose 5-phosphate is converted into a linear isomer. Elsewhere, Dihydroxyacetone phosphate is converted into glyceraldehyde 3-phosphate. Pyridoxal 5'-phosphate synthase subunits pdx1 and pdx2 (*Pdx2* and *pdx1)* then combine the resulting modified ribulose 5-phosphate with the glyceraldehyde 3-phosphate and glutamine to produce pyridoxal 5'-phosphate, simultaneously giving off glutamate as a by-product.

3.1.6 Biotin

Biotin also known as B7 (and formerly as vitamin H) acts as a coenzyme for carboxylase enzymes, involved in the synthesis of fatty acids, and some amino acids and is also involved in reactions involving carbon dioxide[160]. Many microbes, plant and fungi contain the full complement of genes for biosynthesis. In prokaryotes there are three main routes to biotin. One generally seen in gram positive species (not exclusively) involves the breakdown of pimelate by *bioW* into pimeloyl-COA and the conversion of this into 8-amino-7oxononanoate by *bioF*. Another route is through the conversion of long chain acyl-[acp] into pimeloyl-[acp] by *bioI*. Alternatively, a different route involves the production of 8- amino-7oxononanoate by the production of pimeloyl-[asp] by the Fatty acid biosynthesis double enzyme cascade from malonyl[asp]

(*bioC,FabB,FabG,FabZ,FabI* and *bioH*) pimeloyl-[asp] is then converted into the 8-amino-7oxononanoate by *bioF*. For either route, the pathway to biotin then proceeds in the same manner, *bioA* converts the 8- amino-7oxononanoate into 7, 8-diamino-nonanoate, *bioD* then converts this into dethiobiotin and *birA*, also annotated as *bioB* in some species, then converts this into biotin. Alternative routes to biotin also exist from biotin sulfoxide or biocytin, however, these are cyclical and involve biotin as a starting component in the cycle.

3.1.7 Folic Acid

Folic acid is involved in synthesis of DNA, growth and division of cell and the repair and methylation of DNA. The complete biosynthetic pathway is present in both *C. autoethanogenum* and *C. ljungdahlii*. *FolE, folA,* and *folK* are genes involved in a sub pathway enzyme cascade which aids the conversion of GTP to H_2-Pterin-CH_2OPP. Simultaneously, the genes *pabA, pabB* and *pabC* are involved in the conversion of Chorismate to PABA (described in further detail in 3.1.8). Chorismate is also involved in the production of phenylalanine, tyrosine and tryptophan. The gene *folP,* then combines the PABA and the H_2-Pterin-CH_2OPP to produce dihydropteroate and *folC* combines this with glutamate to produce dihydrofolate which is eventually converted into folate by *dfrA*.

3.1.8 Paraminobenzoic Acid

B10, though not always classically included in the vitamin B complex, is nevertheless essential in some organisms and as a result is included in the traditional B vitamin mixes used in media[13]. The active form in organisms is 4-paraaminobenzoic acid (PABA). It acts as an intermediate in the production of folates and is an essential component in the biosynthesis of B9. Chorismate is converted to 4-amino-4deoxychorismate by chorismate synthetase components I

and II (*pabA* and *pabB)* then 4-amino-4-deoxychorismate lyase (*pabC*) converts this into PABA[161] which feeds into the folate pathway as described in 3.1.7

3.1.9 Cobalamin

Used in cobalamin dependent reactions, methionine synthase, ribonucleotide reductase etc., cobalamin is converted to methylcobalamin which is involved the Wood-Ljungdahl pathway. The production of cobalamin in bacteria generally follows one of two routes- either the aerobic or the anaerobic, in *C. ljungdahlii* and *C. autoethanogenum*, the anaerobic is used. It is an incredibly complex pathway involving around 30 enzymes in total, which are all present in the genome of *C. ljungdahlii* and *autoethanogenum*. In brief, the biosynthesis begins with a precorrin 2 complex. This is then converted by SirC *(sirC)* into sirohydrochlorin (factor 2) complex, *cbiK* and *cbiX* encode for enzymes which combine copper into this complex to form cobalt sirohydrochlorin (cobalt factor 2) an enzyme cascade encoded by: *cbiL,H,F,G,D,J,E,T* and *C* then convert this into cobyrinic acid. The genes c*biA,P* and *cobA* encode for enzymes which then turn cobyrinic acid into adenosylcobyric acid. Finally, another large enzyme cascade converts the adenosylcobyric acid into cobalamin. The genes involved in this are c*biP*, *cobD,U,T,C,D,P,U and V*[162].

3.1.10 Lipoic Acid

Although not strictly a B-vitamin (its classification was removed as humans do not require it), lipoic acid, (sometimes also called thioctic acid) is included in standard B vitamin mixes such as Wolfe's B-vitamin solution used for a large number of clostridial growth media[13]. It acts as a co-factor of pyruvate dehydrogenase complex and fatty acid biosynthesis. Generally used as a cofactor for enzymes involved in aerobic respiration, it is possible that despite the

presence of the full complement of genes in both species the requirements for the production of lipoic acid are much lower than perhaps they would be in their aerobic counterparts as the vast majority of their effect is in the citric acid cycle. There are two main routes in the cell for lipoic acid metabolism. The first is biosynthesis in which octanoyl-[acp] is converted into lipoyl-[acp] by lipoyl synthase (*lipA*) and the octanoyl-[acp] is also converted into Protein N6-(octanoyl) lysine by lipoyl(octanoyl) transferase (*lipB*). *LipB* then acts on the lipoyl-[acp] and *lip A* acts on the protein N6(octanoyl lysine) and the two parts of the pathway fuse to create Protein N6-(lipoyl)lysine the active form of lipoic acid which can be converted into oxo-acid dehydrogenase complexes. Alternatively lipoate can be salvaged and converted into protein N6 (octanoyl) lysine by lipoate protein ligase (*lplA*).

3.1.11 Chapter Aims

Before leaving out any key components of a media used for economic production of industrially relevant products, it is essential to characterise the two organisms responses to the lack of certain vitamins in the media, in order to ensure they continue to operate desirably now they have to make the vitamins themselves. We can study the genome and to determine if the genes for biosynthesis of the vitamins are present. However, presence of genes alone does not indicate that the pathway is fully functional, for example if an upstream product is not being produced, the final vitamin may not be constructed. Alternatively, there may be biosynthesis of the vitamin, but it is in such small quantities that the cell must uptake it from external sources in order to be viable. Only after confirmation by studying growth rate and viability, and measuring the products ethanol, acetate,

formate and lactate can we say that the species' do or do not need a certain vitamin.

3.2 Chapter Specific Materials and Methods.

3.2.1 Bioinformatics

BLAST (Basic Local Alignment Searches) searches using NCBI[150] and UNIPROT[153], and KEGG PATHWAY[151] were used to identify if vitamin biosynthesis pathways were present and annotated in the genomes of *C. autoethanogenum* and *C. ljungdahlii* and if they contained all of the genes required for biosynthesis and which had missing genes. If a gene was not annotated, homologues from other closely related species were used to identify whether any unannotated genes were present.

3.2.2 Bacterial Strains

Clostridium autoethanogenum JAI-1 (DSM 10061), and *Clostridium ljungdahlii* (DSM13528T) were obtained from the German Collection of Microorganisms and Cell cultures DSMZ (Braunschweig, Germany).

3.2.3 Media

C. autoethanogenum and *C. ljungdahlii* were cultivated for these experiments in PetC-MES Media, made as outlined in section 2.5.1.3 of the main methods chapters. When present 10g/L of fructose was used in liquid media or 100KPa of CO was added to the head space of the serum bottles. If solid media was required, PetC-MES was used with the addition of 15g/L bacterial agar, and solid YTF media was used as a positive control. The B-vitamin stocks were filter sterilised and added to the media after it was autoclaved. There was a vitamin stock solution containing all of the required vitamins – to be used as a positive

control and separate vitamin stock solutions that lacked one or more key vitamin were made as outlined in section 2.5.3.

3.2.4 Inoculum

For growth on liquid and solid media an inoculum was prepared by serial dilution of an exponential culture of the organism grown in standard PetC-MES media, and diluted in PetC-MES without any vitamins added. The third dilution of the series served as the inoculum.

3.2.5 Growth Experiments

For growth tests on solid medium 50µl was streaked onto: YTF plates, minimal media plates with standard B vitamins, and minimal media with one or more vitamins excepted from the media. Colonies were enumerated after 8 days of growth and the experiment performed in biological and technical triplicate.

Growth experiments in liquid culture for *Clostridium autoethanogenum* and *C. ljungdahlii* were carried out in order to compare the growth profile of a culture growing with and without a key vitamin. Experiments were conducted as mentioned in 2.9.3 in a volume of 50ml of PetC-MES media in 150ml serum bottles, sealed with butyl rubber stoppers. All cultures were inoculated with a 1% inoculum as described above. The pH of the media was 5.8-6.0, and also contained D-cycloserine and cysteine. Samples were taken to analyse OD, to test the pH and for HPLC testing. All growth experiments were performed in triplicate, and used the *Clostridium autoethanogenum* or *Clostridium ljungdahlii* wild type growing in media containing all vitamins as the positive control and no vitamins as the negative control.

3.2.6 HPLC

Ethanol and acetate yield were analysed by using HPLC as shown in methods section 2.10.2. MES masks the peak for 2,3-butanediol, and therefore, the yields for 2,3-butanediol could not be quantified.

3.3 Results

3.3.1 Bioinformatics

Initally, it can be elucidated as to whether *C. autoethanogenum* and *C. ljungdahlii* are likely to possess the capability of biosynthesis of a vitamin by determining whether the genes required for its production are present in the genome. Close examination of our annotated strain of *C. autoethanogenum* and the published genomes for *C. ljungdahlii* revealed the presence and absence of annotated genes for vitamin biosynthesis as outlined in Table 17. All genes were present for the biosynthesis of riboflavin, niacin, pyridoxine, folic acid, PABA, cobalamin and lipoic acid. The pathways for the production of thiamine, biotin and pantothenate were missing key genes, and extensive BLAST searching of genes from closely related organisms revealed no likely putative genes within the organims.

Vitamin	**Genes required for biosynthesis**	**Genes missing in the genome of *C. ljungdahlii***	**Genes missing in the genome of *C. autoethanogenum***
Thiamine(B1)	*thiF*, *thiS*, *thiG*, *thiO*, *thiL*, *dcx*, *thiC*, *thiI* *thiE*, *thiD*, *thiM*, and *pdxK*	*thiS*, *thiG*, *thiO*, *thiL*, *dcx*, *thiC*, *thiD*, and *pdxK*	*thiS*, *thiG*, *thiO*, *thiL*, *dcx*, *thiC*, *thiD*, and *pdxK*
Riboflavin (B2)	*ribA*, *ribB*, *ribG*, *ribD*, *ribH*, *ribBA*	Full complement present	Full complement present
Niacin (B3)	*nadB*, *nadA*, *nadC*, *nadD*, *nadE*, *nadK*,	Full complement	Full complement present in the genome

	nadV, pncA, pncB	present in the genome	
Pantothenate (B5)	*ilvBN, ilvC, ilvD, panB, panC, panD, panE*	*panB, panC, panD*	*panB, panC, panD*
Pyridoxine (B6)	*pdx1, pdx2*	Both genes present in genome	Both genes present in genome
Biotin (B7)	*bioW, bioF, OR bioI, OR bioC, fabB, fabG, fabZ, fabI, bioH, AND bioF, bioA, bioD, bioB, bioY*	*bioW, bioI, bioB,bioD, bioF, bioH, bioA, bioC,*	*bioW, bioI, bioB,bioD, bioF, bioH, bioA, bioC,*
Folic Acid (B9)	*folE, folA, folK, pabB, pabA, pabC, folP, folC, dfrA*	All genes present in genome	All genes present in genome
PABA	*pabA, pabB, pabC*	All genes present in genome	All genes present in genome
Cobalamin (B12)	*sirC, cbiK, cbiX, cbiL, cbiH, cbiF, cbiG, cbiD, cbiJ, cbiE, cbiT, cbiC, cbiP, cobD, cobU, cobT, cobC, cobP, cobU, cobV, cbiA, cobA*	All genes present in genome	All genes present in genome
Lipoic Acid	*lipA, lipB, lplA,*	all genes present in genome	All genes present in genome.

Table 17 Results of bioinformatics research into the presence of genes for biosynthesis of vitamins in the genomes of C. autoethanogenum and C. ljungdahlii.
All genes are present in the genomes of both species for all vitamins except for pantothenate, biotin and thiamine, which are missing key genes.

3.3.2 Plate Viability

A Series of viability tests were made on agar plates, to determine whether *C. autoethanogenum* or *C. ljungdahlii* could grow with and without different vitamins. It was clear for both species that no growth could occur in either species on plates which lacked either pantothenate, biotin or thiamine in the media. However, growth was capable in plates which lacked any other single vitamin and plates which contained no B-vitamins other than pantothenate,

thiamine and biotin. This experiment was repeated each time in biological and technical triplicate (see Table 18).

Table 18: Vitamin requirements of *C. ljungdahlii* and *C. autoethanogenum*.

Medium	*C. ljungdahlii*	*C. autoethanogenum*
YTF	+	+
MM- with all vitamins	+	+
MM – no vitamins	-	-
MM with all vitamins except Thiamine	-	-
MM with all vitamins except Riboflavin	+	+
MM with all vitamins except Niacin	+	+
MM with all vitamins except Pantothenate	-	-
MM with all vitamins except Pyridoxine	+	+
MM with all vitamins except Biotin	-	-
MM with all vitamins except Folic Acid	+	+
MM with all vitamins except PABA	+	+
MM with all vitamins except Cobalamin	+	+
MM with all vitamins except Lipoic acid	+	+

Table 18 Plate viability tests for C. autoethanogenum and C. ljungdahlii when grown with and without different vitamins.
In each instance, one vitamin has been excluded from the media. In all cases growth of over 1000 colonies occurred (if a + is present) but when biotin, thiamine or pantothenate is removed from the media no colonies appear on plates.

3.3.3 Growth Profile with Fructose as a Carbon Source

To examine the impact of different vitamins being left out of the media on the growth profile of each species, *C. ljungdahlii* and *C. autoethanogenum* were studied in a growth experiment in PetC-MES media containing vitamin free Casamino (described in table 2.5.1.3) and 10g/L fructose and amended with complex vitamins solutions that lacked each vitamin. As shown in Figure 9.

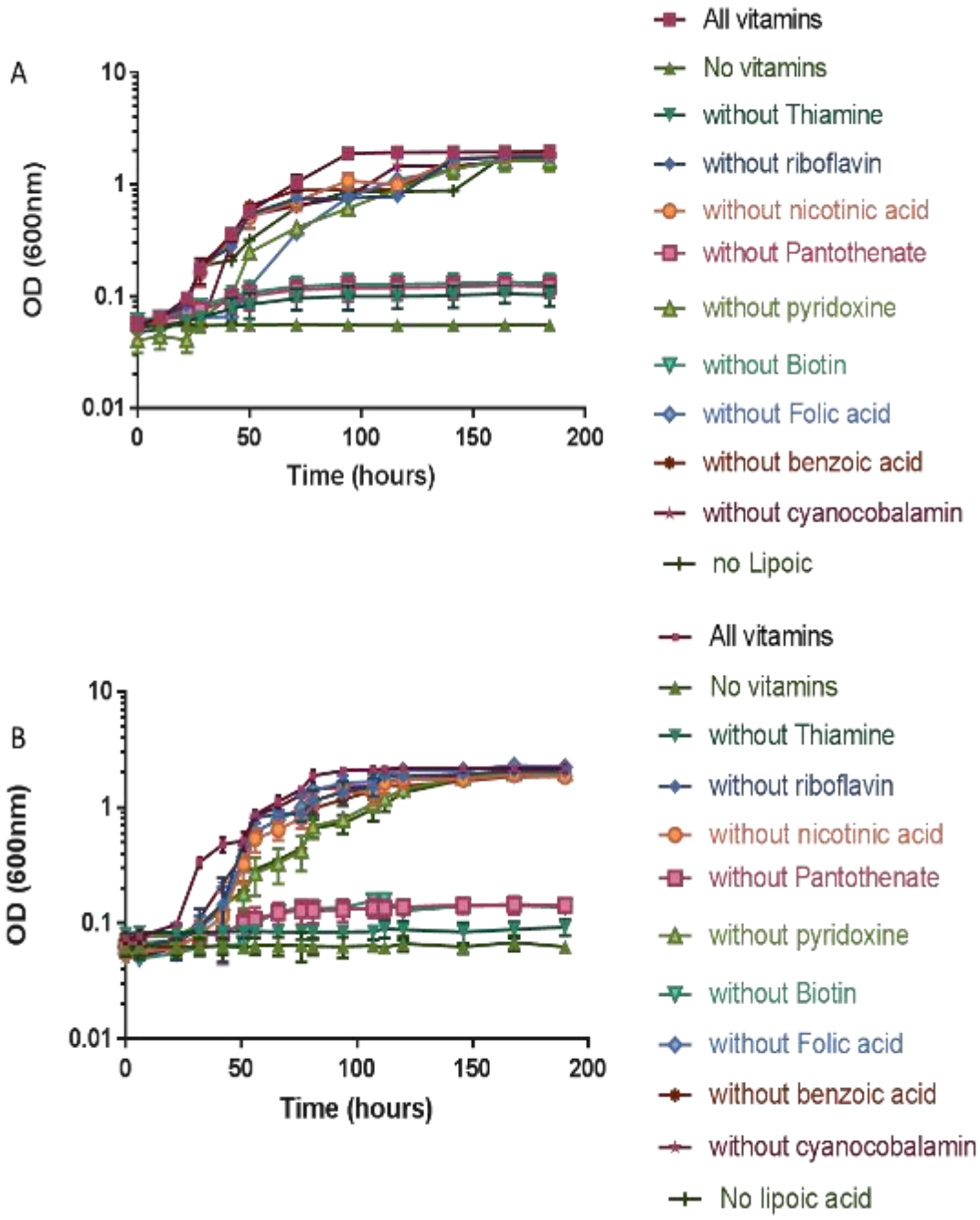


Figure 9 Growth curve analysis of (A) C. autoethanogenum and (B) C. ljungdahlii with one vitamin removed from the media, using fructose as a carbon source.

Growth rate comparison in minimal media analysing the differences in growth rates observed when different vitamins are left out of the media. Highly significant differences were seen in the growth of autoethanogenum and ljungdahlii when pantothenate, biotin and thiamine were not present in the media. Increase in the lag time of C. autoethanogenum was also seen in pyridoxine, folic acid deficient media, and in C. ljungdahlii in lipoic acid and pyridoxine but they eventually reached a similar optical density. Error bars represent the standard error OD the mean of three replicate measurements.

Inoculated medium that lacked pantothenate, thiamine or biotin did not support an increase in turbidity respectively or reliably support growth, in either

organism. In all other resultant profiles there was a strong indication that the vitamin deficient in that test was not essential and the organism was capable of growth with its exclusion, leading to the determination that the "vitamin" was not essential for growth and the organism was autotrophic for the micronutrient. In some cases such as growth in pyridoxine free media (for both organisms) and without folic acid (*C. autoethanogenum*) and without lipoic acid (*C. ljungdahlii)*, it seems that the exception of the chemical from the media leads to a much slower initial growth rate. This could be due to the time it takes to adapt to conditions without the vitamin and for the organism to begin producing the vitamin itself in large enough quantities to support growth. In all cases the vitamins which have full biosynthetic pathways present in their genomes are capable of biosynthesis and viability eventually and those which do not have complete biosynthesis pathways are not able to grow autotrophically.

3.3.4 Growth profile with CO as a carbon source

The process of growth of *C. ljungdahlii* and *C. autoethanogenum* using gas as their carbon source requires a radical upheaval of the cell's metabolism and resources as it enters into utilising the Wood-ljungdahl pathway to convert carbon to Acetyl-CoA instead of going through the process using fructose as its carbon source. As the industrial potential of the organism is based on its ability to utilise the carbon from syngas it is essential in any reasonable study to focus on characterisation the vitamin deficiency profile when the organism is growing in these conditions. The same experiments were run again using a gas adapted strain of each species (shown in Figure 10) but using a bar over atmospheric pressure of Carbon monoxide instead of the addition of sugar and using PetC-MES media as the media in which the cells were growing.

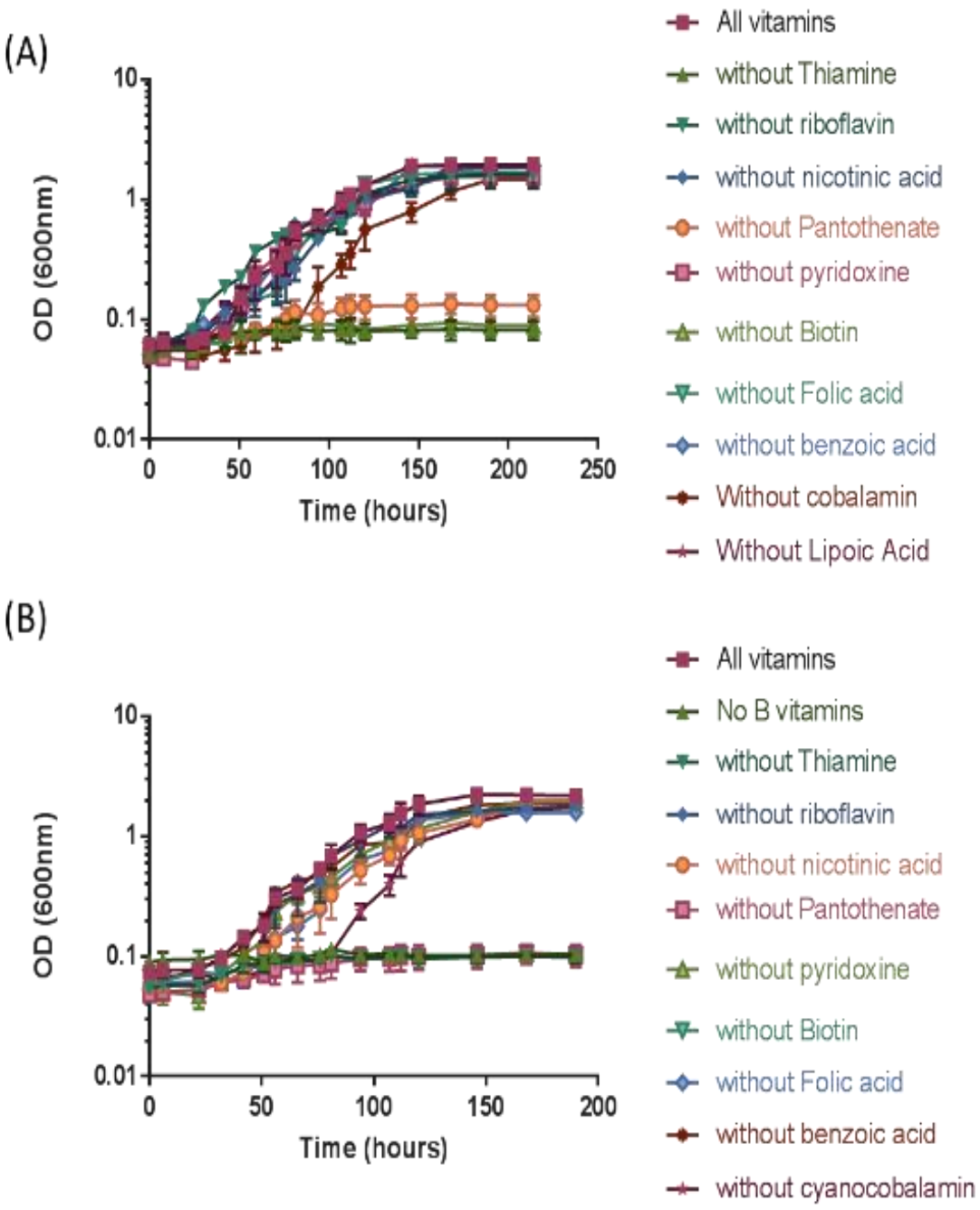


Figure 10 Growth Profile comparison of cultures of (A) C. autoethanogenum and (B) C. ljungdahlii, with individual vitamins removed from the media when grown using CO as a carbon source

Growth profile comparison in minimal media analysing the differences in growth rates observed when different vitamins are left out of the media. Highly significant differences were seen in the growth of C. autoethanogenum and C. ljungdahlii when pantothenate, biotin and thiamine were not present in the media. Initially the growth rate of the species when grown without cyanocobalamin is lower but this eventually speeds up and reaches a similar terminal OD to the positive control. In almost every instance the optical density of each of the other vitamin free media is lower than that of the positive control, but growth is clearly possible and a similar terminal OD is eventually reached. Error bars represent the standard error on the mean of three replicate measurements.

Due to the inability for *C. autoethanogenum* or *C. ljungdahlii* to grow when biotin, thiamine or pantothenate are not present in the media it seems fair to suggest that these are the only three true vitamins for the two species, as substantial growth is possible if any of the other "vitamins" are excepted whether the carbon source is CO or fructose.

3.3.5 HPLC results

In order to ascertain the differences in yields of the industrially interesting products; acetate and ethanol *C. autoethanogenum* produced when grown without each "vitamin" (using CO as a carbon source) in different phases of growth, HPLC samples were taken at time points throughout the growth experiment. These phases were determined by the growth of the wild type *C. autoethanogenum* in media containing all vitamins. The phases were: initial inoculation (0 hours), Early Exponential (48 hours), three exponential phase measurements (58, 96 and 120 hours) and a terminal stationary phase measurement (190 hours). Samples were taken as outlined in section 2.10.2, and analysed as mentioned. Resulting mM concentrations of ethanol and acetate from cultures growing without each vitamin were then compared to the results for a culture growing with full vitamins. The acetate: ethanol ratio was also calculated and compared.

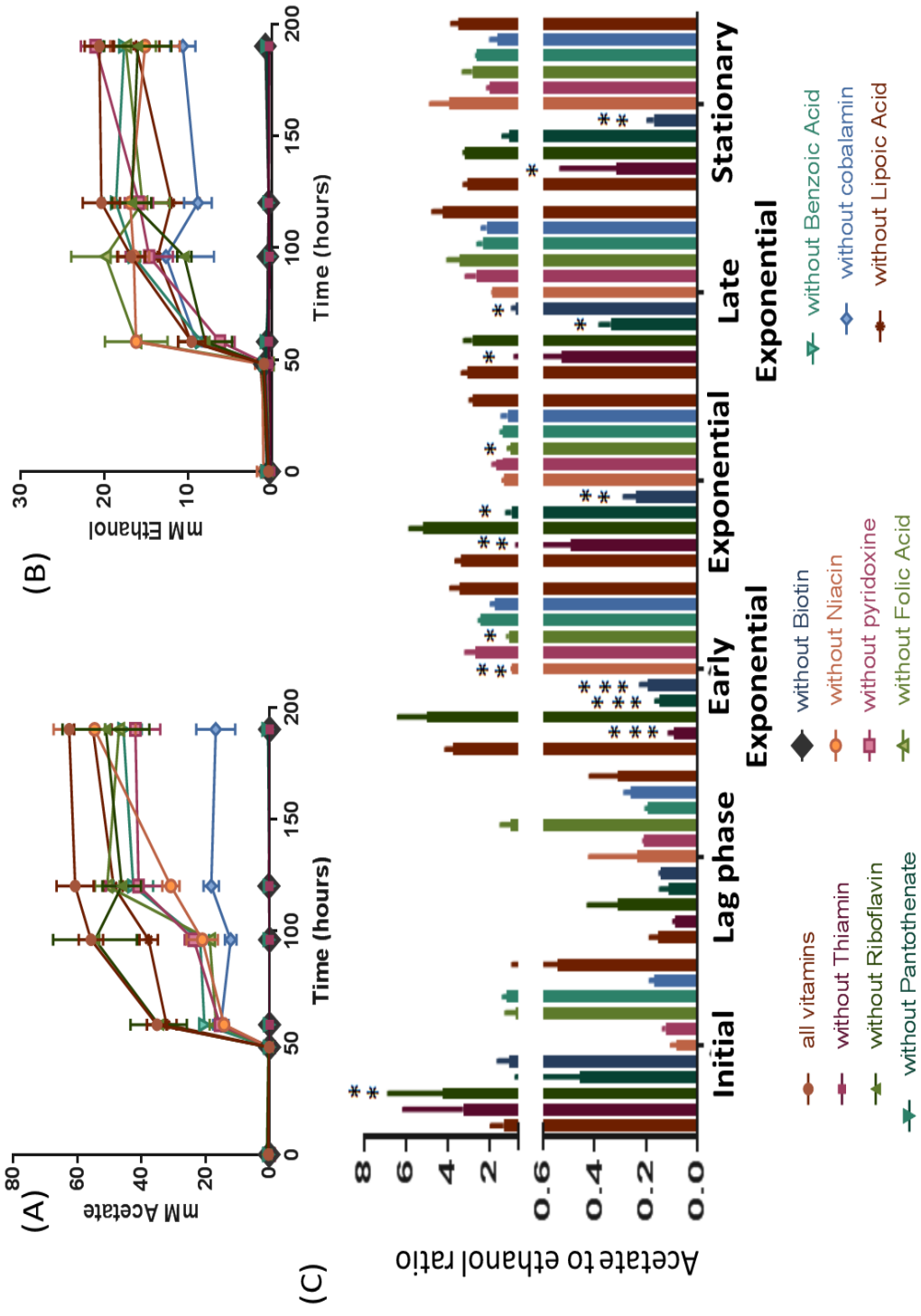


Figure 11 Graphs of HPLC data for growth of C. autoethanogenum using CO as a carbon source. (A) mM acetate yield (B) mM ethanol yield (C) acetate: ethanol ratio - y axis split 0-0.6 and 0.6-8 for clarity of small values. Error bars = Standard error of 3 replicates per trial.(C) Statistical differences between the groups were calculated using two way ANOVA with a posteriori Dunnett's multiple comparisons test. In each instance the column was compared to the column growing with full vitamins in that phase. No significant difference in the acetate: ethanol ratio was observed when compared to growth in full vitamins if no asterisk is present. Statistical significance of differences between groups is indicated with p-values (, P<0.05; **, P<0.01, ***, P<0.001)*

In the results from Figure 11, we can clearly see a huge difference in the yield of ethanol and acetate from cultures growing without thiamine, pantothenate and biotin as except in the first two measurements (which are very similar for all vitamins, including cultures growing with all vitamins) they produce hardly any of either product at all. This difference is due to the huge discrepancy in growth as there are very low optical densities when compared to a culture growing in full vitamins. The vast majority of ethanol is produced in the later phases of growth [99] and as the cultures growing without biotin, pantothenate or thiamine never enter this phase, the production of the products seems almost non-existent.

For the most part, cultures lacking any other single vitamin produce reasonably high quantities of acetate and ethanol. The acetate yields for cultures without niacin, pyridoxine, folic acid and benzoic acid are reasonably similar to each other throughout the different phases. Cultures without riboflavin and lipoic acid initially are much more similar to cultures growing with full vitamins, but after 96 hours (mid exponential phase) the production is more similar to the values for niacin, pyridoxine, folic acid and benzoic acid. Throughout the experiment the cultures growing without cobalamin produce much lower yields of acetate. It is possible this is in part an effect of non-carbon limitation (discussed at length in chapters 4 and 6) but more probably due to the lower optical densities as seen in figure 10.

The ethanol yields showed that the production of ethanol in cultures without niacin or folic acid is significantly higher in the early and mid-exponential phases (58 and 96 hours) than the culture growing with full vitamins. This increase in ethanol, coupled with the lower values for acetate production in these phases than the positive control, could be due in part to the effects of non-carbon limitation

which are characterised by a shift in acetate: ethanol ratios in *C. autoethanogenum*. After the mid-exponential phase, this effect reduces and the yields for the cultures without these two vitamins are more similar to the values of cultures growing without pyridoxine, benzoic acid, riboflavin, and lipoic acid. Again, the yields of cultures growing without cobalamin are much lower for ethanol than they are in any of the other trials (except for those growing without pantothenate biotin and thiamine). Because the cultures also do not produce very much acetate it is more likely that this low production effect is due to the lower OD than non-carbon limitation effects, but it is a possibility.

3.3.6 Pantothenate, Biotin and Thiamine.

Further tests on combinations of the three key vitamins – biotin, thiamine and pantothenate were made as their growth profiles and the plate tests for these deficiencies indicated that they were essential. Tests confirmed that when a combination of just those three vitamins were used then growth would occur, but the exclusion of any of them resulted in the culture not entering into the exponential phase. The lag time was much longer when using just biotin, thiamine and pantothenate, but growth to a similar terminal OD was shown as seen in Figure 12.

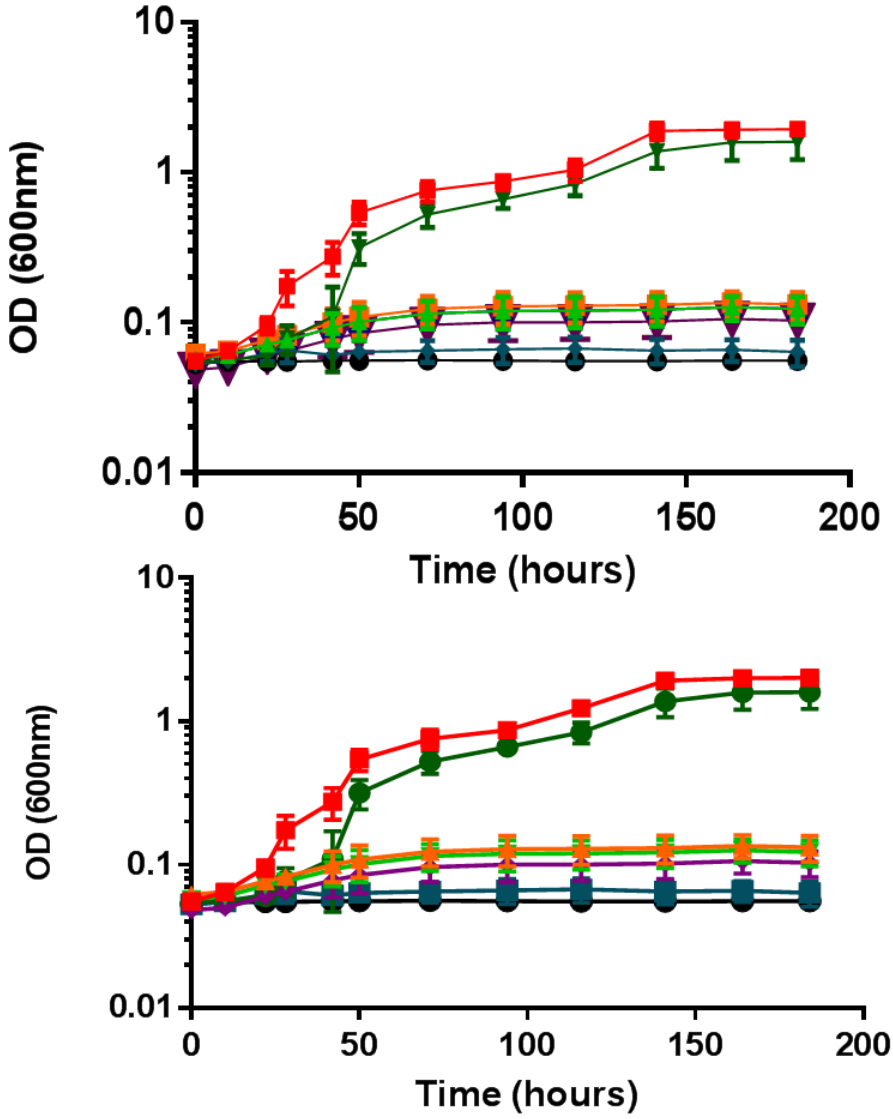


Figure 12 Further growth curve analysis of C. autoethanogenum (top) and C. ljungdahlii (bottom) with missing vitamins when grown on fructose

Growth rate comparison in minimal media analysing the differences in growth rates observed when just biotin, pantothenate and thiamine are left out of the media. Highly significant differences were seen in the growth of cultures when pantothenate, biotin and thiamine were not present in the media. As well as a significant difference between growths with only those three vitamins present and growth with all vitamins except those three vitamins present. The former being very similar to the positive control (all vitamins) and the latter being similar to the negative control. Error bars represent the standard error of the mean of three replicate measurements. Key: red; all vitamins, dark green; only pantothenate, biotin and thiamine in the media. Black; no B vitamins in the media. Light green; all vitamins except pantothenate, purple; all vitamins except thiamine, yellow; all vitamins except biotin, blue; all vitamins except pantothenate, biotin and thiamine.

3.3.6.1.1 Growth using CO

Due to the aforementioned changes to the cell's metabolism when switching from fructose to CO as a carbon source, the results from experiments using fructose (which are much more successful and do not require gas adaptation) must be repeated using CO as the carbon source. Results seen in Figure 13, were very similar to those seen when the species was growing using fructose (Figure 12) as the carbon source.

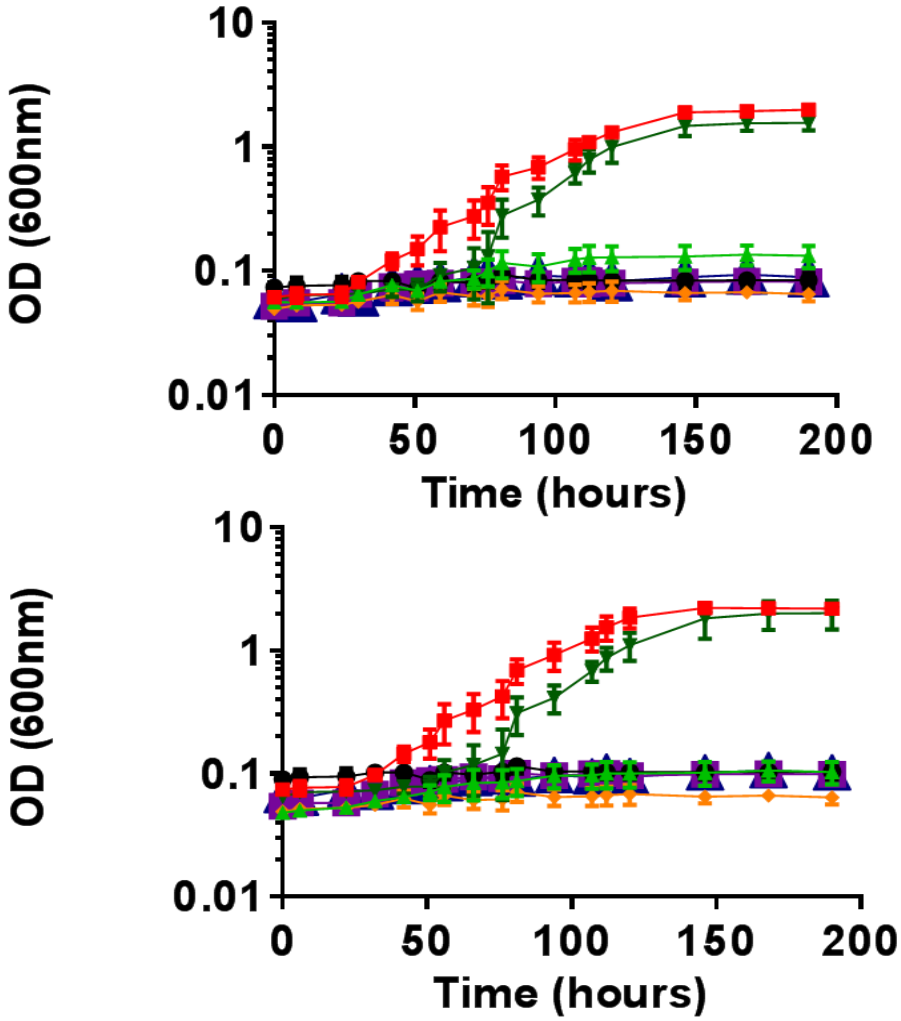


Figure 13 Further growth curve analysis of (top) C. autoethanogenum and (bottom) C. ljungdahlii with missing vitamins when grown using CO as a carbon source.
Growth rate comparison in minimal media analysing the differences in growth rates observed when just biotin, pantothenate and thiamine are left out of the media. Large differences were seen in the growth of both species when pantothenate, biotin and thiamine were not present in the media. As well as a large

difference between growths with only those three vitamins present and growth with all vitamins except those three vitamins present. The former being very similar to the positive control (all vitamins) and the latter being similar to the negative control. Error bars represent the standard error of the mean of three replicate measurements. Key; black, no B vitiamins added, yellow, all B vitamins except pantothenate, biotin and thiamine added, red all vitamins, purple all vitamins except thiamine, dark green just pantothenate, biotin and thiamine, blue all vitamins but without biotin, light green all vitamins except pantothenate.

The resultant graphs indicate that there is a great degree of similarity in the growth rate of both organisms with just pantothenate, biotin and thiamine present in the media when compared to media with all vitamins present, in many time points with no significant differences between the two, but with very little growth in cultures lacking either of the three vitamins and very little growth when all vitamins are present except those three.

3.3.6.2 HPLC results

In order to determine the effects of growth with just pantothenate, biotin and thiamine, samples were collected for HPLC analysis and results are shown in Figure 14. Results showed that there was very little production of either product when cultures were grown with no pantothenate, biotin or thiamine (PBT) but all other vitamins were present. When measuring acetate, cultures grown with only PBT performed similarly to the culture growing with all vitamins, however, the yields were not as high. When measuring ethanol, cultures growing with only PBT produced more ethanol than cultures growing with a full complement of vitamins. As we can see in Figure 14 C, the growth with just PBT has a marked effect on the acetate: ethanol ratio and whilst the ratio is consistently high in growth with all vitamins, when the number of vitamins are reduced to just three, there is a much lower ratio due to the increase in ethanol production. This could be due in part to the effects of non-carbon limitation which will be discussed in later chapters.

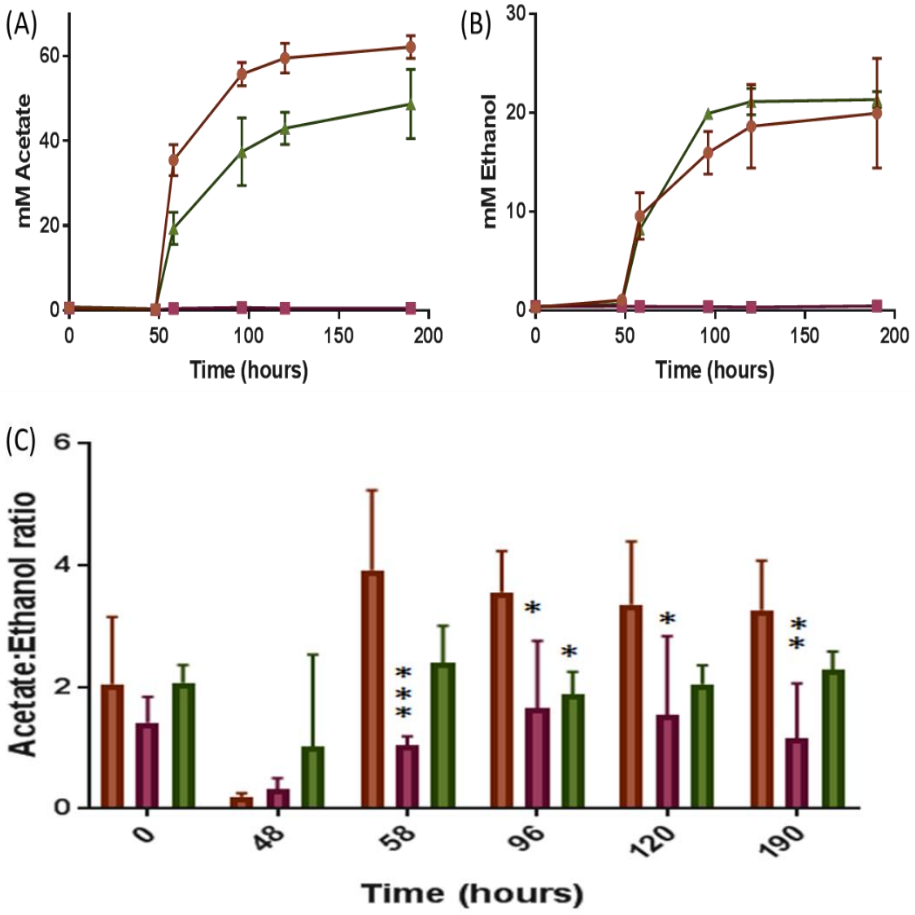


Figure 14 profiles for acetate and ethanol of C. autoethanogenum WT growing using 1 bar CO as a carbon source.
(A) Acetate profile (B) ethanol profile; (C) acetate to ethanol ratio. In (C) Statistical differences between the groups were calculated using two way ANOVA with a posteriori Dunnett's multiple comparisons test. In each instance within each row the column means were compared to the column mean of the control which was growth with all vitamins present. No significant difference in the acetate: ethanol ratio was observed when compared to growth in full vitamins if no asterisk is present. Statistical significance of differences between groups is indicated with p-values (, P<0.05; **, P<0.01, ***, P<0.001) Key; Pink-WT growing with all vitamins except pantothenate, biotin and thiamine, Brown- WT growing with all vitamins, green-WT growing with just pantothenate, biotin and thiamine.*

3.4 Discussion

Reduction of input costs makes production of bulk and fine chemicals via biotechnological routes, as opposed to traditional routes via fossil fuels, more attractive. The more robust an organism, the better it is able to withstand the stresses of industrial fermentation. In this study, in addition to demonstrating the essential nature of pantothenate, thiamine and biotin to the viability and growth of *C. autoethanogenum* and *C. ljungdahlii*, it was shown that the addition of all

other vitamins was not essential for growth of the species whether they were using carbon monoxide or fructose as their carbon source.

In this chapter, we have provided a number of phenotypic characterisation tests to show the viability of these two species when various vitamin mixtures were present, and characterised their growth in comparison to using the normal full complement of vitamins commonly used in the literature and common industrial and academic media recipes for the organism[50, 56, 77]. Crucially, we have shown through the testing by growth in liquid and solid media and using fructose and CO as their carbon source, that growth was indeed possible without most of the vitamins routinely added, and that only pantothenate, biotin and thiamine proved essential for growth. Other vitamins often helped increase the growth rate but were not essential for viability. This is probably largely due to the cells not having to invest in the production of the vitamins, but instead being able to channel more of their efforts into growth and reproduction. Due to this, it could be proposed that these chemicals are in fact not actually "vitamins" by their technical definition, because whilst they are essential for growth they do not need to be obtained from an external source[163] as the species are capable of their production, or survival without their addition. However, in many cases, the lack of their presence in the media shows a slight decrease in growth rate. This was most starkly seen by the lower optical densities seen when using CO as a carbon source in both organisms when cobalamin was left out of the media. During fructose growth experiments this also occurred when pyridoxine was excepted from the media for both organisms and when folic acid was removed from the media for *C. autoethanogenum* and lipoic acid was left out in *C. ljungdahlii*. A change in the product ratios was also seen in some cases, such as the much lower

production of acetate and ethanol by cultures growing without cobalamin. It could be fair to reassign their name to “facultative vitamins” which is a substance that is produced by an organism but it performs better if it does not have to produce it in the first place or supplements the biosynthetic production.

In many instances, although there was some initial growth of cells when pantothenate, biotin or thiamine were present, the cultures did not enter into the exponential phase. This was probably due to some carry-over of vitamins from starter cultures, despite being diluted three times. As shown during serial dilution experiments (included elsewhere) particularly in the case of biotin, the amounts required for growth are so small that some growth can occur from left over vitamins, but once cells enter into the exponential phase these are very quickly depleted.

All vitamin deficiency trials produced reasonably high quantities of acetate and ethanol and in many cases a lower acetate: ethanol ratio was seen than in cultures growing with all vitamins. In some cases, more ethanol than acetate was produced (Acetate: ethanol value <1) such as by cultures growing without niacin in the early exponential phase and those growing without folic acid in the exponential phase, which is in many ways more industrially favourable. It does not seem that the exception of any vitamin from the media apart from biotin, thiamine and pantothenate result in drastically low yields of ethanol or acetate.

Despite differences in the cell metabolisms between using sugar as a carbon source and using carbon monoxide as a carbon source, the vitamin requirements did not change. This is probably largely due to the fact that the B- vitamins are used in processes not involved in the production of Acetyl-CoA (after which

point the cell's processes function in largely the same manner despite carbon source) or are involved downstream of the Acetyl-CoA production, or are being produced in large enough quantities that it is not effecting growth. Pantothenate for instance acts as a precursor for Acetyl-CoA but is required regardless of which system is being used, this is the same with cobalamin.

In some cases vitamins being excepted from the media such as folic acid, pyridoxine or cyanocobalamin, reduced the initial growth rate and the cultures had a much longer lag phase but this is probably largely due to more effort having to be invested in the production of these vitamins, and a switch to their production is required which was not previously necessary when the chemicals were present in the media. This suggests that though the organisms have the capability of production of any of the vitamins, they could be facultative autotrophs[163], and, the chemicals are not true vitamins with the exception of pantothenate, biotin and thiamine.

3.5 Chapter Summary

- *C. autoethanogenum* and *C. ljungdahlii* have the genetic capacity for the majority of vitamin pathway but lack key genes pantothenate, biotin and thiamine pathways and therefore their corresponding enzymes
- In this study we determined that pantothenate, thiamine and biotin are key vitamins for growth and viability of the two species, that the strains are auxotrophic for the vitamins
- In addition to this it was shown that all other B-vitamins currently added are not actually true vitamins in this instance as the species are capable of growth without their presence in the external media.

4 Creation of Pantothenate Prototrophic Strains of *Clostridium autoethanogenum* and *Clostridium ljungdahlii.*

4.1 Introduction

Pantothenate, also known as vitamin B5, is essential for life, and is involved in the production of CoEnzyme A (CoA) [164-166]. It is a water soluble vitamin from the "B" class of vitamins and there is a global annual industrial requirement of roughly four thousand tonnes, primarily used for pharmaceutical and feed additives, as well as fortification of foods for human consumption[167]. CoA is particularly important to acetogens utilising gas fermentation as it is required for the production of Acetyl-CoA from the Wood-Ljungdahl pathway (see figure 2), which provides energy and feeds into downstream pathways which produce an array of industrially interesting products.

4.1.1 CoEnzyme A and Phosphopantetheine

CoEnzyme A and its enzyme bound counterpart the "carrier protein" phosphopantetheine function as acyl carriers and as carbonyl activating groups for Claisen reactions (to form ester to ester bonds) in cells. CoEnzyme A also plays a role in reactions involving amides, esters, and thioesters in the cell. The reactions these cofactors are involved in are key in the formation of non-ribosomal peptides and polyketides and in the breakdown of fatty acids in cells[168]. It has been estimated that at least 4% of enzymes use CoEnzyme A as a cofactor[168]. Carrier proteins utilising a phosphopantetheine prosthetic group are formed by the donation of the phosphopantetheine moiety to proteins from

CoEnzyme A. These can be utilised as a shuttle for key intermediates in the production of fatty acids, peptides and polyketides between active sites. [169]

4.1.2 Biosynthesis of CoEnzyme A

Along with most microorganisms, many species of Clostridium are prototrophic for pantothenate, and the enzymatic steps and corresponding genes for its synthesis are well characterised, and sequences are reasonably well conserved across the genus [2]. CoA is biosynthesized from four components in the cell – aspartate, ketoisovalerate, ATP and cysteine. Nine enzymes are involved in the production of CoA from the aspartate, ketoisovalerate, ATP and cysteine. As outlined in figure 15, aspartate is decarboxylated to form β-alanine using aspartate 1-decarboxylase, which is encoded for by the gene *panD* (Step 1). Ketoisovalerate is hydroxymethylated to form pantoic acid and then reduced (Step 2 and 3) using the enzymes ketopantoate hydroxymethyltransferase encoded for by *panB* and ketopantoate reductase is the enzyme required for the conversion of ketopantoate to pantoic acid and is encoded for by *panE*. The combination of the products from steps 1, 2 and 3 form pantothenic acid using pantothenate synthetase which is encoded for by *panC* which requires ATP (Step 4). After the production of pantothenic acid, CoA is formed by steps 5, 6, 7, 8 and 9. In Step 5, 6 and 7 the 4-phosphopantetheine is generated using the enzymes pantothenate kinase (*coaA*), 4'-Phosphopantothenoyl-cysteine synthetase (*coaB*) and 4'-Phosphopantothenoylcysteine decarboxylase (*coaC*) along with cysteine and CTP. In Step 8, this is then converted to dephospho CoEnzyme A by 4'-phosphopantetheneine adenyltransferase and ATP (*coaD*) and in Step 9 another ATP dependent reaction converts the dephospho CoEnzyme A into CoEnzyme A using dephospho CoEnzyme A kinase (*coaE*). Finally, if the active form of the

CoA is required, this can be formed by phosphopantetheinyl transferase, requiring acyl carrier protein-ser-OH, to form phosphopantetheine[168]. It is important to note that a branch of the pathway (not shown here) from ketoisovalerate is essential in the production of the amino acid valine using the enzyme; branched-chain amino acid aminotransferase (BCAT) encoded for by *ilvE* or ketoisovalerate can be converted by 2-isopropylmalate synthase (*leuA*) into isopropylmalate by and into leucine by BCAT (*ilvE).*

Biosynthesis of Pantothenate and Coenzyme A

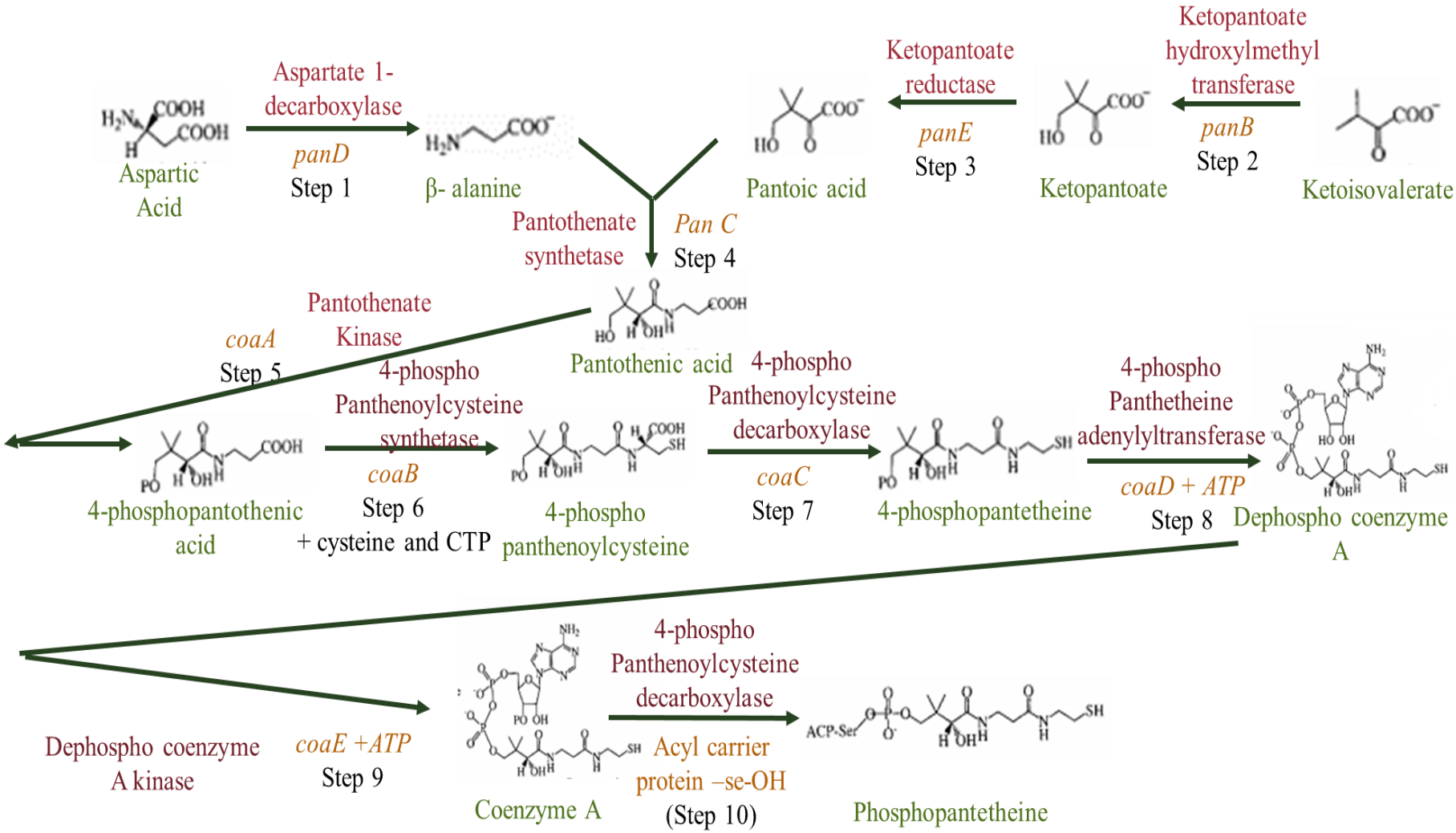


Figure 15 Biosynthetic pathway of Pantothenate and Coenzyme A.
Highly conserved in most species of Clostridia. Most species of Clostridia contain the full complement of genes required for biosynthesis of the vitamin. Green indicates chemical or product name, orange indicates gene name, red indicates enzyme involved in the conversion step.

4.1.3 Genetic capacity for production of CoA and Pantothenate in *C. autoethanogenum* and *C. ljungdahlii*

Clostridium autoethanogenum and *ljungdahlii* have the genetic capacity for the majority of this pathway but lack three key genes *panB, panC* and *panD*, and therefore, their corresponding enzymes, leading us to conclude that it is unlikely that without importing pantothenate from its environment that the bacteria would be able to produce the vitamin. In the previous chapter, it was suggested *panB, panC* and *panD* were essential in the production of pantothenate, and in other species knock outs of the genes have been shown to result in auxotrophy for the production [164, 165, 167]. It has also been shown that a down regulation or knockout of the L-leucine pathway (after ketoisovalerate) results in overproduction of D-pantothenate in some species of bacteria [164, 165, 167].

4.1.4 Methods for conferral of traits by plasmid

Incorporation of exogenous genes by artificial plasmids has been a standard tool for horizontal gene transfer in molecular microbiology since the early 1970's[170]. The artificial plasmids are replicons and replicate autonomously within a host, driving replication of recombinant DNA in order to express a protein within a host organism. A great deal of molecular microbiology occurs due to the transformation of a strain of interest with a recombinant plasmid. Artificial plasmids often contain an antibiotic resistance marker in order to make selection of successful transformants easy, and to provide a selection pressure for the plasmid to be maintained in the cell, when the antibiotic is present in the

media. Due to the fast growth and replication time, artificial plasmids are often generated in strains of *E. coli*, and then transformed into the strain of interest using electroporation, heat shocking of chemically competent cells or conjugation, in order to characterise the effects of the introduction of the genes. Artificial plasmids must, therefore, contain all elements vital for replication and maintenance of the plasmid in the host organism. If a plasmid is to be transferred using conjugation it is essential that a transfer function compatible with the new host strain is present. As outlined in the main introduction the pMTL8000 system of shuttle plasmids is commonly used in the clostridium genus and acts as a modular system containing all of the prerequisite components required. The pMTL8000 system allows for the generation of plasmids from modules in a standard proved method and structure[138].

4.1.5 Methods to confer traits into the genome

Despite the benefits to the incorporation of genes into an organism by replicative plasmids; fast generation, ease of transfer, quick to characterise, conveyance of genes in this method can be transient and without a selective pressure such as antibiotic selection, plasmids can be easily lost. In order to maintain the recombinant DNA in the long term it can be beneficial to insert genes into the chromosomal DNA of the organism. As outlined previously, allelic coupled exchange using the *ΔpyrE* selection method is a powerful system for the incorporation of genetic material into the genome of a host organism. The *pyrE* system works due to a positive and negative counter selective system, in which if the *pyrE* gene is functional, a cell is autotrophic for uracil but produces a toxic compound if 5-FOA is present and if the *pyrE* gene is not functional (*ΔpyrE*) then cells are uracil auxotrophs but do not generate a toxic compound from 5-FOA.

The system is particularly useful for the incorporation of exogenous DNA as a plasmid can be created which will “fix” a previously generated “deficient” *pyrE* gene and will also bring in a payload of genes to be inserted at the *pyrE* locus[149].

4.1.6 Chapter Aims

In the previous chapter we began to determine that pantothenate is a key vitamin for growth and viability of the two species, and that the strains are auxotrophic for the vitamin. Characterisation of the requirement for this vitamin is necessary and by introducing three key missing genes we could restore prototrophy for the vitamin. Given the importance for this vitamin to the crucial processes and cellular viability, any attempts to rectify the auxotrophy could be used to create a more robust organism for fermentation purposes.

4.2 Chapter specific methods and conditions

4.2.1 Bacterial Strains

Clostridium autoethanogenum JAI-1 (DSM 10061), and *Clostridium ljungdahlii* PETC (DSM13528T) were obtained from the German Collection of Microorganisms and Cell cultures DSMZ (Braunschweig, Germany). Both organisms were grown in strictly anaerobic conditions [171]. For the extraction of genes from *Clostridium acetobutylicum*, the original strain was obtained from the American type Culture Collection (ATCC) strain #824.

4.2.2 Bioinformatics

BLAST (Basic Local Alignment Searches) searches using NCBI[150] and UNIPROT[153], and KEGG PATHWAY[151] were used to identify if vitamin biosynthesis pathways were present in the genomes of *C. autoethanogenum* and *C. ljungdahlii* and complete and which had missing genes. Homologues from other closely related species were used to identify whether any unannotated genes were present.

4.2.3 Media

Bacteria were grown in liquid or solid (1% agar) media at 37 °*C*. Growth was measured by optical density at 600nm (OD600nm). *Clostridium autoethanogenum* and *C. ljungdahlii* were cultivated in PetC-MES media. *Clostridium acetobutylicum* was cultivated in YTF (yeast Tryptone fructose) medium. The *E. coli* strains used as intermediate hosts were cultivated in Luria Broth medium and grown aerobically. Growth experiments were carried out in PetC-MES in anaerobic conditions, with vitamin concentrations or contents modified according to experiment. The Casamino acids used were vitamin free.

B-vitamin stocks (1000X concentrated) were filter sterilised and added after autoclaving. There was a vitamin stock solution containing all of the required vitamins – to be used as a positive control and separate vitamin stock solutions were made lacking one of the vitamins. 1 ml of these was added per litre of media.

4.2.4 Plate viability tests

A series of viability tests were made on solid media, to test whether different strains of *C. autoethanogenum* could grow without pantothenate. In order to produce an inoculum without pantothenate, initial cultures of the wild type *C. autoethanogenum* were grown until they reached the exponential phase and then serially diluted 1:10 in liquid minimal media without vitamins three times for each dilution. 40µl was streaked onto on: A, YTF plates, B minimal media plates with B vitamins (+1 negative control, and *C.* minimal media plates with one vitamin excluded but containing all other vitamins. This was performed in biological and technical triplicate (+1 negative control).

4.2.5 Growth Experiments

Growth experiments for *Clostridium autoethanogenum* and *C. ljungdahlii* were carried out to determine the growth profile of strains in order to compare transformed strains with the wild type as outlined in methods section 2.9.3. Cultures were grown with a 1% with an inoculum of growing culture. Before inoculation, the inoculum was washed with the three washing steps detailed in section 4.2.4. Samples were taken to analyse OD, to test the pH and in some experiments for HPLC analysis.

4.3 Development and Characterisation of Strains.

4.3.1 Further characterisation of the Wild Type and elucidation of limitation by pantothenate

Due to missing genes in the pathway it was predicted that *C. autoethanogenum* and *C. ljungdahlii* would be auxotrophic for the biosynthesis of pantothenate and had to obtain the vitamin from its environment. To explore the influence of pantothenate on *C. autoethanogenum* and C *ljungdahlii*, growth in liquid and solid media was examined using chemically defined media. Work was also done to try to elucidate the lower limit of pantothenate required for growth.

Requirements for pantothenate were ascertained using a series of dilution experiments in order to determine the lowest concentration of pantothenate which could be added to the media without limiting growth. Standard B-vitamin solutions often add vitamins in extremely high concentrations in order to ensure growth is not limited by low availability. The serial dilution experiment used vitamin free Casamino acids containing liquid medium, amended with complex vitamins solutions that lacked pantothenate. Experiments were carried out at 37ºC in 25ml Hungate tubes, containing 25ml of media The first tube was inoculated with 200µl of a culture in exponential phase and contained a normal amount of pantothenate present in the media. Cells were serially grown over 5 cultures with one transfer every 48 hours. The inoculum volume was 1%, covering dilution up to 10^{-10}. These tubes contained all other vitamins but lacked any pantothenate save what was transferred during the serial dilution. Growth was indicated by an increase in optical density (600nm), monitored after 8 days, and confirmed by phase contrast microscopy.

As previously shown in chapter three, some cultures are able to grow initially without any pantothenate in the media. This is due to residual amounts of vitamins left in the cells, however, this tends to run out after about 50 hours. Therefore, viability in low concentrations was determined to have been successful if an OD of over 1.5 was reached at 8 days. In this case, cultures containing 100% (5µg/ml) and 10%(0.5µg/ml) of the standard addition of vitamins were able to grow to an OD (600) of over 1.5 in 8 days, but whilst there was some growth when 1%(.05µg/ml) of the standard pantothenate was added there was a much lower increase in optical density in the time frame. In the cultures growing with 0.1 % (0.005µg/ml) and 0.001% (.0005µg/ml) of the pantothenate in the media no growth occurred. Results are shown in figure 16.

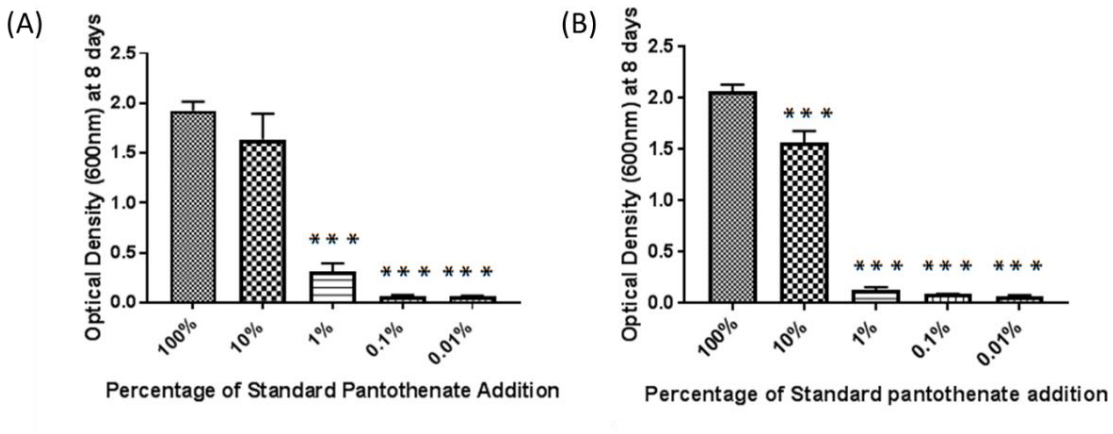


Figure 16 Initial and final optical density of (A) C. autoethanogenum and (B) C. ljungdahlii cultures growing in different concentrations of pantothenate compared to the standard addition (100%).
*After 10% of the standard addition of pantothenate the growth is severely reduced. Green; initial OD, Red; final OD N=3. Error bars show standard error calculated for each data set. Asterisks indicate statistical significance difference to 100% calculated by one-way ANOVA (A) (F (4, 10) =1.941) (B) (F (4, 10) = 1.484 with a posteriori Dunnett's multiple comparisons test. (*** indicates P>0.001)*

Pantothenate requirement for cultures growing on solid media that contained fructose and 100%, 10%, 1%, 0.1% and 0.01% of the standard amount of pantothenate present in the media is shown in Figure 17. Cells were washed and

inoculated onto each plate (each condition performed in triplicate). Colonies were then enumerated, and if a lawn was present this was deemed as over 1000 colonies. Similar to liquid media, growth on solid media was unaffected if there is more than 10% of the normal amount of pantothenate present in the media but lower concentrations lead to much lower colony counts and optical densities and no growth or only one or two colonies were obtained at concentrations of 1% or less. In the case of *C. autoethanogenum* 1 colony was present on plates containing .01% of the original pantothenate concentration whereas in *C. ljungdahlii* no growth occurs after 1%.

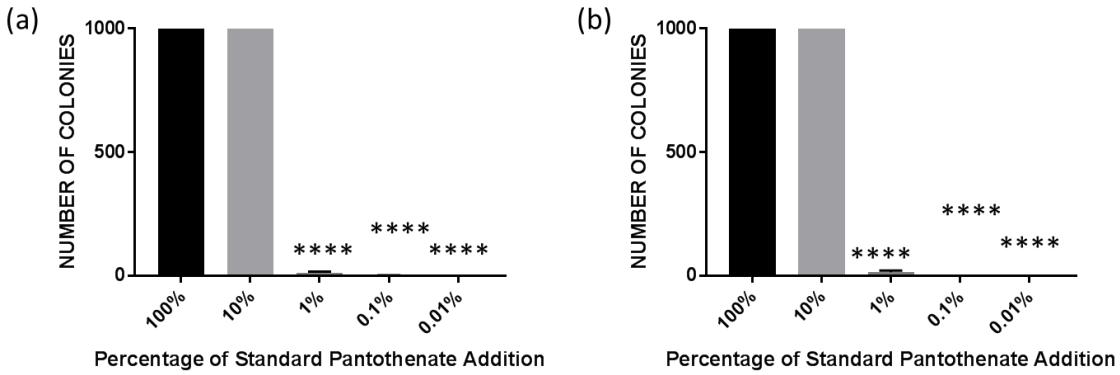


Figure 17 Average number of colonies found on plates containing different concentrations of pantothenate in the media
*(A) Shows results for C. autoethanogenum and (B) shows results for C. ljungdahlii. 100ul of washed culture was plated. (N=3) Asterisks indicate statistical significance difference to 100% calculated by one-way ANOVA (A) (F (4, 10) =3.523, p=0.0001) (B) (F (4, 10) = 2.139, P= 0.0001) with a posteriori Dunnett's multiple comparisons test. (**** indicates P>0.0001)*

4.3.2 Restoration of Prototrophy for Pantothenate Biosynthesis in *C. autoethanogenum* and *C. ljungdahlii* using plasmids.

4.3.2.1 Bioinformatics

Using UniProt, KEGG and NCBI, to analyse the genomes of *C. ljungdahlii* and *C. autoethanogenum* revealed the presence of putative genes responsible for pantothenate biosynthesis pathway, except for three key genes- *panB, panC* and

panD which catalyse three steps of the pathway. *PanB* encodes an enzyme – (ketopantoate hydroxymethyl transferase) which converts ketoisovalerate into ketopantothenate. *PanC* encodes pantothenate synthase – which converts β-alanine+Pantoate into D-Pantothenate and *PanD* encodes aspartate-1-decarboxylase, involved in a precursor reaction which converts L-aspartate into β-alanine. Therefore, as a starting point we attempted to convey pantothenate biosynthesis by synthetic biology to introduce these three genes from a closely related organism –*Clostridium acetobutylicum,* which was identified as the most suitable donor, due to the degree of relatedness and as shown in figure 18, it possesses the three genes in a single cluster (*panB* CA_C2914; *panC* CA_C2915; *panD* CA_C2916).

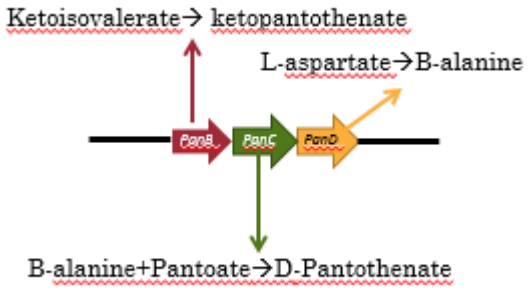


Figure 18 Role of each of the three key missing genes in the pantothenate biosynthesis pathway naturally occurring as a cluster in C. acetobutylicum.

4.3.2.2 Construction of pMTL-FJAL3

The plasmid pMTL_FJAL3 was constructed in order to insert the *panBCD* cluster into *C. autoethanogenum* and *C. ljungdahlii*, using the pMTL modular system outlined in the introduction. The pantothenate cluster genes *panB-panC-panD* were PCR-amplified using primers *PanBCD*1Fwd and *PanBCD*1Rev from the genome of *C. acetobutylicum.* Top10 *E. coli* was transformed with

pMTL_FJAL3. The Top10 strain containing the plasmid was then inoculated into LB media with chloramphenicol supplementation to select for plasmid presence. Successful ligation reactions were confirmed by restriction enzyme analysis and Sanger sequenced, after plasmid extraction from Top10 (subsequently known as "conformation method 1").

4.3.2.3 Transformation of *C. ljungdahlii* with pMTL_FJAL3

The plasmid pMTL_FJAL3, was isolated by plasmid Miniprep from TOP10 E.*coli*. 5µl of the plasmid was then transformed into 100µl of electrocompetent *C. ljungdahlii* cells as outlined in methods section 2.5.8.4. The transformed cells were allowed to recover in YTF for 20 hours before plating onto YTF plates containing an appropriate amount of thiamphenicol. Only colonies containing the plasmid were able to survive the selection pressure. Colonies were restreaked and purified on YTF plates containing thiamphenicol, and single colonies used to inoculate 15ml of YTF. 10ml of this culture was aliquoted into 1ml cryogenic eppendorfs and stored with 10% glycerol in the -80 degree freezer. The remaining 5ml was used to perform a plasmid Miniprep, and restriction digested and the region containing the *PanBCD* fragment, sanger sequenced to ensure the plasmid was still correct (subsequently known as "transformation method 1").

4.3.2.4 Transformation of *C. autoethanogenum* with pMTL_FJAL3

The plasmid pMTL_FJAL3, was isolated by plasmid Miniprep from TOP10 *E.coli*. 5µl of the plasmid was then transformed into 100µl of electrocompetent Sexpress cells, which were selected for by growing overnight in LB

supplemented using thiamphenicol and tetracycline. The next day cultures containing cells with both plasmids were centrifuged and washed twice with anaerobic PBS and the supernatant aspirated each time. The pellet was then transferred to the anaerobic cabinet and resuspended using 500µl of wild type *C. autoethanogenum,* and then recovered in 15ml of YTF media without supplementation for 20 hours, before plating onto YTF plates containing an appropriate amount of thiamphenicol and D-cycloserine (to select against any surviving *E.coli* cells). Only colonies containing the plasmid were able to survive the selection pressure. Colonies were restreaked and purified on YTF plates containing thiamphenicol, and single colonies used to inoculate 15ml of YTF. 5ml was used to perform a plasmid Miniprep, and the plasmid was sent for Sanger sequencing which showed no deviation from the expected sequence (subsequently known as "transformation method 2). If strains were to be grown using CO as a carbon source they were gas adapted as outlined in the methods section 2.5.5.1.

4.3.2.5 Characterisation of transformed strains.

The transformation of *C. autoethanogenum* and *C. ljungdahlii* with the plasmid pMTL_FJAL3 led to a large increase in growth in both species when grown in conditions without pantothenate compared to the wild types. When cultivated in medium without pantothenate, the transformed strains performed similarly to the wild type with pantothenate in the medium during growth profiles, (Figure 19) and showed good viability on plates with and without pantothenate in the medium. The expression of these three genes on plasmid confers adequate pantothenate biosynthesis to the cells for them to become autotrophic for the vitamin.

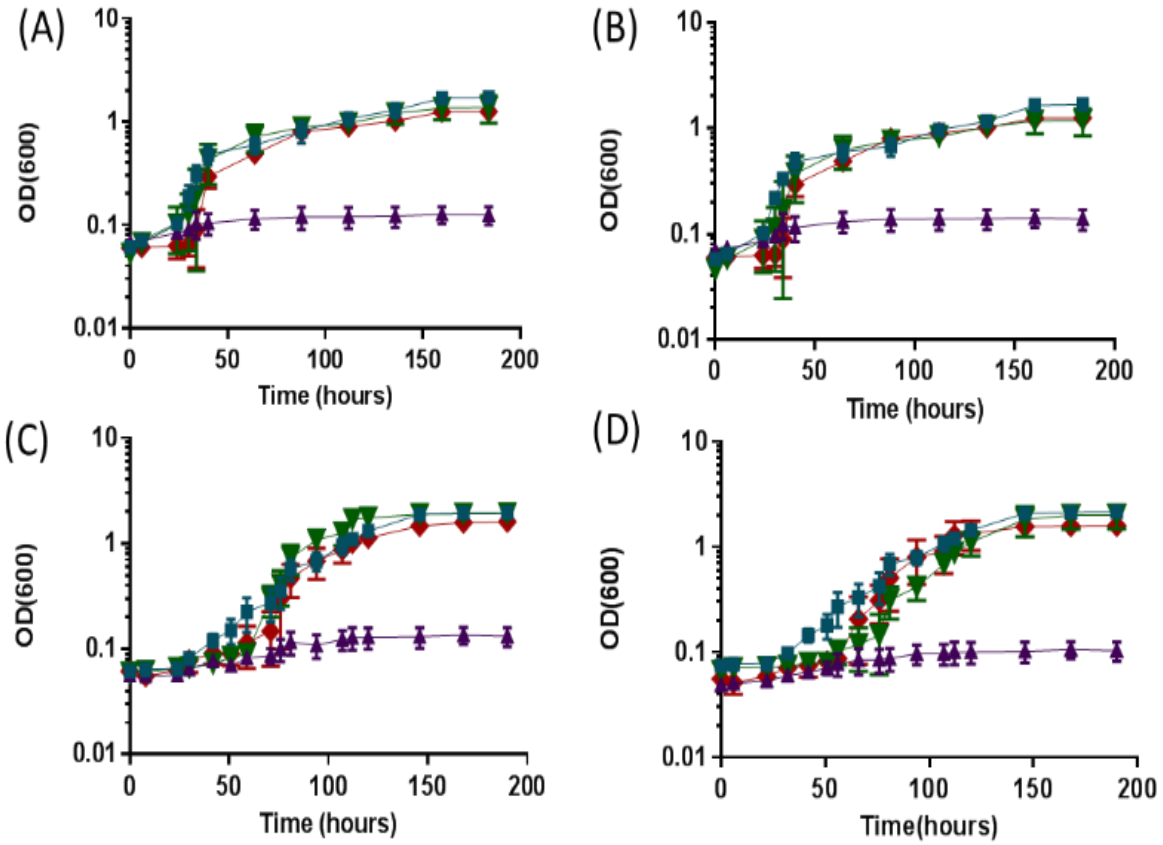


Figure 19 Comparison of performance when growing with and without pantothenate in the media, after the transformation of the strains with the plasmid FJAL3.
(A) comparison of WT C. autoethanogenum and C.a::p_FJAL3 when grown with and without pantothenate in the media using fructose (B) comparison of WT C. ljungdahlii and C.l::p_FJAL3 when grown with and without pantothenate in the media using fructose (C) comparison of WT C. autoethanogenum and C.a::p_FJAL3 when grown with and without pantothenate in the media using CO (D) comparison of WT C. ljungdahlii and C.l::p_FJAL3 when grown with and without pantothenate in the media using CO. Blue; WT + pantothenate, Purple; WT – pantothenate, Red; strains transformed with pMTL_FJAL3 +pantothenate, Green; strains transformed with pMTL_FJAL3 – pantothenate.

4.3.3 Determining gene importance

To ensure all three of the inserted genes were essential, we ran tests to ensure that the strains could not be transformed by just one of the genes or a mixture of two and still have the prototrophy conveyed. Each of the different gene combinations were transformed into the two species by transformation method 1 and 2 and viability was determined on plates.

4.3.3.1 Construction of pMTL_FJAL4

The plasmids shown in table 19 were constructed to ascertain whether various genes or different combinations of genes were essential for biosynthesis of pantothenate. Gene fragments were amplified using specific primers and then ligated into their respective lineralised plasmids.

Successful insertion of the fragments was ascertained by conformation method 1 and plasmids were transformed into *C. autoethanogenum* and *C. ljungdahlii* by transformation method 1 or 2, before being recovered and characterised

4.3.3.2 Characterisation of Gene Importance

In order to characterise the "essential" nature of each of the genes to the organisms, the transformed strains were grown in cultures with and without pantothenate to determine if prototrophy had been conveyed. Resistance to the antibiotic was seen as a marker of successful transformation. Successful colonies were restreaked for purity and then after 48 hours of growth were resuspended in anaerobic PBS. 100µl was tested on plates with the antibiotic and with and without pantothenate in the media, and the rest was inoculated into 5ml YTF media for over nights to plasmid Miniprep to check that the plasmid was still present and correct. Results were identical for both *C. autoethanogenum* and *C. ljungdahlii*, (Table 19). Unless all three genes were present in the strain, no prototrophy was conveyed for pantothenate, and if it was not present in the media there was no viability.

Plasmid name	Genes inserted	Promotor	Resistance to antibiotic		Viability without Pantothenate in liquid and solid media	
			C. ljungdahlii	*C.* auto	*C. ljungdahlii*	*C.* auto
None	None	None	-	-	-	-
pMTL_FJAL3	*PanBCD*	Pthl	+	+	+	+
pMTL_FJAL6	PanB	Pthl	+	+	-	-
pMTL_FJAL7	PanC	Pthl	+	+	-	-
pMTL_FJAL8	PanD	Pthl	+	+	-	-
pMTL_FJAL5	PanBC	pthl	+	+	-	-
pMTL_FJAL4	PanCD	pthl	+	+	-	-
pMTL_FJAL4 +pMTL_FJAL6	panCD + panB	pthl and fdx	++	++	+	+
pMTL_FJAL5+ pMTL_FJAL8	panBC + panD	Pthl and fdx	++	++	+	+

Table 19 table showing the results of transformation of different gene combinations of panB, panC and panD in order to determine whether all are essential for biosynthesis of pantothenate.
All transformations were successful as the antibiotic resistance is conveyed by the plasmid but unless all three genes are present no growth occurs if pantothenate is not present in the media

4.3.3.3 Alternative routes.

As no growth was possible if *panB*, *panC* or *panD* was excepted from the plasmids, it was hypothesised that production of pantothenate could be achieved with the introduction of intermediates as "pro-vitamins". To this end strains were tested containing pMTL_FJAL5 with β-alanine (usually converted by *panD* which is missing from the cluster in this plasmid) in the media, pMTL_FJAL4 with pantolactone (which can act in place of the ketopantoate produced by Ketopantoate hydroxymethyl transferase encoded by *panB*) and pMTL_FJAL7 with pantolactone and β-alanine in the media. As demonstrated in Table 20, in each instance, if the plasmid contained the genes for part of the pathway and an appropriate intermediate was added then viability was possible even if pantothenate was not in the media, as the pathway was complete as the intermediates bypassed the requirement for the gene.

Plasmid name	Genes inserted	Intermediate added	Resistance to antibiotic		Viability without Pantothenate in liquid and solid media	
			C. auto	*C. ljungdahlii*	*C.* auto	*C. ljungdahlii*
None	None	None	-	-	-	-
pMTL_FJAL3	*PanBCD*	None	+	+	+	+
pMTL_FJAL7	PanC	Pantolactone and β-alanine	+	+	+	+
pMTL_FJAL5	PanBC	β-alanine	+	+	+	+
pMTL_FJAL4	PanCD	pantolactone	+	+	+	+

Table 20 table to display viability of both species when grown with genes inserted by plasmid and with an intermediate in the media.
Both strains are viable if they contain the genes and an intermediate.

4.3.4 Insertion of *panBCD* into the genome of *C. autoethanogenum*

We decided to try to fix the pantothenate biosynthesis pathway in the genome in order to convey long term autotrophy, as the plasmid can be easily lost, and in order to maintain the genes it is important to fix the genes in the genome. To this end, the Δ*pyrE* system was implemented and a Δ*pyrE* strain of *C. autoethanogenum* was developed.

4.3.4.1 Development of the Δ*pyrE C. autoethanogenum* strain.

In order to develop the *pyrE* deficient strain, the previously developed pAnne12 plasmid was used (Dr. A. Henstra, University of Nottingham). This plasmid (as shown in figure 20) contained the capacity for deletion of the *pyrE* C-terminus in *C. autoethanogenum*. The plasmid was developed and supplied by Dr. Anne Henstra (University of Nottingham), and based on the pMTL8000 system using the backbone vector 84151. The plasmid contained a 1219 base pair long homology arm and a 303 base pair short homology arm, separated with the *lacZα* gene, this allows for the deletion of 227 base pairs at the C-terminus of *pyrE* and its replacement with the *lacZα* gene. The *lacZα* gene is used because it is small

and has no deleterious effect on strain after it insertion. A heterologous *pyrE* imported from C. *acetobutylicum* with a ribosomal binding site (RBS) is also present on the plasmid, 18 base pairs downstream of the *catP* gene (thiamphenicol and chloramphenicol resistant) to ensure the loss of plasmid when using 5-fluoroorotic acid (FOA) as counter-selectable marker, the heterologous *pyrE* is similar enough that it still functions to produce the toxic 5-FUMP product, but not similar enough to cause accidental recombination.

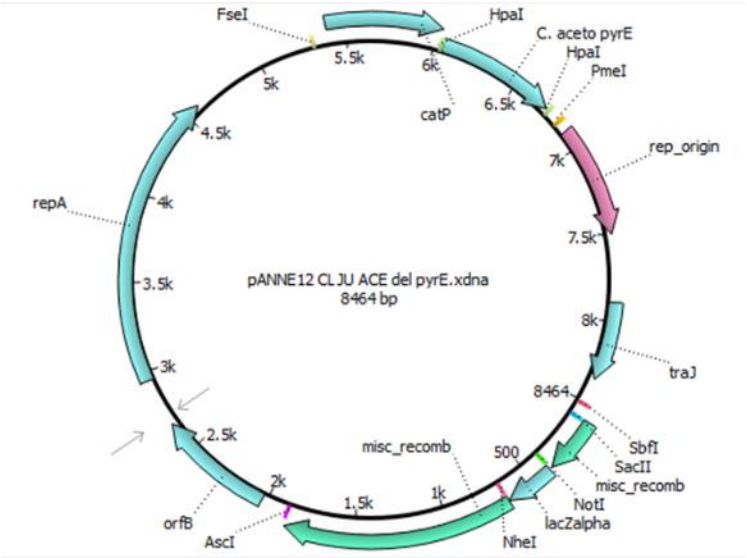


Figure 20 Map of pAnne12 for the deletion of pyrE gene in C. autoethanogenum.

The plasmid was transformed into CA434 containing E. *coli* strains and then inserted in to Wild Type *C. autoethanogenum* by conjugation. Successful colonies that emerged on solid YTF plates supplemented with thiamphenicol and trimethoprim were restreaked and purified on further YTF plates with thiamphenicol and trimethoprim. Larger, faster growing colonies were then replated onto YTF agar, and labelled. Larger faster growing colonies were selected because the plasmid is defective in the gram positive replicon, and so will replicate very slowly limiting resistance and growth of colonies, when there

is a single crossover event the limitation is relieved as replication of the plasmid is under the control of the organism instead of the plasmid, so colonies can grow larger and quicker than their unintegrated counterparts. These colonies were subjected to further rounds of streaking onto YTF supplemented with thiamphenicol. gDNA was isolated from several of the fastest growing thiamphenicol resistant colonies and subjected to colony PCR screening using an internal primer (CMH 1) annealing to a region located on the plasmid, and the downstream flanking primer (ACE *pyrE* RSP), which generated a roughly 1800 base pair fragment if the strain contained a single crossover (Figure 21).

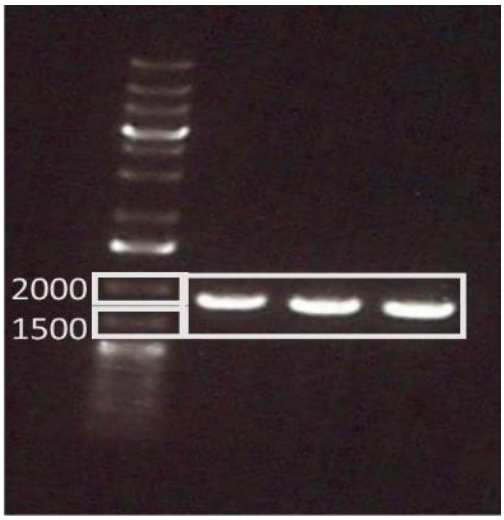


Figure 21 amplifications using the primers CMH1 and ACE pyrE RSP.
Successful single cross over mutants showed an 1800bp band. Gene ruler used –1log ladder.

Colonies which had successfully shown the single crossover event were partially stored in 10% glycerol in the -80ºC freezer for holding and also resuspended in 100 µl PBS. All 100 µl of resuspension were plated onto YTF plates containing FOA (1mg/ml) and uracil (20 µg/ml) and plates wrapped in foil to prevent degradation of FOA by light in order to select for a double crossover event. Colonies which were able to grow on FOA plates were replated onto SDMM-C (semi-defined minimal media, containing Casamino acids) plates with and

without uracil to test for auxotrophs (i.e. no functional *pyrE*), the most promising strains were then plated onto plates with and without uracil. Colonies which were auxotrophic for uracil were inoculated into 4ml of YTF culture overnight and gDNA was extracted. The double crossover event was then screened for using the primers ACE-*pyrE* FSP and M13 FSP (WT would show no band, mutant would show a 600bp band shown in Figure 22 (A)). A separate PCR was also run using the primers ACE *pyrE* FSP and ACE *pyrE* RSP to isolate the *pyrE* deletion region for Sanger sequencing to confirm replacement of the 3' end of *pyrE* with *lacZα*. Colonies which had shown the correct 2 kilo base band (Figure 22 (B)) were sent for Sanger sequencing (sequencing in supplementary material) to confirm the deletion with the primer ACE *pyrE* FSP. Serial passage on non-selective YTF medium was then performed to induce the loss of the plasmid by removal of selective pressure. After three passages the loss was confirmed by replica plating onto YTF agar medium supplemented with thiamphenicol. Successful mutants were sent for whole genome sequencing and the strain containing the least SNP's (Made by Ronja Breitkopf) was used in subsequent experiments.

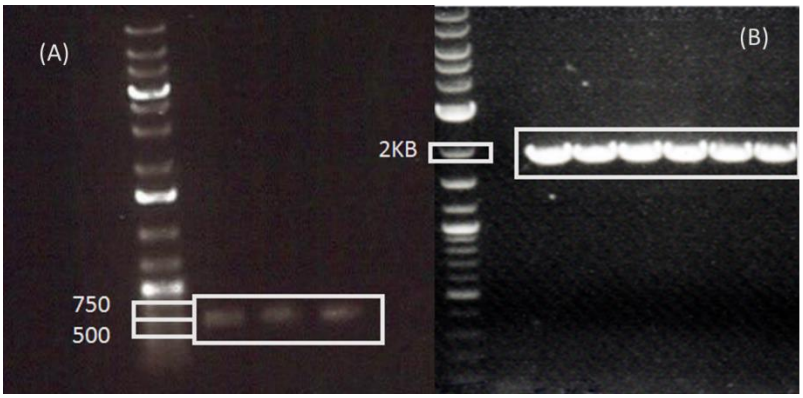


Figure 22 Double knockout of pyrE in C. autoethanogenum pyrE mutants.
(A) Double crossover event (600bp) ruler-Ugene 1-log ladder (B) sequencing over the pyrE deletion region of the genome to confirm truncation of pyrE and insertion of lacZα (2kb) ladder- NEB 2-log DNA ladder.

4.3.4.2 Insertion of *PanBCD* into the genome

The major advantages of using ACE in the generation of knockout mutants such as the *ΔpyrE* strain is the possibility of restoring the deleted part of the *pyrE* gene back to its wild type genotype. This is better than many other plasmid based complementation because the gene dosage remains the same and hopefully the only altered components of the genome are intentional ones. Furthermore, there is no need for plasmid control or an antibiotic selection pressure to maintain the plasmids containing the genes. To repair a *ΔpyrE* mutant, the transformation of a plasmid containing the missing 3'end of the gene can be inserted and restoration can be selected for by uracil prototrophy.

For the restoration of the Δ*pyrE* mutant the plasmid CH23 was used (figure 23), originally made by *C.*M. Humphreys (University of Nottingham). The plasmid was made using the pMTL backbone pMTL84152. The plasmid has the same long homology arm as pAnne12 but a longer short homology arm (526 base pairs long instead of 303 base pairs) in order to "fix" the deleted *pyrE* portion of the Δ*pyrE* strains. In order to "fix" the deleted *pyrE* portion of the *ΔpyrE* strains and restore it to its original unscarred self. Unlike pANNE12 the plasmid does not contain a *pyrE* gene from *C. acetobutylicum* so no autotrophy for uracil production can be attributed to the heterologous gene.

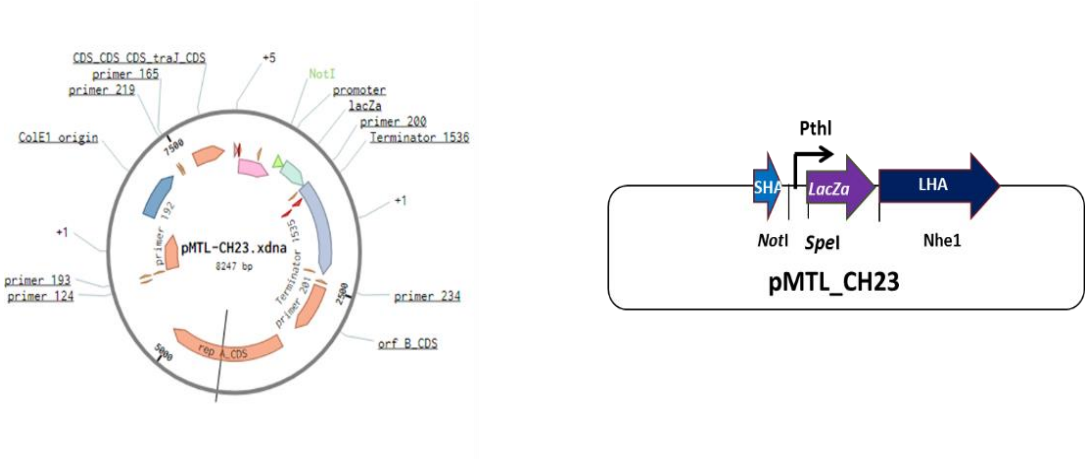


Figure 23Plasmid map and schematic of unaltered pMTL_CH23

The CH23 plasmid contains a *lacZα* gene between the two homology arms. Importantly this can be excised and another gene inserted in order to in a single stroke, repair the *PyrE* deficiency and bring in a payload of genes to be inserted into the genome.

4.3.4.3 Generation of pFJAL10

The plasmid pMTL_FJAL10 (figure 24) was created in order in insert the *panBCD* gene cluster from *C. acetobutylicum* into a *pyrE* deficient mutant of *C. autoethanogenum* created for this project as outlined in methods section 2.6.2. The plasmid was made using the backbone of the thiamphenicol resistant CH23 plasmid. The placeholder gene *lacZα* was removed by restriction digest and the *panBCD* gene cluster discussed in section 4.3.2.2 was amplified from *C. acetobutylicum* using the primers *panBCD*2fwd and *panBCD*2rev and the backbone and insert were ligated, and confirmed by confirmation method 1.

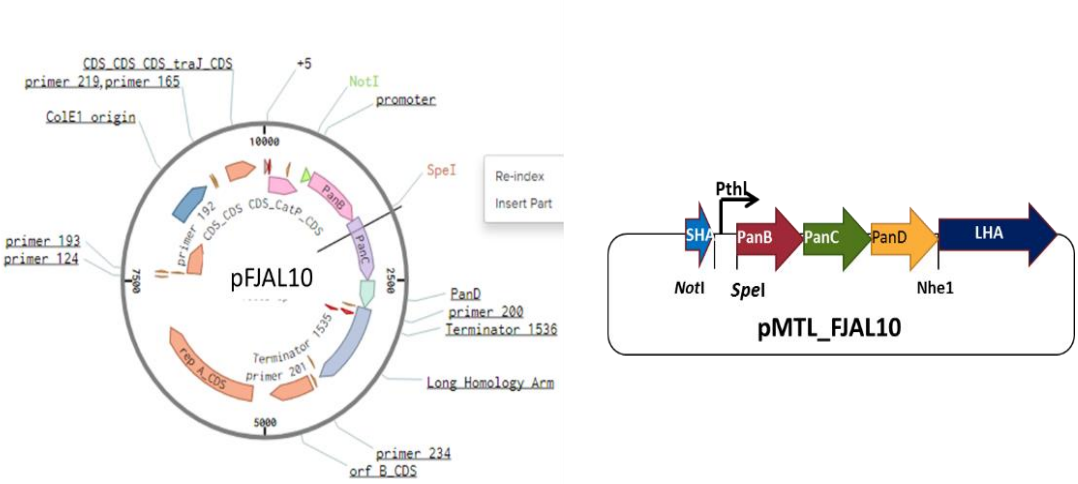


Figure 24 Schematic diagram of pMTL-FJA10 plasmid.
This plasmid has been designed to introduce the genes panB, panC and panD into C. autoethanogenum to determine if growth is possible if the genes are present on plasmid and biosynthesis is possible if the genes are present in the genome.

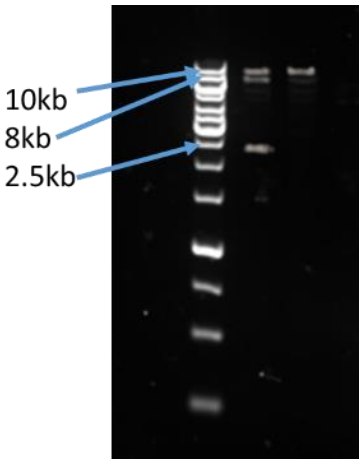


Figure 25 Confirmation of pMTL-FJAL10 structure by restriction enzyme analysis, visualised by gel electrophoresis.
(1) Dual digest of FJAL10 with SbfI and FseI resulting in two bands of 2451 and 7537 which add up to the predicted length of 9988 bp, implying the insertion of the genes has occurred as without the insertion the second band length would 5412bp (2) Single digest of pMTL-FJAL10 with SbfI restriction enzyme, results in a band of lineralised plasmid of 9988bp.

4.3.4.4 Transformation of *ΔpyrE C. autoethanogenum* with FJAL_10

Successful pMTL_FJAL10 plasmids were then transformed into Sexpress electrocompetent cells as described in 2.5.7.2., and conjugated into *pyrE* deficient *C. autoethanogenum* as mentioned in 2.5.8.3. The thiamphenicol resistance, large fast growing colonies were streaked onto solid SDMM media with and without

uracil to select for restored autotrophy. Uracil autotrophs were then PCR screened using the primers ‘ACE-*pyrE*-F’ and ‘ACE-*pyrE*-R’. Colonies with a repaired *pyrE* locus are demonstrated by the presence of a band which is the size of the inserted genes *panBCD* (2055base pairs) + 2 kilo base pairs for the restored *pyrE* resulting in an expected band of 4055bp as seen in the gel in figure 26. The resultant PCR products were also Sanger sequenced to verify the restoration of the wild type *pyrE* genotype, with the insertion of the *panBCD* cluster, sequencing results shown in supplementary material.

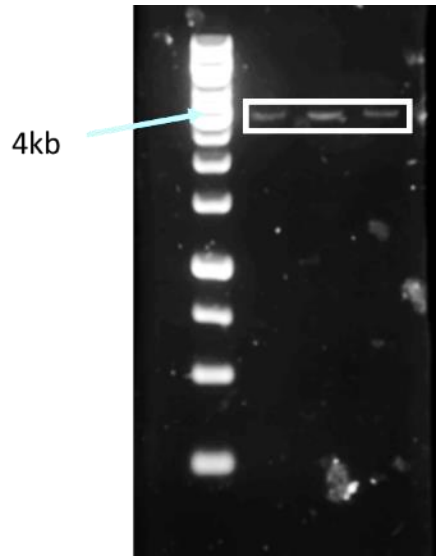


Figure 26Restoration of C. autoethanogenum pyrE mutants.
PCR screening of fully restored pyrE mutants with the integration of the panBCD genes with a band of roughly 4125

4.3.4.5 Transformation of *ΔpyrE C. ljungdahlii* with FJAL10

A previously developed *C. ljungdahlii* strain deficient in *pyrE* was donated by Dr. A. M. Henstra. The homology arms required to “fix” *ΔpyrE* are identical and so the plasmid pMTL_FJAL10 can be used to transform both species to bring in the *panBCD* cluster and repair the mutant *pyrE* gene. Electrocompetent *ΔpyrE ljungdahlii* cells were created and the pMTL_FJAL10 plasmid was inserted into these cells. After recovery, testing for successful transformants occurred as

outlined in methods section 2.6.2. Colonies which were uracil autotrophs and could not survive on plates with 5-FOA were PCR screened using the primers 'ACE-*pyrE*-F' and 'ACE-*pyrE*-R' and if colonies contained a 4kb band, these were sent for Sanger sequencing to verify the restoration of the wild type *pyrE* genotype, with the insertion of the *panBCD* cluster. Intermediate primers were used to ensure full read coverage over the 4KB section.

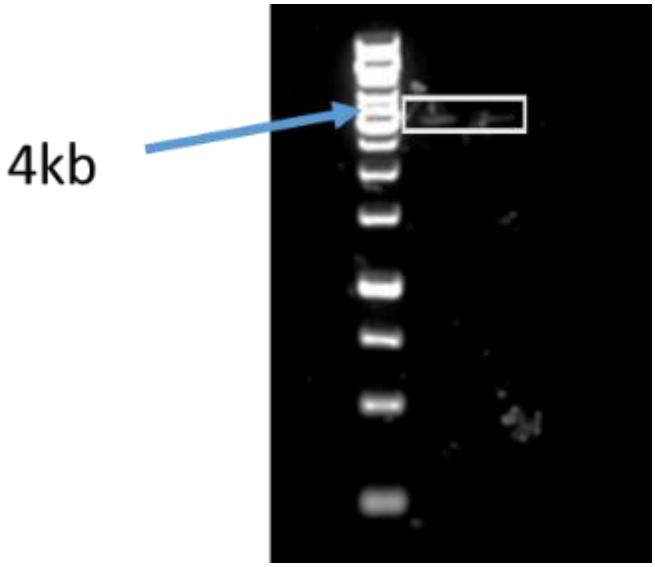


Figure 27 Restoration of C. autoethanogenum pyrE mutants.
PCR screening of fully restored pyrE mutants with the integration of the panBCD genes with a band of roughly 4125

4.3.5 Characterisation of *panBCD* Cluster into the Genome

The decision to try to fix the pantothenate biosynthesis pathway in the genome was made in order to convey long term prototrophy, as the plasmid can be easily lost and in order to maintain the genes it is important to fix the genes in the genome. Using the *ΔpyrE* system, the plasmid pMTL_FJAL10 was conjugated into *C. autoethanogenum* and *C. ljungdahlii* and the *panBCD* cluster successfully integrated as well as the deleted section of the *pyrE* gene repaired to the wild type genotype, to create the strains known as "+*pantothenate*". Once this had successfully occurred, and strains to be grown using CO as a carbon source were

"gas adapted," the strain was tested in conditions with and without pantothenate to see if the auxotrophy had been fixed and full biosynthesis for pantothenate was now present in the cells.

4.3.5.1 Growth on solid media of +*pantothenate* strains

As seen in figure 28, when grown on fructose containing solid media plates the insertion of these genes into the genome seems to have restored prototrophy and biosynthesis of pantothenate seems possible as the altered strain is able to grow regardless of whether pantothenate is present in the media. The wild type is not capable of growth if pantothenate is omitted from the media. Growth of prototrophic strains is slower on media lacking pantothenate but they are capable of prototrophic growth which the wild type is not. The slower growth may be because a longer adaptation phase needed from the transformed strains which have to produce the pantothenate to grow rather than taking it from the surrounding media.

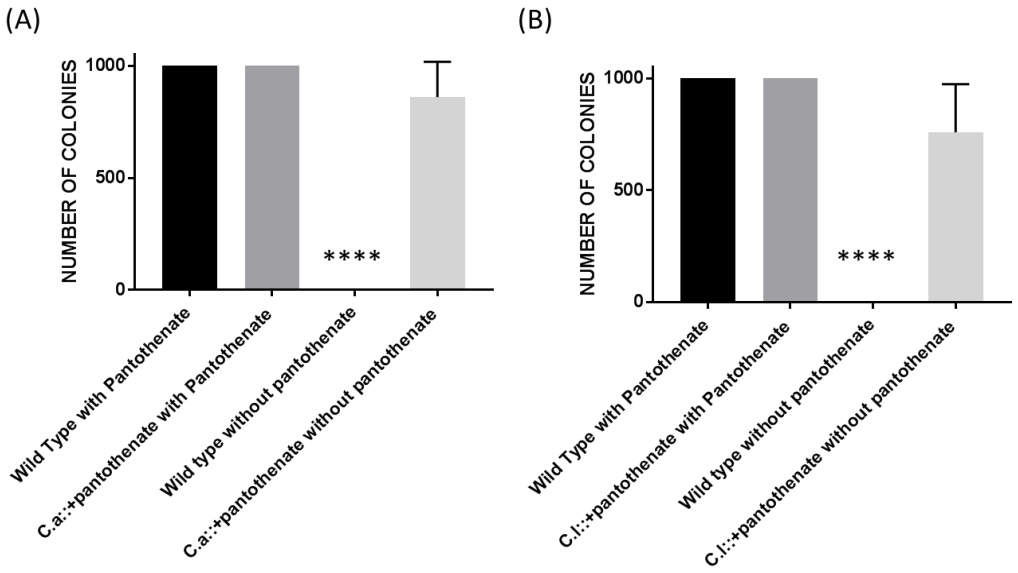


Figure 28 (A) average number of colonies found on plates when wild type and (A) C. autoethanogenum and (B) C. ljungdahlii +pantothenate strains are grown with and without pantothenate in the media. 100ul of washed culture was plated. (N=3)

*Asterisks indicate statistical significance difference to 100% calculated by one-way ANOVA (A) (F (3, 8) =3.125,) (B) (F (3, 8) = 2.27) with a posteriori Dunnett's multiple comparisons test. (**** indicates P>0.0001)*

Over 1000 colonies were present on plates containing pantothenate whereas there was none for the wild type strain on plates without any pantothenate.

4.3.5.2 Growth in liquid media with fructose or CO as a substrate of *+pantothenate C.autoethanogenum*

The impact of pantothenate being left out of the media on the growth capability of the gas adapted *+pantothenate C. autoethanogenum* strain or the gas adapted wild type *C. autoethanogenum* strain was studied in a growth experiment with vitamin free Casamino acids and using fructose or 1 bar of CO as the carbon source. As shown in figure 29, with either carbon source, the wild type cells which were grown in conditions without pantothenate showed very little signs of growth after 40 hours, and remained at a similar optical density throughout the experiment after 40 hours, however, the wild type culture with all of the vitamins reached an OD (600) over 2.0 with both substrates after 120 hours. *+pantothenate* was able to grow with and without pantothenate in both conditions, although in both cases terminal OD is lower when grown without pantothenate in the media, however, the cells are still capable of significant growth. Inoculated medium that lacked pantothenate did not support an increase in turbidity or reliably support growth for the wild type in either instance, however, in both carbon sources the transformed strain could grow without pantothenate showing that pantothenate is required for autotrophic growth in the wild type but not in the strain transformed with the *panBCD* cluster. Interestingly the growth of the transformed strain without pantothenate when growing on CO is actually more similar to the wild type growing with pantothenate in the media than its counterpart is when growing in fructose. This could be because all cultures required a longer adaptation phase

than they did when growing in fructose, so there is more time for the cultures to start the biosynthesis of the pantothenate. Both carbon sources would require pantothenate to grow as shown in previous experiments as without it Acetyl CoA cannot be formed, so no growth will occur. Perhaps as the species grows slower when using CO as a carbon source in batch culture serum bottles, the requirement for pantothenate is lower, and so the cultures can produce enough pantothenate to match the requirements from the wild type growing in CO with abundant pantothenate.

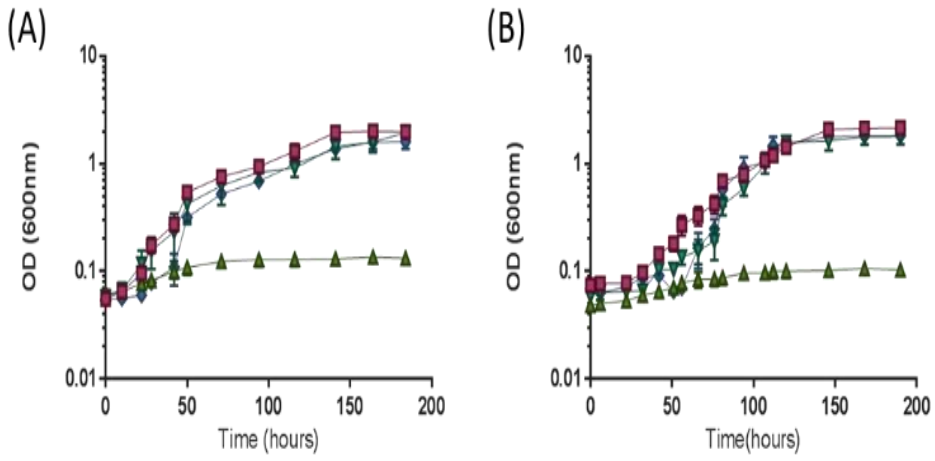


Figure 29 Comparison of performance when growing with and without pantothenate in the media, after the transformation of the strains with the plasmid FJAL10.
(A) The growth curves of +pantothenate C. autoethanogenum vs. wild type when grown with and without pantothenate in the media using fructose (B) the growth curves of +pantothenate::C. autoethanogenum vs wild type when growing on CO as a carbon source.
Pink; wild type + pantothenate, Green; wild type – pantothenate, Turquoise; +pantothenate strain grown with pantothenate, Blue; +pantothenate strain grown without pantothenate. Error bars show standard error. N=3

4.3.5.3 Fermentation products.

In order to ascertain the differences in yields of industrially interesting products *C. autoethanogenum* produced when in different phases of growth whether the vitamin was present in the media or not with either strain, samples were taken from growth experiments at four distinct phases. These phases were determined by the growth of the wild type *C. autoethanogenum* in media containing

pantothenate. The phases were: initial inoculation (0 hours), Early Exponential (42 hours), two exponential phase measurements (94 and 120 hours) and a terminal stationary phase measurement (214 hours). Samples were taken as outlined in 2.10, and analysed as mentioned. Resulting concentrations of ethanol and acetate from cultures growing without pantothenate were then compared to the results for a culture growing with full vitamins. The acetate: ethanol ratio was also calculated and compared. In the results from figure 30, we can clearly see a huge difference in the yield of ethanol and acetate from +*pantothenate* strains grown without pantothenate when compared to wild type cultures grown without pantothenate (used as a negative control). Interestingly the culture of the transformed strain growing without pantothenate initially produced similar quantities of acetate but in the later phases, produced less acetate than the wild type or the transformed strain and produces comparatively more ethanol.

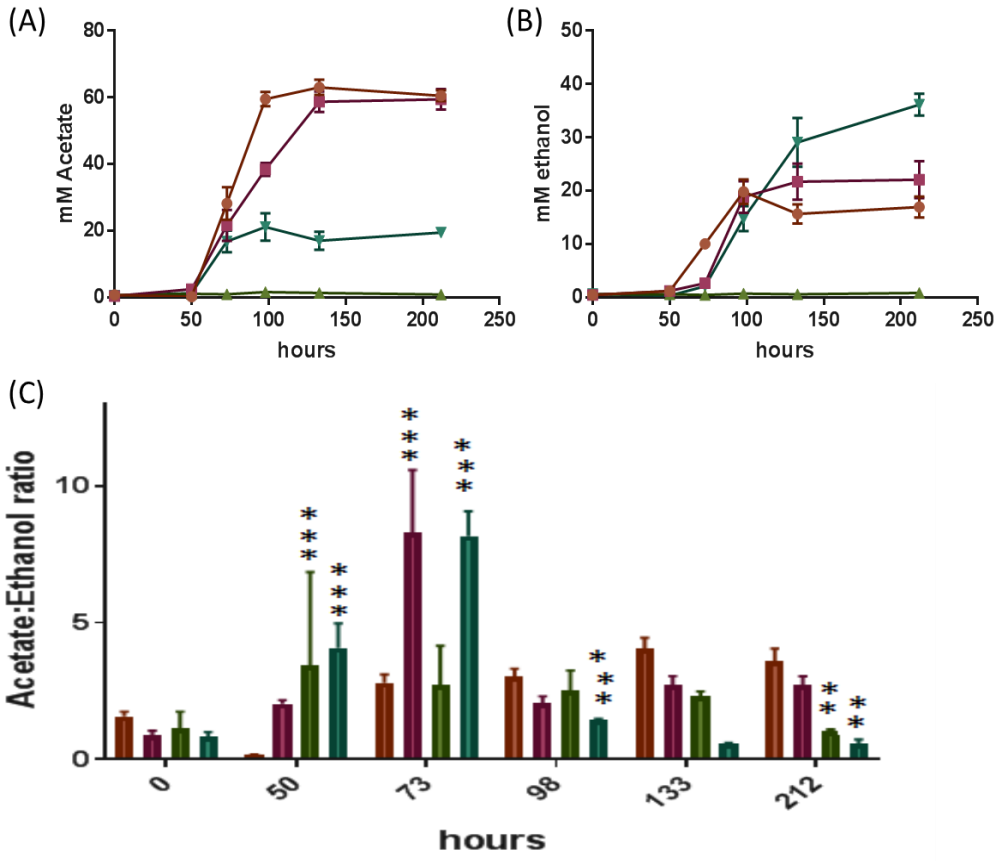


Figure 30 HPLC results when a +pantothenate strain and a wild type strain of C. autoethanogenum is grown in pantothenate containing and pantothenate free media.
*(A) mM acetate production (B) mM ethanol production (C)acetate: ethanol ratio. Brown;: wild type strain in media containing pantothenate, green: wild type strain in pantothenate free media, pink: +pantothenate strain in media containing pantothenate and Blue: +pantothenate strain in media without pantothenate. All using 1 bar of CO as a carbon source (N=3). Asterisks indicate statistical significance difference to wild type growing in 100% vitamins in each phase. Calculated by two-way ANOVA (F (15, 48) = 10.78, p = 0.0001) with a posteriori Dunnett's multiple comparisons test. *** indicates p>0.0001*

Wild Type Cells are not capable of exponential growth without pantothenate present in the media however, the transformation of the genome to include the *panBCD* cluster of genes allows for biosynthesis of pantothenate and prototrophic growth is possible in these strains.

4.3.5.4 Potential limitations of the introduction of the *panBCD* Cluster into the genome.

The plate viability, growth curves and HPLC results all showed that the introduction of *panBCD* into the genome resulted in prototrophy for pantothenate, without particularly adverse effects on the production of acetate and ethanol or the growth profile of either species. However, it is possible that the introduction of the cluster may cause potentially adverse effects on the production of other biosynthetic molecules in the cell, leading to the replacement of one auxotrophy for another. In order to potentially mitigate this problem and to see if there was any benefit to the yields of economic products of the cultures if the *panBCD* cluster was under the control of a weak promotor, the plasmid pMTL_FJAL11 (figure 31) was produced. The promotor Sac_fdx, a ferredoxin promotor from the *Clostridium saccharoperbutylacetonicum* was capable of increasing the copy number of the genes under its control, was demonstrated to be weaker than the fdx promotor commonly used in the pMTL system (Bart Pander, unpublished). In order to construct this plasmid, the pFJAL10 plasmid was digested with Spe1 and Not1 to remove the Pthl promotor and

dephosphorylated using Antarctic phosphatase. The sac_fdx promotor was excised from a plasmid donated by Bart Pander, by digestion with Spe1 and Not1 and ligated into the pMTL_FJAL10 plasmid upstream of the *panBCD* genes. Confirmation method 1 was used to confirm structure and successful plasmid inserts were sent for Sanger sequencing.

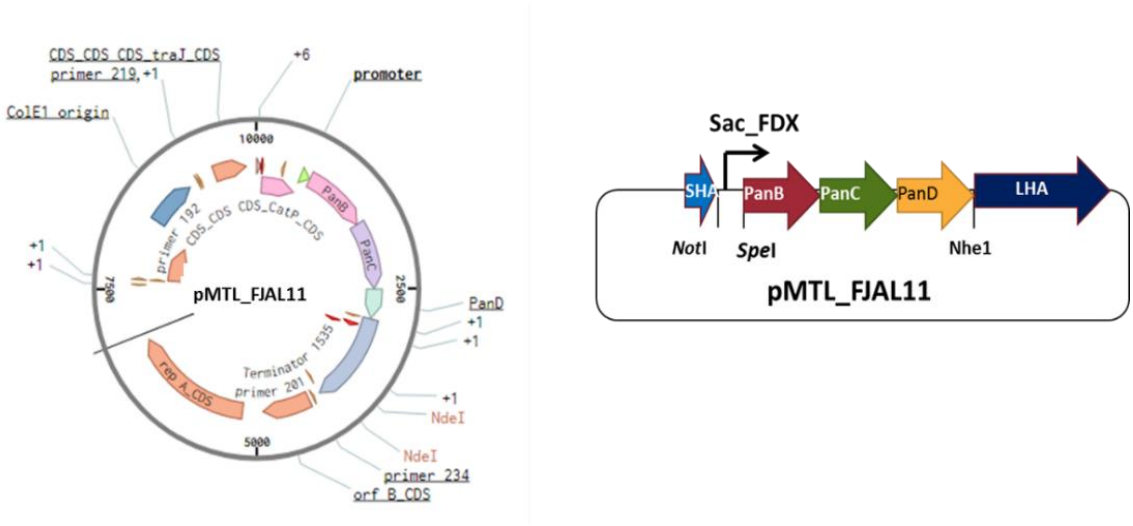


Figure 31pMTL_FJAL11 plasmid map.
Plasmid still contains long and short homology arms to repair deleted part of pyrE, however, the only difference between pMTLFJAL10 and pMTL_FJAL11 is pMTL_FJAL11 contains the weak promotor sac_fdx instead of the strong promotor Pthl

The plasmid pMTL_FJAL11 was then transformed into *ΔpyrE C. autoethanogenum* mutants. Promising colonies were subjected to the *pyrE* repair protocol as outlined in methods sections 2.6.2 Colonies with a repaired *pyrE* locus and the gene cluster are demonstrated by the presence of a roughly 4kb band shown in figure 32. The resultant PCR products were also Sanger sequenced to verify the restoration of *pyrE* genotype and presence of the *panBCD* genes. Sequencing results in supplementary materials. The strain was subsequently known as "*+pantothenate-weak*"

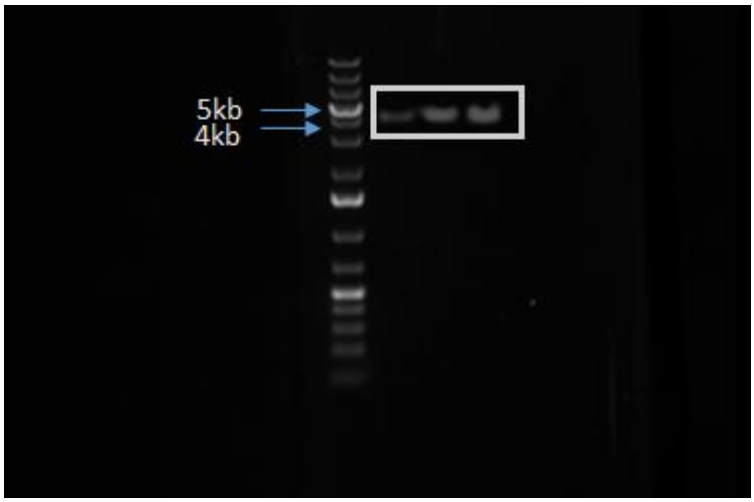


Figure 32Restoration of C. autoethanogenum pyrE mutants whilst bringing in the panBCD genes under the control of a weak promotor.
panBCD + complemented pyrE is roughly 4kb. O'GeneRuler 1kb plus DNA ladder.

4.3.5.5 Characterisation of +pantothenate-weak

Limited characterisation of *+pantothenate-weak,* post gas adaptation, showed that the introduction of the weak promotor led to a reduction in number of colonies present on plates, and a lower optical density at 8 days (shown in figure 33AB), as well as changes to the product profile of the cultures shown in figure 33CDE. Whilst it was clear that growth was possible, and therefore, enough pantothenate was being produced by the cells for viability in both species, the amount produced was likely not as great as that by the strong Thiolase promotor in *+pantothenate* and this may have had a negative impact on the culture growth profile. Light microscopy also showed cultures with a higher proportion of dead or unhealthy cells (rough edges, lighter in colour) and the presence of a large number of longer than average cells. Interestingly, there was a change in the HPLC product profiles of the cells and the strains containing the weak promotor did not produce as much acetate but they did produce more ethanol than the wild type strain. Differences in cell density between *+pantothenate* and *weak pantothenate* may have resulted in lower yields.

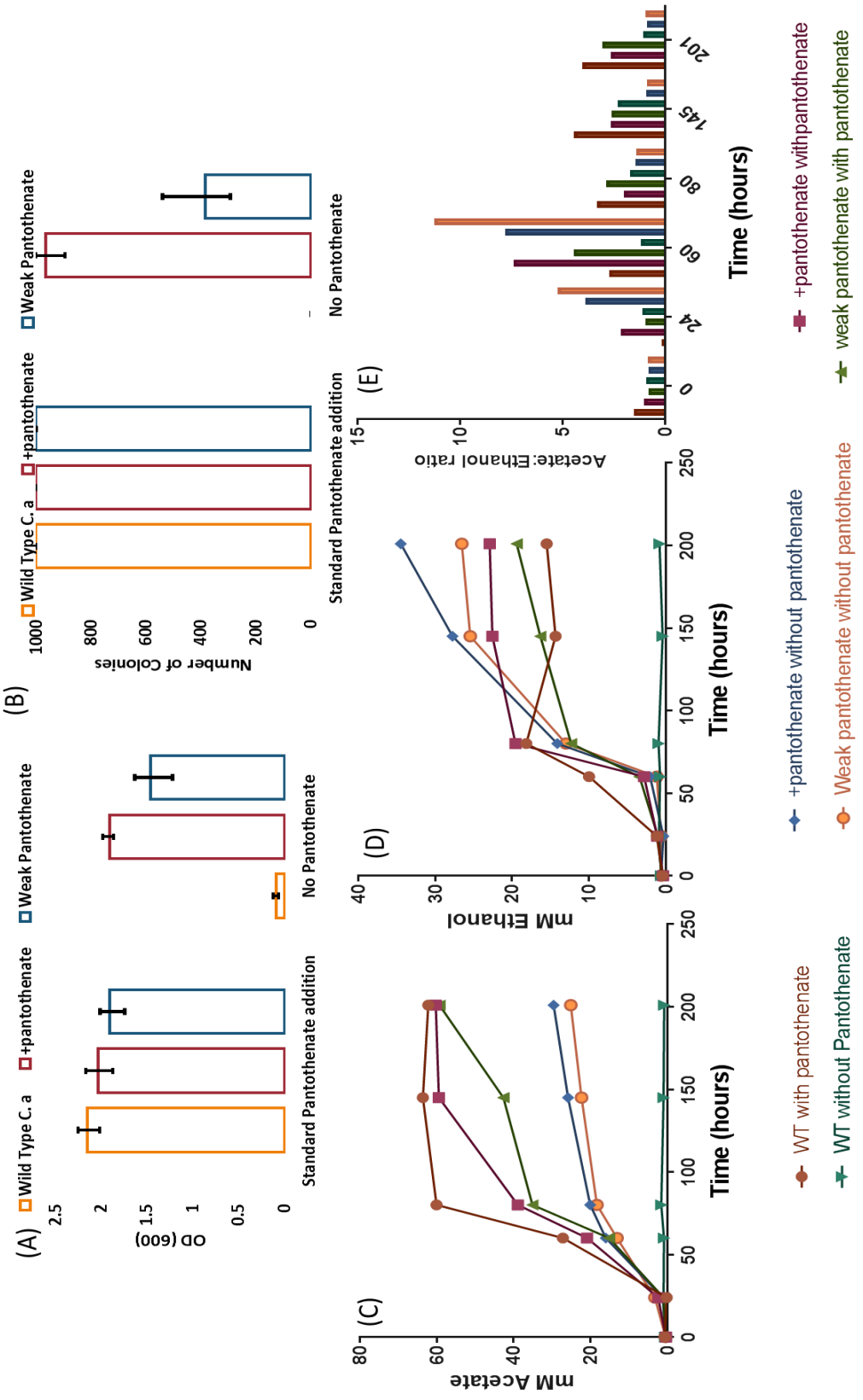


Figure 33 Limited characterisation of FJAL11 (weak pantothenate) strains,
(A) Shows terminal optical density of cells after 8 days (N=3) (B) Number of colonies on plates (N=3) (C) Acetate production (D) Ethanol production (E) acetate to ethanol ratio

4.3.5.6 Valine and leucine

Two potential essential products which may be effected by the introduction and strong regulation of pantothenate production are the production of valine and leucine as these are also downstream products of ketoisovalerate. Shown in the diagram of the pathway in figure 34. Work in other species has shown that a knockout of the leucine pathway by a deletion of the *ilvE* gene has resulted in marginally higher concentrations of pantothenate production[167].

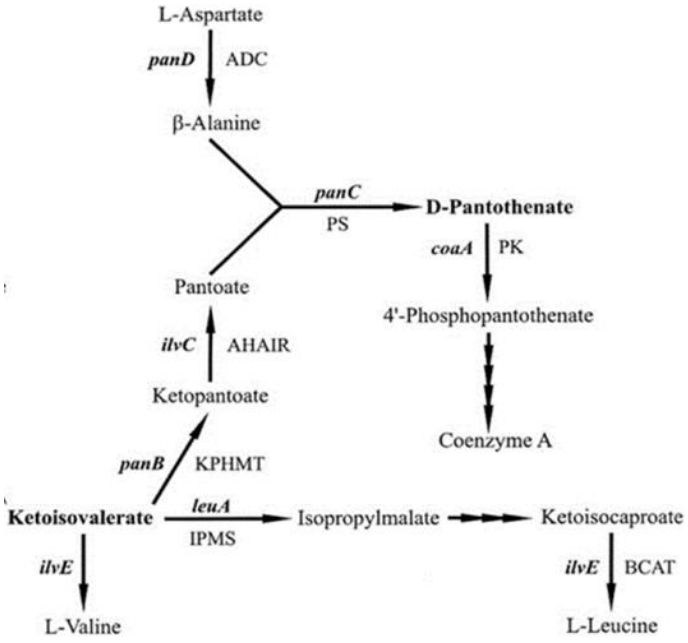


Figure 34Ketoisovalerate and its downstream products

The genes *ilvE,* and *leuA* are both present in the genome. In order to ascertain whether the amino acids were still being produced in large enough quantities for viability the *C. autoethanogenum* strain; +*pantothenate* was grown in media without either of these amino acids present. This was achieved by altering the SDMM recipe to contain separately added amino acids rather than Casamino acids normally utilised in the production of media. Media was made with and without each of the amino acids. As shown in Figure 35, cultures growing with CO as a carbon source were all capable of reaching an OD_{600} of over 1.5 within

8 days (B) and able to produce over 1000 colonies on plates with and without the amino acids present (A), suggesting that the transformed strains still contain full biosynthetic capability for the amino acids in high enough thresholds for growth.

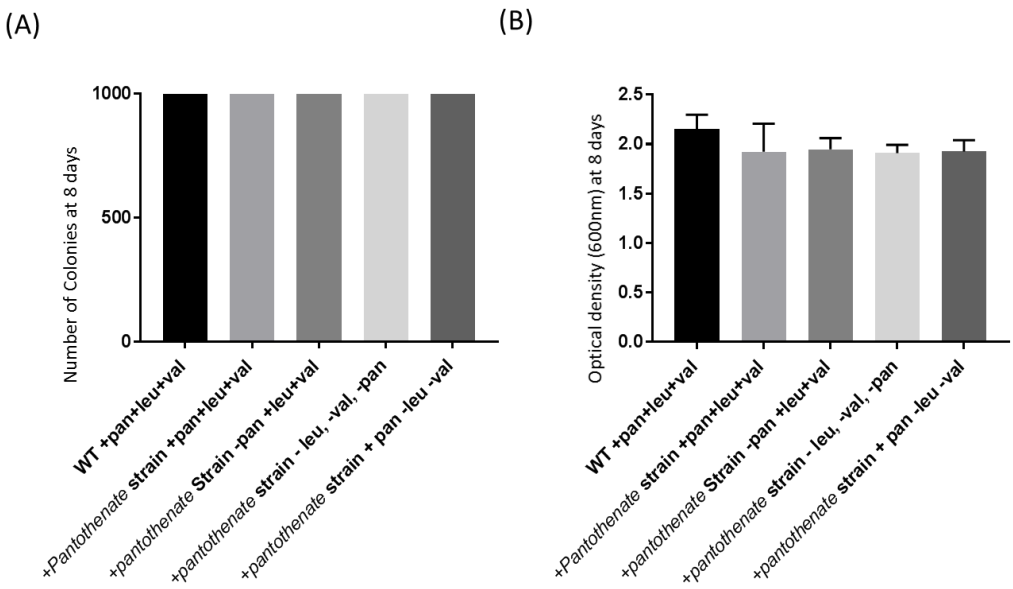


Figure 35 Characterisation of the effects of growth of C. autoethanogenum with and without valine and leucine
(A) Comparison of number of colonies on plates when transformed C. autoethanogenum is grown with and without pantothenate valine and leucine in the media when compared to the wild type. In each instance more than 1000 colonies were present on plates, confirming viability whether pantothenate was present and if valine and leucine are missing showing there is no auxotrophy replacement. (B) Comparison of terminal optical density at 8 days. (n=3) One-way ANOVA Test comparing means of +pantothenate strain in different conditions to the mean of the wild type growing with pantothenate, leucine and valine showed no significant differences in the means in any condition.

In media deficient in pantothenate, valine or leucine growth was comparable in strains which had been transformed to contain the *panBCD* genes under a strong promotor than the wild type *C. autoethanogenum* growing in full vitamin media, insinuating that the introduction of the genes did not result in a deleterious effect on the production of the amino acids.

4.3.6 Development of non-antibiotic based selection system for plasmids

The "all or nothing" capability of *C. autoethanogenum* and *C. ljungdahlii* to produce pantothenate if all three of the *panBCD* genes are present led to the

question of whether plasmids could be maintained without antibiotics present in the media as a selection pressure for the cells to keep the plasmid, but using the prototrophy of pantothenate as a selection mechanism instead. To this end 100µl of *C. ljungdahlii* and *C. autoethanogenum* transformed with pFJAL3 were plated onto PetC-MES plates without pantothenate but containing thiamphenicol. Once successful colonies were established on these plates they were picked and streaked onto plates without any pantothenate or any thiamphenicol. Colonies were established on these plates and were still viable in plates without pantothenate or thiamphenicol eight passages later, indicating that pantothenate is being produced and also the lack of pantothenate is acting to maintain the plasmid in the cells. The plasmid containing cells were then plated onto PetC-MES plates without pantothenate and with thiamphenicol and colonies were still viable (see Figure 36), so the whole plasmid must have been maintained, which was confirmed by extraction and digestion of the plasmid. As a control, in tandem, the strains were also streaked on plates with pantothenate in each passage and the plasmid remained for all eight passages if antibiotics were present in the media, but was lost after four or five passages if not pantothenate or antibiotics selection pressure was present.

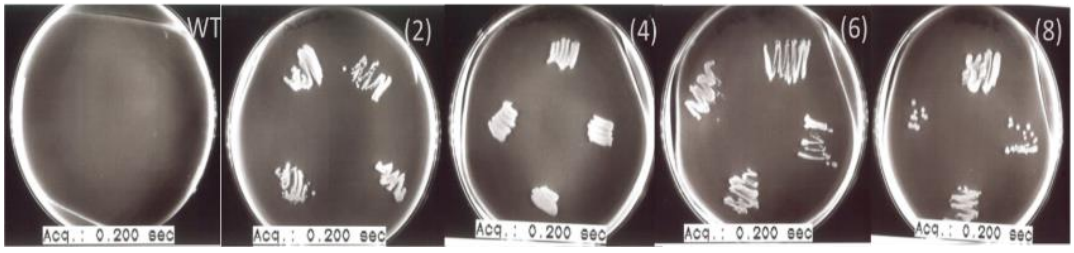


Figure 36 Alternative selection pressure plates.
Strains containing the pMTL_FJAL3 plasmid were capable of growth on plates containing thiamphenicol after eight passages on plates lacking antibiotics or pantothenate. Colonies were taken from plates without thiamphenicol but without pantothenate (as the selection pressure) after the (n) number of passages and plated onto plates containing thiamphenicol shown in the image. The wild type (WT) was not able to grow on plates without pantothenate. The pMTL_FJAL3 contains the gene for thiamphenicol resistance. The

plasmid is maintained by lack of pantothenate and is not lost, therefore after 8 passages it is still capable of growth on plates with thiamphenicol.

Further development on the work shown in table 19 and 20 shows that the strains containing pMTL_FJAL4 (*panCD*) and pMTL_FJAL6(*panB*) and the other strains containing pMTL_FJAL5 (*panBC*) and pMTL_FJAL8 (*panD*) are capable of maintaining both plasmids and their antibiotic resistance factors (thiamphenicol and erythromycin) after 8 passages in medium without pantothenate, and it could be possible to use this system as a mechanism for selection by inserting genes into either or both plasmids and maintaining the plasmids inside the organism without using antibiotics but instead using pantothenate prototrophy as the selection pressure.

4.3.7 Effects of the Reduction of Pantothenate to 10%

Following the results from chapter seven it was decided that it would be valuable to test to see if the mutant +*pantothenate C. autoethanogenum* strain would produce similar results in 10% pantothenate as seen in the bioreactor. Due to time and resource constraints it was not practical to do this experiment in a chemostat, however, bottle reactions (shown in figure 37) were run in order to ascertain the effects on the product yields in 100% and 10% of the standard pantothenate addition. To some extent, similar results were seen and there is a strong reduction of acetate production and the overall acetate: ethanol ratio when the +*pantothenate* strain is grown using 10% or 0% of the pantothenate in serum bottles, however, the production of ethanol is best when the strain is grown in 0% pantothenate in the media possibly due to the stronger limitation effects discussed further in chapter 7.

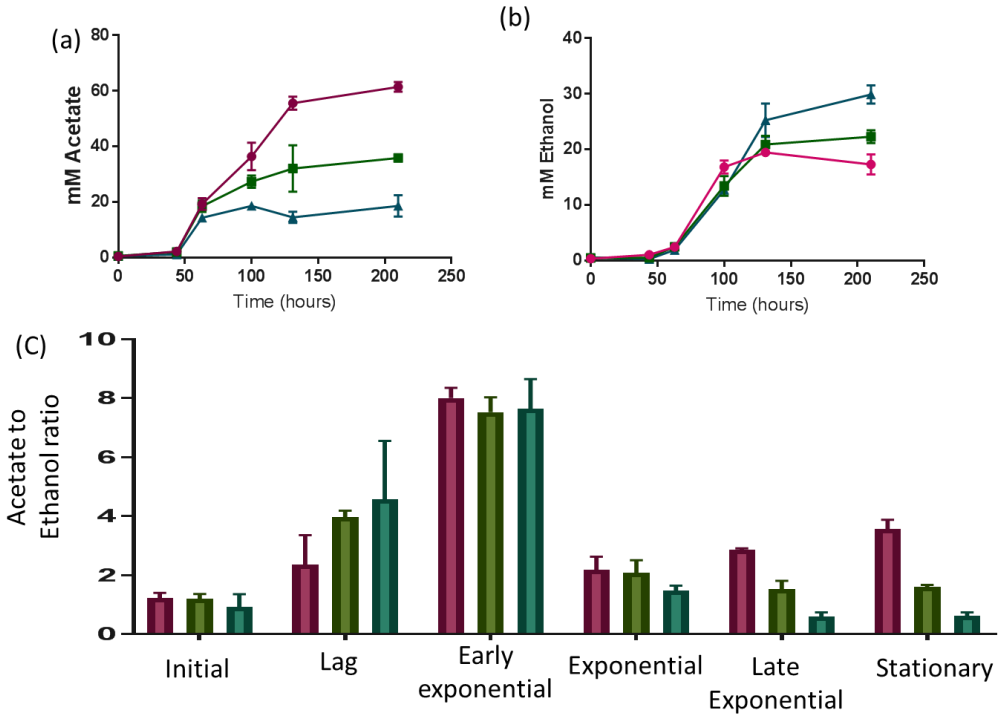


Figure 37 HPLC analysis of samples from +pantothenate strain growing in 100%, 10% and 0% standard pantothenate addition.
(A) Acetate production (B) ethanol production (C) acetate: ethanol ratio. Pink: 100% Green: 10% Blue 0%

4.4 Discussion

In this chapter we have presented a range of data to support the theory that the introduction of the *panBCD* gene cluster from *C. acetobutylicum* restores prototrophy for pantothenate production in *C. autoethanogenum* and *C. ljungdahlii*. Pantothenate is required for the production of CoA. Without CoA the Wood-Ljungdahl pathway, which is essential for the growth of the bacteria when using carbon monoxide and carbon dioxide as its carbon sources, is unable to function, and therefore, Acetyl-CoA is not formed, and so it cannot be converted into cellular carbon or to generate industrially interesting products.

4.4.1 Defining requirements for the wild type strains

In this study, building on the demonstration in chapter 3 of the essentiality of pantothenate to the viability and growth of *C. autoethanogenum* and *C.*

ljungdahlii, we have shown that the current addition of pantothenate commonly used in most media was over 10 times the amount required for the same amount of growth, and while reasonable growth was possible with just 1% of the standard concentration of pantothenate in the media, this was not as successful as 10%. If no lower limitation requirements have been established, it makes sense to add more than the required amount of pantothenate in the media in order for the vitamin to not be a limiting factor in growth, however, due to the finding that 10% of the standard addition will result in comparable growth, product ratios and viability on plate to the normal addition of pantothenate, any further addition is not necessary.

4.4.2 Restoration of prototrophy by plasmid

We reasoned it might be possible to temporarily restore pantothenate biosynthesis by the insertion of three key genes – *panBCD,* in the pathway into the organisms' genomes. Literature studies, and pathway analysis suggested that these three genes were essential for the production of pantothenate[165]. We demonstrated that the insertion of the missing genes into the organism by replicative plasmid restored the biosynthetic pathway of pantothenate allowing the organisms to grow in media with and without the vitamin, to a similar degree as the wild type with standard pantothenate addition. We also demonstrated that all three genes were required for pantothenate prototrophy. However, the addition of part of the cluster and an appropriate intermediate –*panBC* and β-alanine, *panCD* and pantolactone and *pan*C and pantolactone and β-alanine in the media conveyed enough pantothenate biosynthesis for growth. Use of these components and intermediates could be used as an additional form of selection pressure. The best form of this selection would be a system based on the addition of

pMTL_FJAL4 with β-alanine in the media instead of pantothenate as only cells containing the plasmid would be able to produce pantothenate and therefore grow. The pMTL_FJAL4 plasmid would be the best candidate as the genes *panC* and *PanD* are smaller. Therefore, larger plasmid inserts could be used whilst still maintaining good transformation efficiency without the plasmid.

4.4.3 Long term conveyance of prototrophy by augmenting the genome

After it was confirmed *panBC* and *D* were required to convey prototrophy, work was undertaken to insert these three genes into the genome of the two organisms. In order to do this, however, first a *ΔpyrE* strain had to be successfully established. A *ΔpyrE* strain for *C. ljungdahlii* was available (AM Henstra (University of Nottingham), unpublished), but work had not been done to develop a *C. autoethanogenum* mutant for the group which could be freely used. It is essential to any genetic engineering effort that a reliable method of introducing foreign DNA into the host cells exists, and the development of this strain allowed the addition of foreign genes into the genome whilst repairing the genotype to the wild type for the *pyrE* gene.

Once a successful Δ*pyrE* mutant had been developed it was possible to integrate the *panBCD* cluster into the genome by ACE whilst fixing the *ΔpyrE* gene. In both organisms, the introduction of the cluster into the genome resulted in prototrophy for the vitamin and comparable growth to the wild type growing with full amounts of pantothenate. However, the wild type was not able to grow without pantothenate in the media. Work in other organisms to overproduce pantothenate for the synthetic vitamin market frequently confirm the essentiality of these three genes and any attempt to delete them in other organisms has resulted in auxotrophy for the vitamin, whilst overexpression of the genes –

particularly the *panB* and *panC* genes results in greater production of pantothenate[164, 165, 167, 172]. The product ratios, growth profile and viability of the +*pantothenate C. autoethanogenum* strains in both CO and fructose trials showed a much greater similarity to the wild type grown with pantothenate than when the prototrophy was conveyed by plasmid. Given the reproducibility of the results, and the very minimal error bars generated in every assay, we can be reasonably confident the results are due to the biosynthesis of pantothenate by the inserted gene cluster.

Yields of the industrially interesting products acetate and ethanol show a marked difference in the production of key products when transformed strains are compared to wild type strains growing without pantothenate present, mostly due to their incapability to enter the exponential phase. Whilst the yields are not at high as the wild type growing in a full vitamin solution, transformed strains produce comparable product yields, and certainly greater than its untransformed counterparts without pantothenate. Acetate: ethanol ratios are different in conditions without pantothenate and more ethanol and less acetate is produced than the wild type produces when grown in standard concentrations of pantothenate. The theory behind this increase in ethanol is discussed in detail in chapter seven, but hinges on the implication that whilst the +*pantothenate* strains are producing enough pantothenate to allow for growth, the amount of pantothenate available to cells may still be limiting to some extent which is making the conversion of acetate to ethanol by AOR more favourable to the cell. It is interesting to note that in the absence of pantothenate in the media the ethanol production is higher.

4.4.4 Weak vs. Strong Promotors

Work was established to see if there was any particular difference in the characteristics of the transformed +*pantothenate C. autoethanogenum* strains if the gene cluster was under the control of the weak sac_fdx promotor or the strong thiolase promotor (used in +*pantothenate* strains). The growth profile and viability tests revealed that control of the introduced pantothenate genes under the weaker promotor resulted in a comparatively lower number of colonies on plates when compared to the +*pantothenate* strain with a strong promotor and a slower growth rate, however, enough pantothenate must have been produced by the cell to allow for some viability. HPLC results showed that there was a change in the acetate to ethanol ratio in cells containing the weak Sac_FDX promotor as they did not produce as much acetate, but did produce more ethanol than their wild type counterparts when grown in pantothenate free media, but was not as successful as the strain with genes under a stronger promotor. In many species it has also been shown that once requirements for pantothenate within the cell have been met, there is not a particularly greater uptake of available ketoisovalerate for the conversion into pantothenate[165]. This could explain why the results using the weak promotor (sac_fdx) instead of the strong thiolase promotor are not more starkly different.

4.4.5 Valine and Leucine interference

Whilst studies in other species have shown that a deletion or downregulation of the *ilvE* gene for the production of valine and leucine from ketoisovalerate leads to an increase in the production of pantothenic acid[167], there seems to be no discernible impact caused to the production of the amino acids – valine and leucine, as the cultures are able to grow without valine and leucine present in the

media. This is likely due to the requirement for the production of pantothenate being relatively low, so the redistribution of resources does not impact the production of these essential amino acids significantly. It has been shown in several species that the quantities of valine and leucine produced natively are much higher than required, and there is a 10 fold higher flux of ketoisovalerate towards valine rather than towards pantothenate[165] in complete pathways. Knockout of the *ilvE* genes does not lead to a 10 fold higher production of pantothenate[165]. Once cells have produced enough pantothenate to remove the limitation they do not tend to naturally overproduce pantothenate. However, we have shown that without the two amino acids in the media there is no major effect on viability or growth profile.

4.4.6 Part fixing system

Further development on the work shown in figure 36 shows that the strains containing pMTL_FJAL 5 (panCD) and pMTL_FJAL7 (panB) and the other strains containing pMTL_FJAL6 (panBC) and pMTL_FJAL8 (panD) are capable of maintaining both plasmids and their antibiotic resistance factors (thiamphenicol and erythromycin) after 8 passages without pantothenate, and it could be possible to use this system as a mechanism for selection by inserting genes into the plasmids and using pantothenate prototrophy as a positive selective marker. It is probable that this would be best completed by inserting genes into the *panD* containing plasmid pMTL_FJAL8 and transforming this into a strain already containing the plasmid pMTL_FJAL6. PMTL_FJAL8 is the best plasmid to use with any inserted genes as it is the smallest gene and therefore the overall plasmid will be smaller, potentially improving transformation efficiency, and success of the system[173]. The major advantage of this system is that the

addition of antibiotics or pro-vitamins as a selective pressure is not needed which could be a major reduction in cost.

4.4.7 Similarity of results in the two organisms

The results in almost all cases are similar for both *C. autoethanogenum* and *C. ljungdahlii*. In practically every instance they respond the same to the introduction of the different genes and the changes to growth profiles and viability in response to the changes made to the organism and the different conditions they are exposed to are in tandem. This is most likely due to the high degree of similarity in the two species[54], and also due to the relatively conserved nature of pantothenate biosynthesis. Pantothenate production and its conversion to CoA is so stable and conserved genetically across species that it has been suggested to have prebiotic origins [85]. Many different studies have found the introduction and upregulation of these three genes to have the same effect regardless of species [86, 166, 169], it follows that two so closely related species would respond in an almost identical manner.

4.4.8 Reduction in pantothente addition to 10%.

Due to the results seen in chapter seven, in which there is a marked difference in the product ratios when the wild type is grown in 10% of the standard addition of pantothenate and 100% of the standard addition, it was deemed prudent to see if this effect was also seen when the *+pantothenate* strain was grown in similar conditions in batch cultures. Experiments in the *+pantothenate* strain of *C. autoethanogenum* showed a difference in the production of products in 10% pantothenate and 100% pantothenate. For reasons elucidated further in chapter seven there is an increase in ethanol production in the *+pantothenate* strain growing in 10% and 0% pantothenate. The results shown here for the strain

growing in 10% pantothenate are more closely aligned to those seen in Steady state five in chapter 7 in which it is supposed that the pantothenate concentration is close to 10% but not quite, leading to an intermediate concentration of products in which ethanol and acetate production are nearly equal.

4.4.9 Pantothenate production

Characterisation of exact pantothenate production is generally done by the method set out in Dusch et al, 1998[164], using the pantothenate assay medium for *Lactobacillus*. However, since the intrinsic pantothenate production of *C. autoethanogenum* and *C. ljungdahlii* strains were expected to be 100- to 1,000-fold lower than that of the *E. coli* strains due to their requirements, the measurement of pantothenate production by using the Lactobacillus assay would be practically impossible, a sadly common occurrence in species with low pantothenate production[164].

4.4.10 Conclusion

In this chapter, building on work from the previous chapter, we have provided a number of phenotypic tests to elucidate the essential nature of the *panB panC* and *panD* genes for the conferral of pantothenate prototrophy. In conclusion, our results demonstrated the auxotrophy the organism had for pantothenate, and that this could be fixed by inserting missing genes into the organism via a range of synthetic biology techniques, either by conferring the genes on plasmid or inserting them into the genome and showed vastly increased performance in pantothenate free medium when compared to the unmodified wild type organisms. Crucially, we have shown that genome integrated strains possess comparable growth characteristics to the wild type strain. The finding that the pantothenate biosynthesis pathway can be restored in this organism shows the

potential for this strategy to be employed to restore other auxotrophies of the organisms.

4.5 Chapter summary

- *C. autoethanogneum or C. ljungdahlii* are capable of growing comparably in only 10% of the standard addition of pantothenate to the media
- Addition of the gene cluster *panBCD* from *C. acetobutylicum* whether conferred in the genome or by plasmid to either *C. autoethanogenum* or *C. ljungdahlii* allowed for the biosynthesis of pantothenate and allowed transformed cells to grow autotrophically in pantothenate free media
- Addition of panothenate genes does not interfere negatively with the production of valine or leucine.
- *panB, panC* and *panD* genes are all essential.
- Growth is possible with the addition of one or two of the genes from the *panBCD* cluster and a provitamin.
- The prototrophy was stable over several passages without pantothenate in the media and the exception of pantothenate from the media is a strong enough selection pressure to maintain plasmids without antibiotics.

5 Creation of Biotin Prototrophic Strains of *Clostridium autoethanogenum*

5.1 Introduction

Biotin, also known as vitamin B7, and variably throughout its history as CoEnzyme R or Vitamin H, had a convoluted discovery and it is not easily attributed to one person. The full structure and function of the vitamin was not defined until the early 1940's[174], although the name "biotin" was coined in 1935 by Fritz Kogl and Paul Gyory, and the unknown growth factor "bios" was mentioned by Wildiers in 1901[174]. It is a water soluble vitamin of the "B" Class of vitamins all of which are cofactors or coenzymes. CoEnzyme R is required by all organisms as it is involved in the production of fatty acids, cell growth and some essential amino acids. Due to its essential nature for growth and human development, since the publication of the structure in the 1940's, efforts have been made to develop a process for chemical synthesis of the vitamin for commercial production. This was made possible just nine years after the elucidation of the structure. As a result of this work, the vast majority of biotin added to media, supplemented in our foods and pharmaceuticals and used in biotinylation assays is produced by chemical synthesis, rather than harvesting the vitamin from biological sources[175].

5.1.1 CoEnzyme R

In microorganisms, biotin is key for cell growth, fatty acid synthesis and branched chain amino acid catabolism[176]. There are four main classes of enzymes which directly interact with biotin: biotin-dependent carboxylases- such as the biotin-carboxylase carriers; biotinidase, which is involved in the recycling

of biotin; biotin dependent decarboxylases and the biotin binding proteins adavin and streptavidin. It has also been suggested that biotin plays a role in some regulatory circuits such as glucokinase.[175]

There are five main biotin dependent carboxylases acting in solvetogenic species, and they are highly conserved across the domains of life. These are: Acetyl-CoA carboxylase which acts with bicarbonate and Acetyl-CoA to form malonyl-CoA which is involved in fatty acid synthesis. Pyruvate carboxylase forms oxaloacetate from pyruvate and HCO_3-. Propionyl-CoA carboxylase catalyses steps in the metabolism of amino acids, and odd chain fatty acids and can be involved in carbon dioxide fixation. Additionally, Methylcrotonyl-CoA carboxylase is also biotin dependent and involved in the degreation of leucine, and urea carboxylase acts to carboxylate urea to make allophanate which can be converted into ammonium ions and bicarbonate[86, 160, 175].

To function as a coenzyme, biotin is attached to the amino group of certain lysine residues in carboxylase enzymes. This process is known as biotinylation and requires ATP and the enzyme holocarboxylase synthetase. Biotin can be covelently bound to the biotin carboxyl carrier protein by biotin protein ligase (*bioB*)[160].

5.1.2 Biosynthetic pathway of Biotin

The generation of biotin, the enzymatic steps and the genes involved in biosynthesis, have been studied in bacteria, with a particular focus on *Bacillus subtilis[177]* – the gram positive model organism and *Escherichia coli*, the gram negative model organism[178]. There are two phases of biosynthesis of biotin.

Firstly, the generation of the pimeloyl moiety and secondly the ring assembly of the pimelate into biotin as shown in Figure 38. [179]

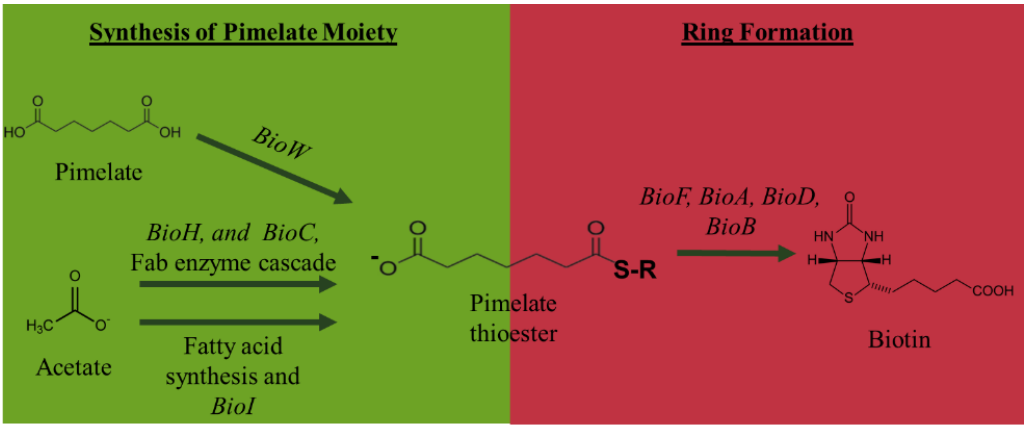


Figure 38 Different Pathways that lead to the biphasic biosynthesis of biotin.
The synthesis of the Pimelate moiety is variable but the pathway for ring formation are highly conserved.

There are several different potential routes to the production of the precursor moiety- pimeloyl. These vary between classes of bacteria and are often poorly characterised and understood[160]. As shown in figure 39; three main routes exist; (1) through the "double Fatty acid biosynthesis (FAB) enzyme cascade" from the precursor malonyl [asp], (2) from pimelate by *bioW* into pimeloyl-CoA and (3), through the conversion of long chain acyl-[acp] into pimeloyl-[acp] by *bioI[151]*.

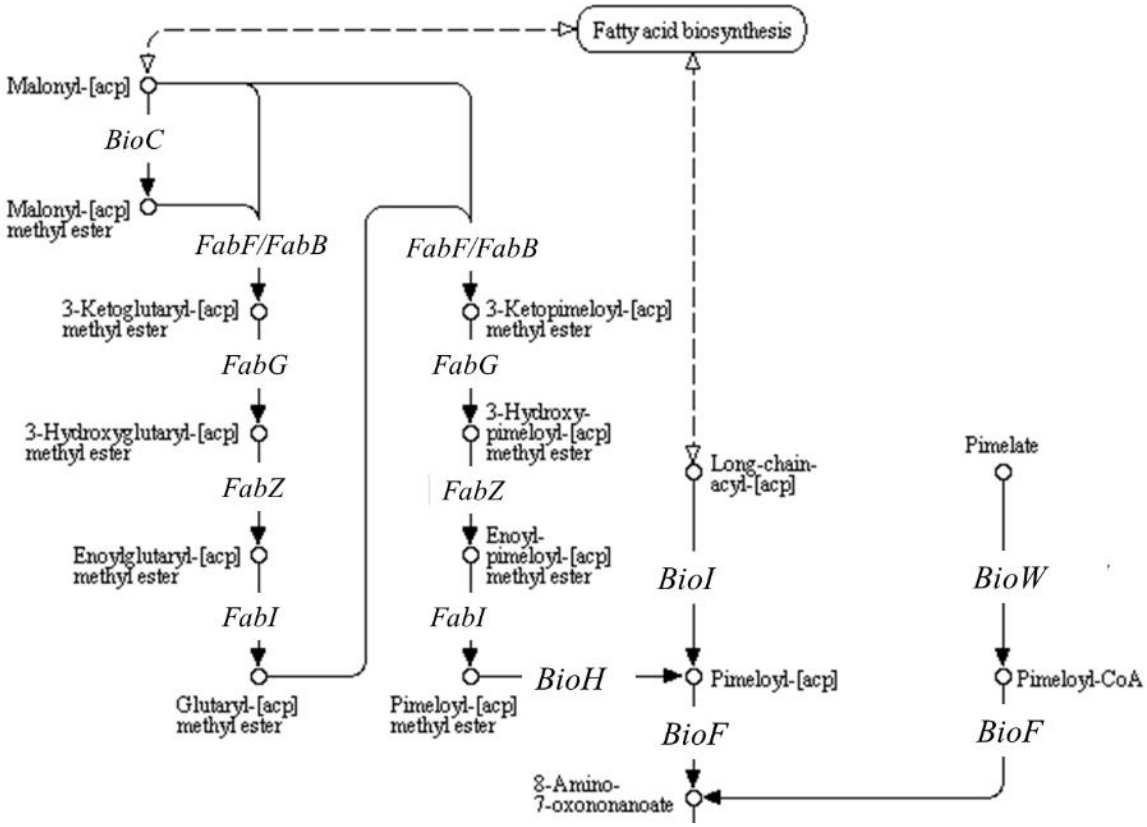


Figure 39Three main routes to the pimelate thioester moiety.
Adapted from KEGG pathway 00780 last updated 9/1/15[151]

However, after the synthesis of the pimeloyl precursor, the following enzymatic steps for its conversion to biotin are reasonably well conserved across species in bacteria. Pimeloyl is converted into 8-amino-7-oxononanoate by 8-amino-7-oxonanoate synthase (*bioF*). Then, 7, 8-diaminopelargonic acid synthase (encoded for by *bioA*) converts the 8-amino-7-oxononanoate into 7, 8-diamino-nonanoate. Dethiobiotin synthase (encoded for by *bioD*) then converts this into dethiobiotin and biotin synthase (*birA*, or *bioB*) then converts this into biotin as shown in figure 40.

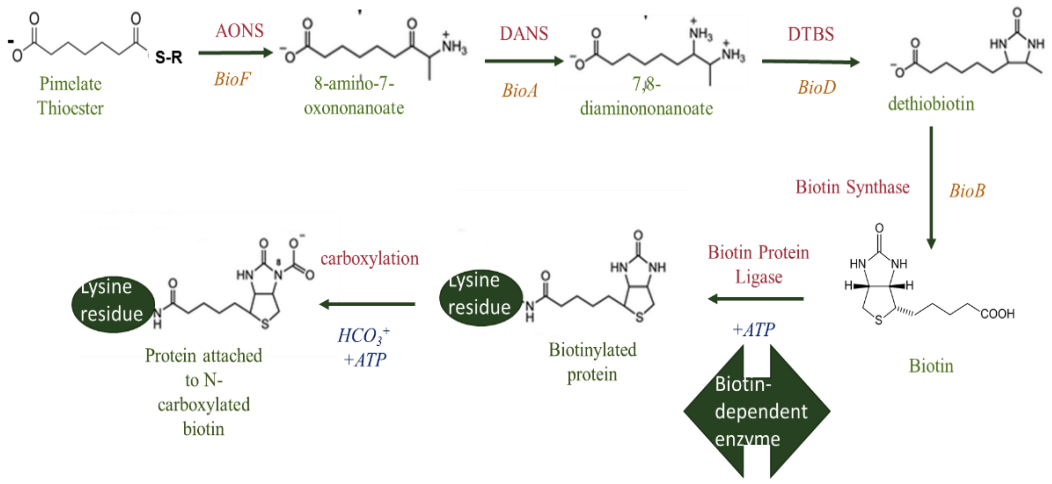


Figure 40 Pathway and associated enzymes and genes for the biosynthetic ring formation of the Pimelate thioester to biotin and its activated coenzyme forms.

BioF, BioA, BioD and BioB have all been overexpressed in species which are closely related to *C. ljungdahlii* and *C. autoethanogenum*, resulting in increased production of biotin[176]. *BioY* encodes for a biotin transport system substrate-specific component, which acts as a transporter for biotin. Many species have incomplete biosynthetic pathways for biotin, and whilst many solventogenic clostridia, such as *C. acetobutylicm* are capable of autotrophic growth, media is often supplemented with biotin to ensure normal cell growth[180]. The concentration of biotin within an organism is also augmented by a particularly effective scavenging pathway from biotin sulfoxide or biocytin[179] ("used" forms of biotin), so in many organisms if any biotin is present in the growth medium, the organism may not biosynthesize the vitamin, but merely recycle the used components, this can be energetically costly, so if biotin is present an organism may preferentially use "new" biotin[180].

5.1.3 Regulation of biotin production

Gene expression for the production of biotin is regulated at transcriptional level by the *bioB* or *birA* (homologous in function –*bioB* present in *C. autoethanogenum*) gene. The gene encodes for a unique bifunctional repressor protein as it is also a ligase for biotin to produce the holocarboxylase enzyme. Biotin protein ligase activates biotin (with ATP) to produce biotinyl-5'-AMP, which transfers the activated biotin into the biotin-carboxylase carrier protein. At the same time high concentrations of biotinyl-5-AMP repress transcription by a classic feedback loop mechanism [175].

5.1.4 Genetic capacity of Biotin Biosynthesis in *C. autoethanogenum*

In chapter 3, it was demonstrated that *Clostridium autoethanogenum* is auxotrophic for the production of biotin and unless it is present in the media, growth is impossible. The biotin biosynthesis pathway is incomplete. The genes *bioD, bioA, and bioF* are missing in the second phase of biosynthesis and lack of *bioW, bioI, bioH, bioC,* make all three pathways to pimeloyl incomplete.

5.1.5 Chapter Aims

In this study attempts were made to introduce missing genes from other organisms into *C. autoethanogenum* both by plasmid and by introduction into the genome. It was then characterised whether this resulted in viability, the effects on growth rate, and the effects on the economic product titres by comparing a prototrophic strain of *C. autoethanogenum* which had biotin biosynthesis conveyed, to wild type *C. autoethanogenum*.

5.2 Chapter Specific Methods

In many instances the methods used are identical to those outlined in chapter 4.

5.2.1 Bacterial Strains

Clostridium autoethanogenum JAI-1 (DSM 10061), was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and grown in strictly anaerobic conditions [171]. For the extraction of genes from *Clostridium acetobutylicum*, the original strain was obtained from the American type Culture Collection (ATCC strain #824). Genomic DNA from *Desulfotomaculum nigrificans* (DSMZ 14880) was obtained from the DSMZ for this project.

5.2.2 Serial dilution tests

Requirement for biotin by *C. autoethanogenum* was studied in a serial dilution experiment as outlined in Section 2.9.2. Tubes contained all other vitamins but lacked any biotin save what was added during the serial dilutions. A 1%v/v inoculum was used.

5.2.3 Growth Experiments

Growth experiments for *Clostridium autoethanogenum* were carried out as outlined in Section 2.9.3.

5.2.4 Genome insertions

In order to insert in the genome of *C. autoethanogenum* to the heterologous biotin genes, the Allele coupled exchange (ACE) system was used [139]. The *C. autoethanogenum* strain *ΔpyrE*, developed in the previous chapter, was used. Modified pMTL_CH23 (*pyrE* correct) Plasmids were then transformed into Sexpress *E. coli* electrocompetent cells as described in 2.5.7.2, and conjugated

into *pyrE* deficient *C. autoethanogenum* as outlined in 2.5.8.3. Thiamphenicol resistant, large fast growing colonies were streaked onto solid SDMM media with and without uracil to select for restored autotrophy. Genomic DNA was extracted from Uracil autotrophs, which were then PCR screened using the primers 'ACE-*pyrE*-F' and 'ACE-*pyrE*-R'. Colonies with a repaired *pyrE* locus are demonstrated by the presence of a band which is the size of the inserted genes + 2 kilo base pairs for the restored *pyrE*. The resultant PCR products were also Sanger sequenced to verify the restoration of the wild type *pyrE* genotype, with the insertion of any genes. Intermediate primers were used to ensure full read coverage over the section.

5.3 Strain Development and Characterisation

5.3.1 Further elucidation of Limitation by Biotin

Due to a lack of genes to encode for the full biosynthetic pathway of biotin, and results from chapter 3, it was determined that *C. autoethanogenum* was auxotrophic for the biosynthesis of biotin and had to obtain the vitamin from its environment. To explore the influence of biotin on *C. autoethanogenum*, the growth in liquid and solid media was examined using chemically defined media that contained different conditions. Work was also done to try to elucidate the lower limit of biotin which needed to be added.

5.3.1.1 Limiting biotin concentration for Wild Type *C. autoethanogenum*

In order to determine the limiting concentration of biotin for *C. autoethanogenum* a serial dilution experiment was conducted as outlined in section 4.3.1. As previously shown in chapter three, some cultures are able to grow initially without any biotin in the media, as there is some residual amount left in the cells,

and this, and many other organisms are inordinately effective at scavenging converted biotin and recycling it[175]. However, the residual biotin is not enough for cultures without biotin in the media to enter the exponential phase and growth levels off after 48 hours.

In the serial dilution experiments, seen in figure 41A, viability was determined if an OD_{600} of over 1.5 was reached in 8 days. Cultures that contained 100%, 10%and 1% of the standard addition of biotin were able to grow to an OD (600) of over 1.5 in 8 days. Whilst the culture growing with only 1% of the standard addition of biotin grew to over 1.5 in 8 days, the optical densities were lower than those in 10% and 100% which reached very similar terminal ODs. In the cultures growing with 0.1% and .01% of the biotin in the media growth was minimal. Results were confirmed using SDMM plates as shown in the methods, containing fructose and an increasingly lower concentrations of biotin. Plates contained the full amount of biotin (2µg/ml) and then 10%, 1%, 0.1% and .01% of the full amount of biotin. The full amount and 10% thereof resulted in a lawn, whilst individual colonies were obtained at 1%. A maximum of two colonies was formed on plates with less than 0.1% of the standard addition of biotin (Figure 41B) as we can see for both conditions – liquid and solid media growth there is comparable viability if there is 1% or more of the normal amount of biotin present in the media but below that, viability drops considerably.

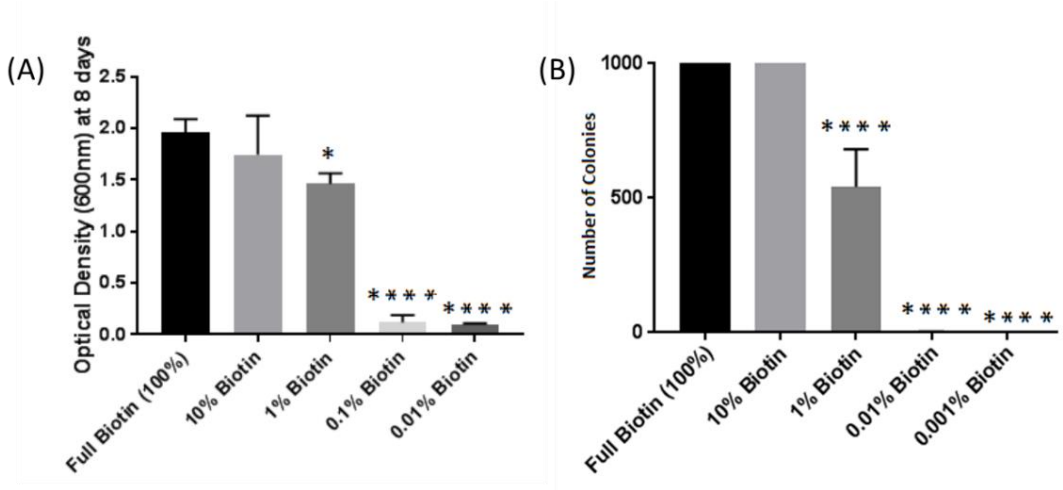


Figure 41Biotin limitation of C. autoethanogenum.
*(A) Initial and final optical density of C. autoethanogenum liquid cultures growing in different concentrations of biotin compared to the full addition (100%). After 1% of the standard addition of biotin, the growth is severely reduced. N=3. Error bars show standard error calculated for each data set. (B) Average number of colonies of C. autoethanogenum found on plates containing different concentrations of biotin. 100ul of washed culture was plated. (N=3) Asterisks indicate statistical significance difference to mean of growth in 100% standard addition of biotin. Calculated by one-way ANOVA (A) (F (4, 10) = 1.59 p = 0.0001) (B) F (4, 10) = 3.354 p = 0.0001 with a posteriori Dunnett's multiple comparisons test. ****, P<0.0001 *, P<0.05;*

5.3.2 Addition of missing genes in the pathway by synthetic biology to restore prototrophy for Biotin biosynthesis in *C. autoethanogenum*.

5.3.2.1 Bioinformatics

Using UniProt, KEGG and NCBI it was shown that the biosynthetic pathways for biotin biosynthesis were incomplete and the bioinformatics research showed us that the genes missing in the biotin pathway were *bioW, BioI, BioA, bioD, BioF, BioH, and BioC* and all other genes in the pathway were present, including the Fab enzyme cascade shown in Figure 39 and the biotin scavenging genes. Further genomic data mining showed full pathways were not present in many close relatives of *C. autoethanogenum*. Literature has shown that *C. acetobutylicum* can produce enough biotin to survive, and overexpression of *bioY, bioD BioA* and *bioB* (CA_C1360 CA_C1361 CA_C1362, respectively) genes resulted in increased biotin production. There are no genes annotated as

bioW, BioI, bioH or bioC genes labelled in *C. acetobutylicum*, however, autotrophic growth is possible in biotin free media, which is amplified when the *bioYDA* and *B* genes are overexpressed. The pathway for the production of the Pimeloyl component in C. *acetobutylicum* is unknown[176]. The addition of *bio W, bioI and bioF* from *Bacillus subtilis* did not increase the amount of biotin produced by *C. acetobutylicum* [176]. The genes *C. autoethanogenum* is missing from the biotin biosynthesis pathway (*bioD, bioF, bioH, bioC and bioA)* were all present in the species *Desulfotomaculum nigrificans* as *Desca_0053 BioB; Desca_0054 BioD; Desca_0055 BioF; Desca_0056 BioH;Desca_0057 BioC; Desca_0058 BioA.* Although *bioB* is not missing from the genome of *C. autoethanogenum* the position of *bioB* in the gene clusters in D. *nigrificans* makes it prudent to include it.

5.3.2.2 Transformation of *C. autoethanogenum* by the insertion of *BioYDA* Cluster from *C. acetobutylicum*

Promising work from the Key Laboratory of Synthetic Biology in Shang Hai has shown *C. acetobutylicum* is capable of biotin synthesis without any of the three putative biotin synthesis genes for the initial enzymatic steps present (*bioI, BioW, BioF, BioH, BioC*) for Pimelate moiety production within its genome, and is capable of growth in biotin free media without any of the putative genes for Pimeloyl production[176]. The closely related nature of *C. acetobutylicum* and *C. autoethanogenum* led to the question of whether the transformation of the strain with the genes *bioYD* and *A* from *C. acetobutylicum* which are overexpressed in [176] could relay some degree of biotin synthesis, and if not, at least with the addition of the Pimelate intermediate convey prototrophy for the second half of biotin synthesis. *C. autoethanogenum* already contains the *bioB*

gene for the enzyme biotin synthase and therefore, we attempted to convey biotin biosynthesis by artificially adding the three genes *bioYDA* from *Clostridium acetobutylicum*, which possess the three genes in a single cluster.

5.3.2.2.1 Construction of pMTL-FJAL20

The plasmid pMTL_FJAL20 was constructed in order to insert the *bioYDA* gene cluster from *C. acetobutylicum* using the pMTL modular system outlined in the introduction. The backbone vector was pMTL_82252. The biotin genes inserted into this vector to create pMTL_FJAL20 were CA_c1360, CA_c1361, and CA_c1362 were PCR-amplified using the primers BioYDAF1 and BioYDAR1 from the genome of *C. acetobutylicum*. This plasmid was subsequently transformed into TOP10 E.*coli* and confirmed using conformation method 1. The confirmed plasmid was then transformed into *E. coli* Sexpress competent cells by electroporation and then the plasmid was conjugated into the wild type *C. autoethanogenum* strain, using transformation method 2.

5.3.2.3 Effect of the genes bioYDA on growth of C. *autoethanogenum* without Biotin

Once successful transformations were confirmed, attempts to characterise the strain were made. Attempts were made to grow the strains in biotin free media. However, despite the cells containing the plasmid, and their ability to grow on plates containing erythromycin, no growth was shown in transformed strains on biotin free media, but the transformants were able to grow on media with biotin. Further tests were performed to see if the transformed strains could grow in biotin free liquid media with fructose, and also with carbon monoxide as the carbon source, but in every instance, without biotin in the media, growth never entered the exponential phase whether strains are transformed or the wild type.

Pimeleic acid was obtained from Sigma Aldrich and added to the media in plates and in liquid media as a pro-vitamin. Further characterisation attempts were made of the strains transformed with pMTL_FJAL20, and these showed successful growth, growing over 1000 colonies on plates and achieving optical densities of over 1.5 within 8 days, when grown using carbon monoxide as a carbon source whether biotin was present or not. As seen in figure 42, the cultures did not perform significantly differently when biotin was present in the media which suggests that biotin synthesis in *C. autoethanogenum* with *bioYDA* and pimelate is sufficient. Wild type cultures were not able to grow with just Pimelic acid present and so the introduced genes must be converting the pimelic acid to biotin successfully.

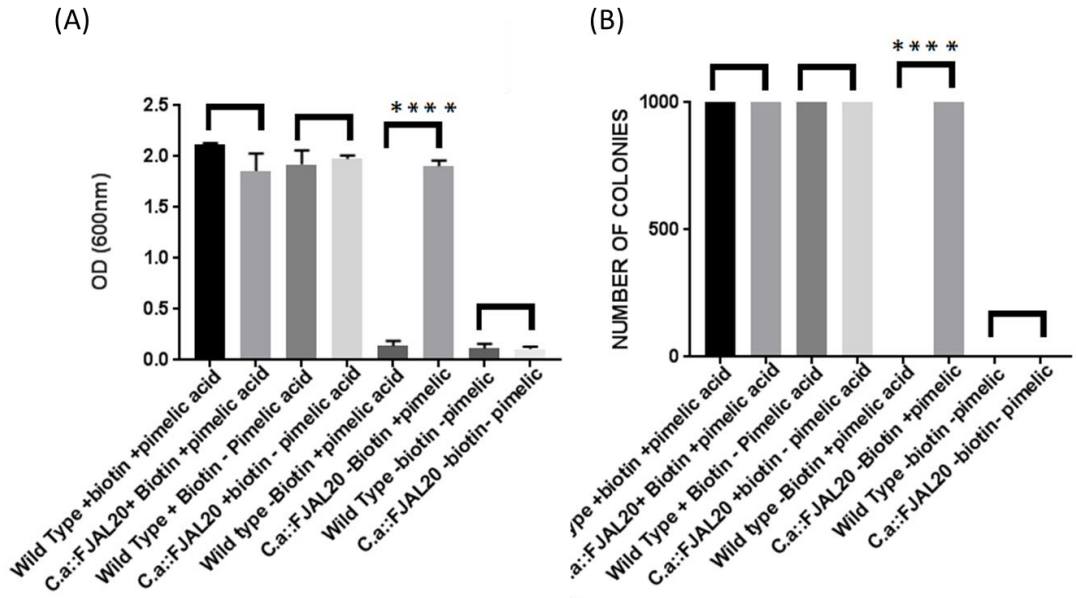


Figure 42 Biotin and Pimelate growth experiments
*(A) Final optical density of wild type and strain C. autoethanogenum transformed with pMTL_FJAL20 cultures growing in different combinations of +/- Biotin and +/- pimelic acid. Growth observed in transformed strains with just pimelic acid but not without. No growth observed for wild type if biotin is not present. N=3. Error bars show standard error calculated for each data set. (B) Average number of colonies found on plates containing different combinations of pimelic acid and biotin for transformed and untransformed strains. 100ul of washed culture was plated. (N=3) Statistical differences between the groups were calculated using the one tailed students paired t-test. There was a significant difference observed between the numbers of colonies grown on plates and the terminal optical density when the wild type was grown without biotin and with pimeleic acid when compared to the strain growing in the same conditions but transformed with pFJAL20 with a confidence interval of > 99.99% (P value< 0.0001). Error bars represent standard error of the mean of three replicate measurements. Statistical significance of differences between groups is indicated with p-values ****, P<0.0001*

5.3.3 Introduction of genes for the full biosynthetic pathway of biotin

Due to the success of growing cultures transformed with the *bioYDA* genes, which when coupled with the *bioB* gene already present in the genome and Pimelate was able to facilitate growth, it seemed appropriate to look for ways to confer full prototrophy for biotin by introducing genes which would allow the conversion of acetate to biotin, without the need for an intermediate. After extensive BLASTing and searches against the wild type genome, no obvious candidates with homology were found for *bioW, bioI, bioF, bioH, or bioC* and no closely related species possessed these genes. Any clostridial species that possessed any of the candidate genes were either recalcitrant or the genes were spread throughout the genome in multiple loci (*C. botulinum, a C. pasteurianum* strain under patent, *C. thermocellulum etc*.). The gram positive species *Desulfotomaculum nigrificans subsp. carboxidovorans* possesses the genes in a convenient cluster: *Desca_0053 BioB; Desca_0054 BioD; Desca_0055 BioF; Desca_0056 BioH; Desca_0057 BioC; Desca_0058 BioA[153]*. These genes in conjunction with the *FAB* enzyme cascade already present in the genome could fulfil the genetic requirements for biotin synthesis.

Six genes is a large number to attempt to convey into an organism and the total size of the gene cluster is 5758 base pairs. Initially it seemed sensible to split the cluster onto two separate plasmids – one with the genes *bioBDF* and one with the genes *bioHCA*, and attempt to transform both into one strain.

5.3.3.1 Creation of the plasmid pMTL_FJAL21

To insert the genes *bioBDF* from *Desulfotomaculum nigrificans (Desca_0053 BioB; Desca_0054 BioD; Desca_0055 BioF)* the genes were amplified from gDNA of the species obtained from the DSMZ using PCR and the primers

BioBDFF1 and BioBDFR1. This fragment was inserted into the backbone vector pMTL82252. The plasmid pMTL_FJAL21 was transformed into top10 *E. coli* and confirmed using conformation method 1 and transformed into *C. autoethanogenum* using transformation method 2.

5.3.3.2 Creation of pMTL_FJAL22

The creation of pMTL_FJAL22 was identical to the creation of FJAL21 with the exception that the primers BioHCAF1 and BioHCAR1 were used to amplify the genes *bioHCA* from *D. nigrificans (Desca_0056 BioH; Desca_0057 BioC; Desca_0058 BioA)* and the backbone the fragment was ligated into was pMTL 83153. Successful colonies and correct plasmids with an insert of roughly 3KB were transformed into Sexpress cells.

5.3.3.3 Transformation of *C. autoethanogenum* with pMTL_FJAL21 and pMTL_FJAL22.

After the transformation of the plasmids pMTL_FJAL22 and pMTL_FJAL21 into Sexpress *E. coli,* the 5ml of LB was inoculated overnight and the next day cultures containing cells with both plasmids were centrifuged and washed twice with anaerobic PBS and the supernatant aspirated each time. Pellet from each strain (Sexpress with pFJAL22 and pFJAL21) were then resuspended in 100µl of PBS and mixed together before a final centrifugation and aspiration step. The combined pellet was then transferred to the anaerobic cabinet and resuspended using 500µl of wild type *C. autoethanogenum,* and then recovered in 15ml of YTF media without supplementation for 20 hours, before plating onto YTF plates containing an appropriate amount of thiamphenicol, erythromycin and D-cycloserine (to select against any surviving *E.coli* cells and for both plasmids). Only colonies containing both plasmids were able to survive the selection

pressure (transformation by two donor plasmids will be subsequently known as transformation method 3). The success rate was extremely low, however after three attempts, 8 successful colonies were seen on plates. Colonies were restreaked and purified on YTF plates containing thiamphenicol and erythromycin and single colonies used to inoculate 15ml of YTF. 5ml was used to perform a plasmid Miniprep, and the plasmid was sent for Sanger sequencing which showed no deviation from the expected sequence.

5.3.3.4 Characterisation of *C. autoethanogenum*:: FJAL21+22

The transformation of *C. autoethanogenum* with the plasmids pMTL_FJAL21 and pMTL_FJAL22 led to a large increase in growth in both species in biotin free media compared to the wild types. When cultivated in biotin free media, transformed strains were able to show a reasonable growth profile, but growth was markedly slower compared to the wild type strain grown in normal media (figure 43A). This could be due in part to the strains necessity to overcome the pressure of two antibiotics which can lead to a decrease in growth rate. Successfully transformed strains showed good viability on plates with and without biotin in the media, but without biotin did not form over 1000 colonies, however, the wild type was incapable of producing any colonies in the absence of biotin (figure 43 B) The expression of *bioBDF* and *bioHCA* genes on plasmid seems to confer adequate biotin biosynthesis without supplementation of an intermediate for the cells for them to become prototrophic for biotin.

.

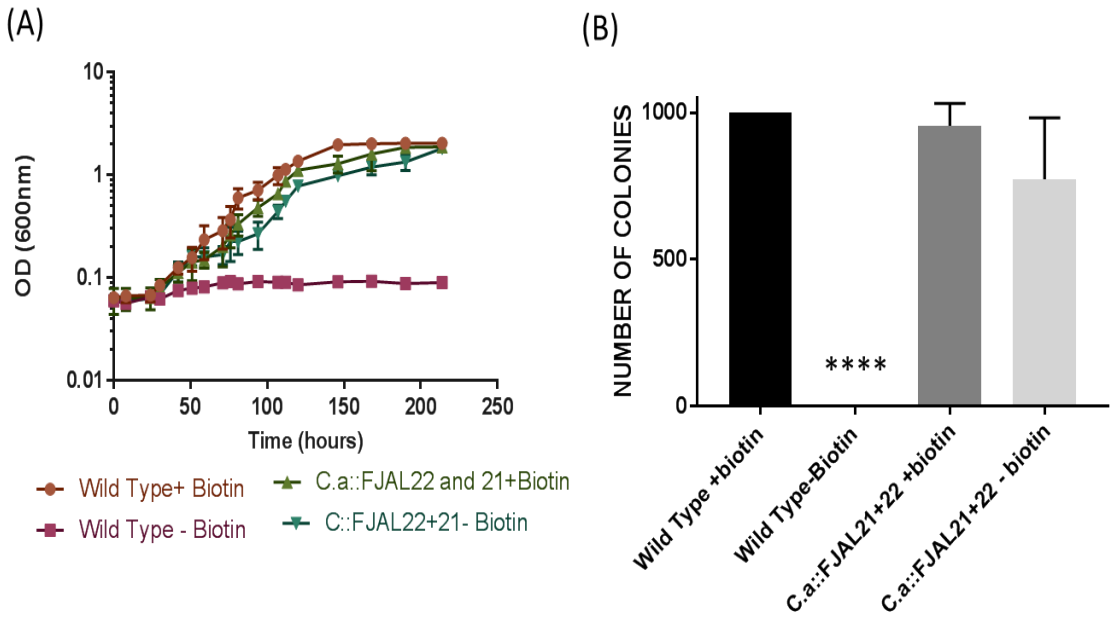


Figure 43 Comparison of performance when growing with and without biotin in the media, after the transformation of the strains with the plasmid FJAL22 and 21
*(A) Growth profile using CO as a carbon source (B) average number of colonies on plates. Statistical differences between the groups in (B) were calculated using one way ANOVA with multiple comparisons. . There was a significant difference observed between the numbers of WT colonies growing on biotin free media when compared to the wild type growing in media containing biotin with a confidence interval greater than > 99.99% (P value< 0.0001). Error bars represent standard error of the mean of three replicate measurements. Statistical significance of differences between groups is indicated with p-values ****, P<0.0001)*

Growth of C.a:: *FJAL21*+22 on plates, or in liquid media was not possible after two passages in media without biotin or antibiotics, and when replated on plates containing the antibiotic no growth was observed. This was probably due to loss of one or both plasmids in the absence of the antibiotic selection pressure. Replication of the plasmid was costly to the cells so they were lost without maintenance.

5.3.4 Conferring Biotin Synthesis genes into the genome of *C. autoethanogenum*

Although the production of enough biotin for growth was possible by transformation of *C. autoethanogenum* with genes from *D. nigrificans* on

plasmid, the growth profile and number of colonies on plates was significantly lower than the wild type and relied on two antibiotic selection pressures to maintain the two costly plasmids. As demonstrated by the results in the previous section, the 6 genes from *D. nigrificans* in conjunction with the FAB gene cascade already present in the genome, convey the ability for growth, without a pro-vitamin addition. The next stage was to try to "fix" the biotin biosynthesis pathway by the insertion of the *D. nigrificans* genes into the genome in order to convey long term prototrophy. Genes were inserted into the *pyrE* locus of a Δ*pyrE* strain of *C. autoethanogenum* by ACE as outlined in the last chapter.

5.3.4.1 Development of the plasmid pMTL_FJAL23

The transformation of such a large number of genes is difficult, and many different methods and plasmid combinations and strains were attempted without success before using a strain transformed with pMTL_FJAL23. The plasmid pMTL_FJAL23 used the backbone vector CH23. In order to ensure full translations of the inserted genes two promotors were used, the P_{Thl} promotor upstream of the *bioBDF* genes and the P_{fdx} promotor upstream of the *bioHCA* genes as shown in figure 44. To create the plasmid the "Gibson Assembly" protocol was used from New England Biolabs and their NEBuilder online generator for primers and protocol was used. However, prior to using this the *bioHCA* genes had to be extracted containing the P_{FDX} promotor. To do this the primers M13R and bioHCAR1 (with an Nhe1 site) were used to amplify the fragment from the plasmid pMTL_FJAL22, then the required PCR products were formed from the primers determined by NEB builder, and the assembly carried out as per the protocol, and ligated plasmids transformed into dh5α competent cells.

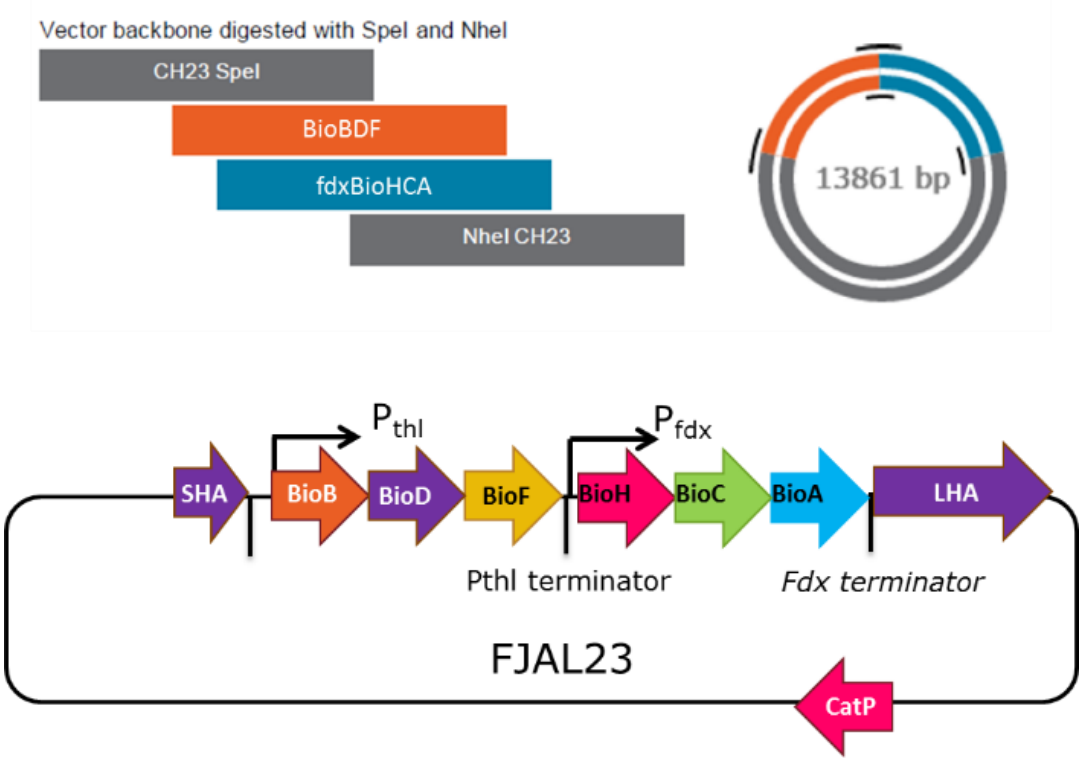


Figure 44 NEBuilder diagram and plasmid map of FJAL23 containing the bioHCA and BioBDF fragments

5.3.4.2 Transformation of FJAL23 into *ΔpyrE C. autoethanogenum*

Once the successful plasmid had been confirmed to show that a 6 kb band was present the plasmid was transformed with difficulty into Sexpress cells and then conjugated into *ΔpyrE* deficient *C. autoethanogenum* cells. The thiamphenicol resistant, large fast growing colonies were streaked onto solid SDMM media with and without uracil to select for restored autotrophy. Uracil autotrophs were then PCR screened using the primers 'ACE-*pyrE*-F' and 'ACE-*pyrE*-R'. Colonies with a repaired *pyrE* locus are demonstrated by the presence of a band which is the size of the inserted genes *bioBDF* and *BioHCA* (5769base pairs) + 2 kilobase pairs for the restored *pyrE* resulting in an expected band of around 8kb as seen in figure 45. The resultant PCR products were also Sanger sequenced to verify the restoration of the wild type *pyrE* genotype, with the transformation of large *bioBDFHCA* cluster. Intermediate primers were used to ensure full read

coverage over the 8KB section –results in supplementary materials. The successful strain was known as *+biotin*.

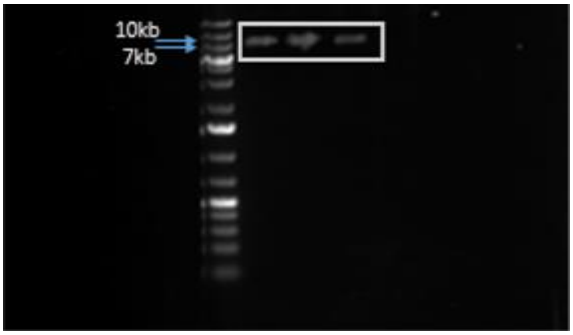


Figure 45Restoration of C. autoethanogenum pyrE mutant whilst carrying in the genes bioBDFHCA. PCR screening of complemented pyrE mutants with concomitant integration of bioBDFHCA. O'GeneRuler 1kb plus used.

5.3.4.3 Characterisation of biotin+ *C. autoethanogenum*

Once the *+biotin* strain had been developed and successfully gas adapted for situations with growth on CO, the strain was tested in conditions with and without biotin to see if the auxotrophy had been fixed and full biosynthesis for biotin was now present in the cells.

5.3.4.3.1 Plate viability tests

The *+biotin* strain showed very good viability on plates with and without biotin and performed to the same degree as the wild type grown with all vitamins (figure 46). The transformation of the strain with these genes has restored prototrophy for biotin. Transformed strains grow equally well with or without biotin on solid media plates.

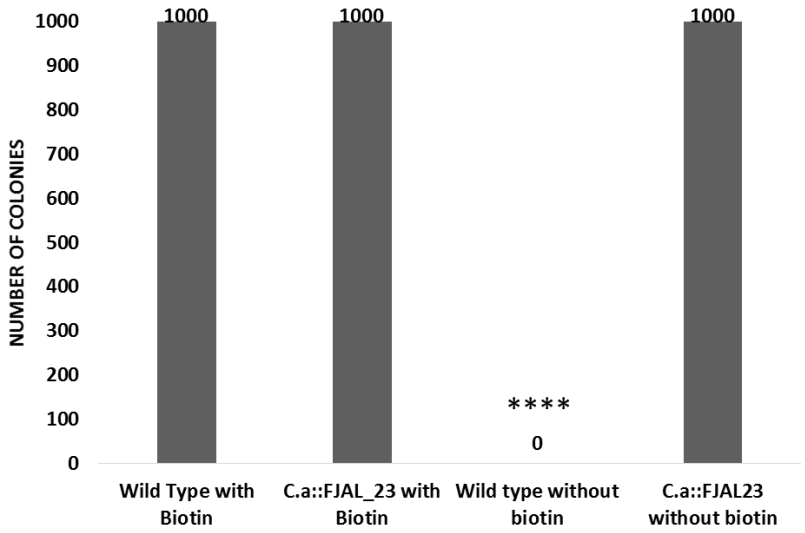


Figure 46 comparison of the average number of colonies grown with or without biotin for C. autoethanogenum or +biotin
*(n=3) 1000 = >1000 and a full lawn. Statistical differences between each of the different groups and the wild type growing with biotin were calculated using the one tailed students paired t-test. There was a significant difference observed between the numbers of colonies of wild type growing in biotin free media compared to wild type growing in media with biotin with a confidence interval of > 99.99% (P value < 0.0001). Error bars represent standard error of the mean of three replicate measurements. Statistical significance of differences between groups is indicated with p-values ****, P<0.0001*

5.3.4.4 Growth in liquid medium

The *+biotin* strain was grown in liquid media in order to characterise the growth profile. Performance of the wild type and the *+biotin* strain were similar in media containing biotin. The *+biotin* strain in biotin free media performed similarly to the wild type growing in standard media, with the exception of a much longer lag phase. Growth of the mutant was much more significant than the wild type growing in biotin free media (figure 47 A). Furthermore, the product profile of the transformed strains was comparable to that of the wild type growing in biotin containing media, so it seems there is not a significant hindrance to product production using the *+biotin* strain in biotin free media (figure 47 BCD).

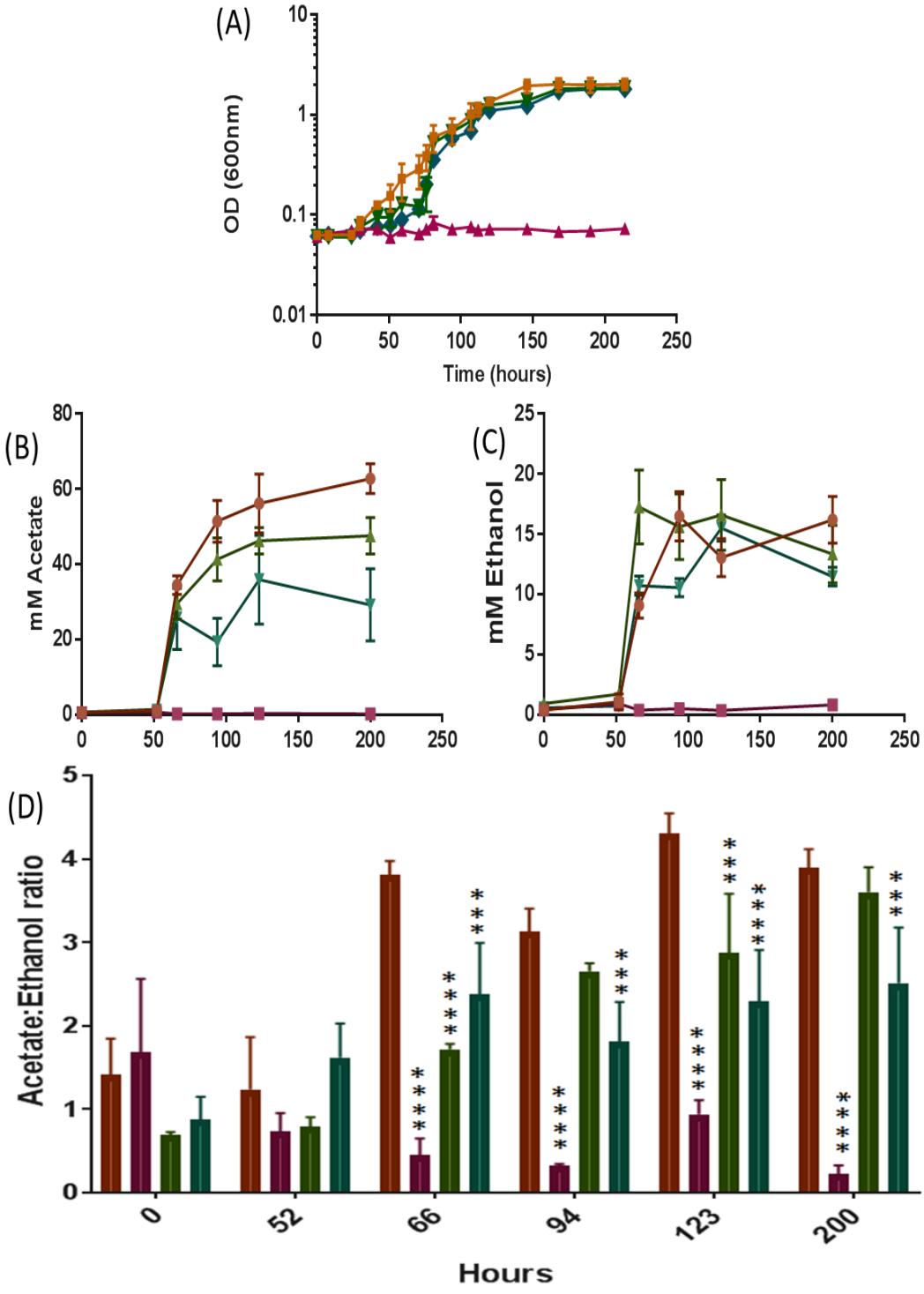


Figure 47 Characterisation of +biotin strain.
Comparison of growth profile and acetate and ethanol production of Wild Type C. autoethanogenum and C. autoethanogenum +biotin (A) Growth profile (B) Acetate production (C) ethanol production (D) acetate: ethanol ratio. Brown; wild type grown with biotin, Pink;: wild type grown without biotin, Blue: +biotin strain grown without biotin, Green: +biotin grown with biotin in media. Asterisks indicate statistical significance difference to the wild type growing in standard vitamin mixtures at each time point calculated by two-way ANOVA ($F(15,48) = 12.66$, $p < 0.0001$) with a posteriori Dunnett's multiple comparisons test

The acetate: ethanol ratio of the transformed strains in biotin containing and biotin free media and the wild type growing in biotin containing media varied quite significantly, however, the growth rate and actual acetate and ethanol production is very similar. The conferral of the genes from *D. nigrificans* into the genome seems to have successfully given rise to biotin biosynthesis in reasonable quantities for viability and good production of acetate and ethanol.

5.3.5 Effects of Biotin Reduction to 10%

Following results from chapter seven in which 10% of the standard pantothenate addition allowed for similar growth results but marked change in the product ratios it was decided to test the *+biotin* strain in 10% biotin cultures to see if growth was possible, but there was a product shift. As we can see from the results above when grown with no biotin the product yields for the *+biotin* strain is similar to those of the wild type when grown with 100% biotin. From the results shown in figure 48 below we can see that although there is a difference in yields when the *+biotin* strain is growth with 0%, 10% or 100% of the standard addition of biotin, all strains are capable of producing ethanol and acetate in reasonably high quantities in all conditions.

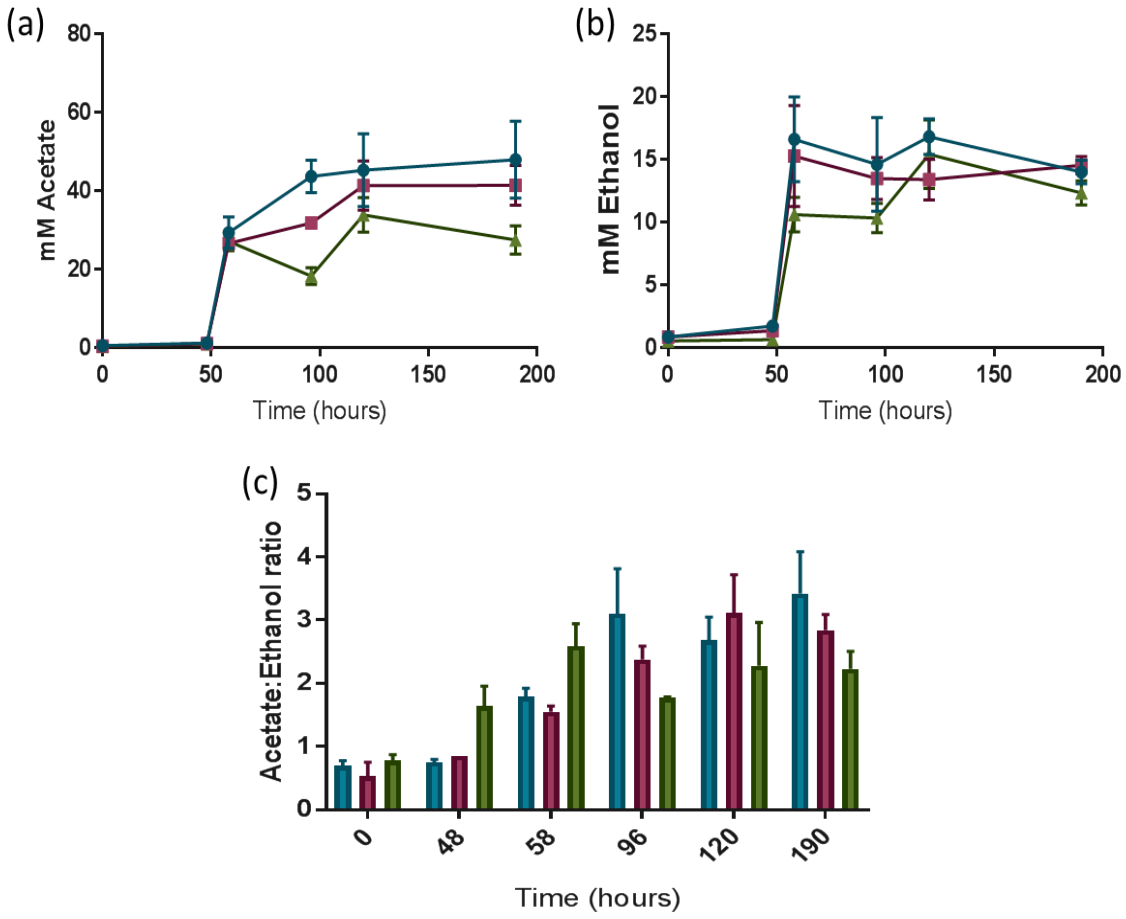


Figure 48 +biotin strain grown in 100%, 10% and 0% of the standard addition of biotin. (A) mM acetate yield, (B) mM ethanol yields and (C) acetate: ethanol ratio in each phase of growth. Blue; growth in 100% biotin, Pink; 10% biotin green: 0% biotin.

5.3.6 Biotin and pantothenate strain.

Following the successful conferral of prototrophy for biotin with the transformation of the genome with the genes *bioBDFHCA*, and the conferral of prototrophy for pantothenate from the successful inclusion of the gene cluster *panBCD*, attempts were made to produce a strain which was autotrophic for all vitamins except thiamine. In order to do this the Wild type *C. autoethanogenum* was transformed with *bioBDFHCA* and *panBCD* genes borne on plasmids. The plasmids used were pMTL_FJAL3, which contains the *panBCD* cluster under the control of a thiolase promotor and is conveys resistance to thiamphenicol and a

new plasmid pMTL_FJAL24 containing *bioBDFHCA* and a *catP* resistance gene.

5.3.6.1 Creation of FJAL24

The plasmid pMTL_FJAL24 was designed to include the *D. nigrificans* gene cluster for biotin under the control of fdx and thiolase promotors, but not to insert these genes into the genome. The plasmid backbone pMTL83251 was used and the "bigbio" fragment (*bioBDFHCA*) from pMTL_FJAL23 was removed by restriction digest. The pMTL83251 vector was used as the plasmid backbone and the backbone and insert were then ligated to form the plasmid pMTL_FJAL24, which was transformed into top10 and confirmed using conformation method 1.

Sexpress cells containing pMTL_FJAL3 and Sexpress cells containing pMTL_FJAL24 were then prepared for conjugation as stated in transformation method 3.

5.3.6.2 Characterisation of strains containing pMTL_FJAL3 and pMTL_FJAL24

Colonies capable of growth on plates containing erythromycin and thiamphenicol were very rare, but several colonies were successful and capable of growth. These successful transformants were restreaked onto further plates with the two antibiotics and colonies from the purification were inoculated into YTF containing the antibiotics to grow for extraction of the plasmid, to determine if both plasmids were there.

Transformed strains were then tested for their ability to grow on solid and liquid media with and without pantothenate and biotin in the media on plate and in liquid media to an OD of over 1.5 in 8 days. Although growth was slower, and

full lawns were not observed on plates lacking pantothenate or biotin there was still much more growth and viability with the transformed strain than the wild type strain in pantothenate and vitamin free media. This confirmed that prototrophic growth for both vitamins is possible when *C. autoethanogenum* has been transformed with both gene sets (figure 49 and 50).

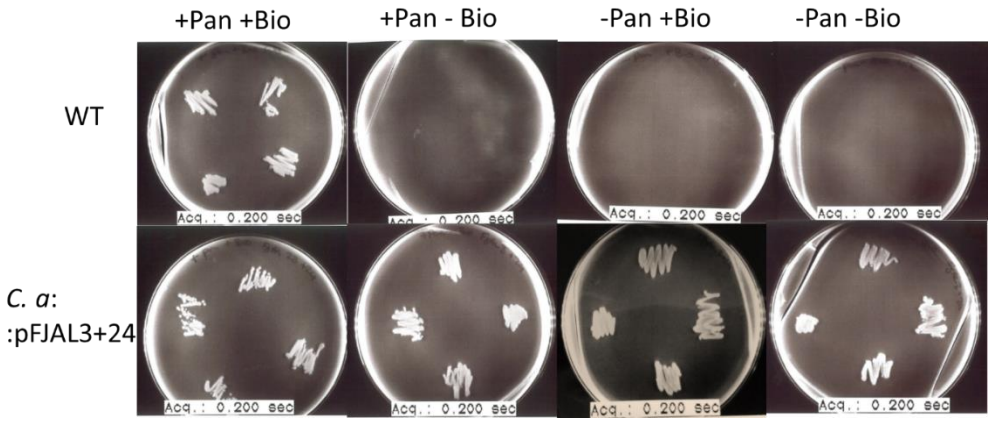


Figure 49 +biotin, +pantothenate growth with and without biotin and pantothenate.
C. autoethanogenum Wild type strain and Strain containing pFJAL3 and pFJAL24 on plates with and without biotin and pantothenate. The wild type can only grow on plates with both vitamins but the transformed strain can grow prototrophically for both vitamins.

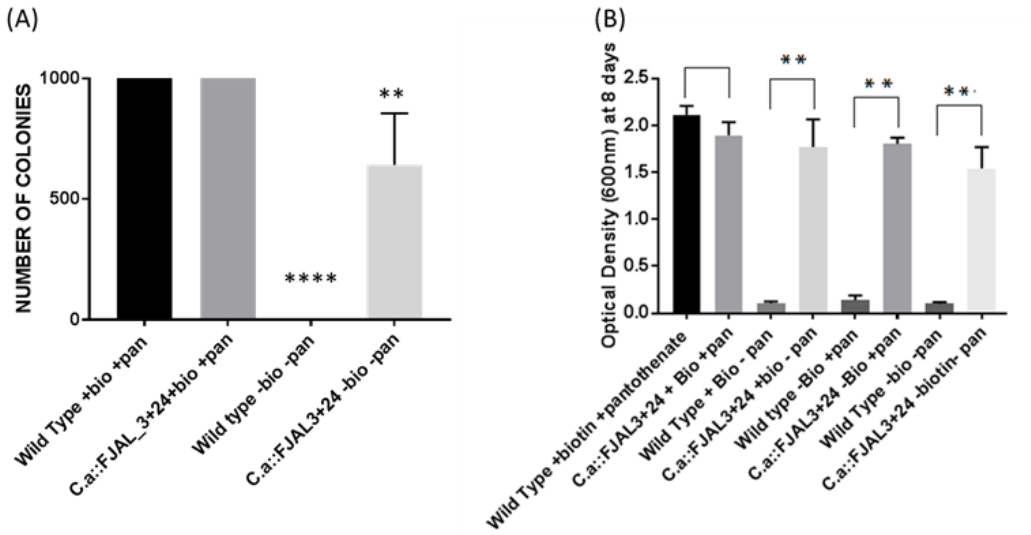


Figure 50 Graphs to compare growth with and without pantothenate and biotin in the media for wild type C. autoethanogenum and C.a::FJAL3+24
(A) Average number of colonies grown on solid media with and without biotin and pantothenate in the media. (B) Final OD for cultures growing with and without biotin and pantothenate in the media. Statistical differences between the groups in (A) were calculated using a one way ANOVA test ($F(3,8) = 3.985$, $p<0.0001$) with a posteriori Dunnett's multiple comparisons test. Asterisks indicate statistical significance difference to wild type growing with both biotin and pantothenate in the media. In (B) statistical differences were calculated using the one tailed students paired t-test. There was a significant difference observed between the optical densities shown in cultures of wild type C. autoethanogenum growing in media lacking one or both vitamins and the transformed strain growing in the same conditions. There was a confidence interval of > 99% (P value = 0.0094, 0.0011 and 0.0088 (left to right)). Error bars represent standard error of the mean of three replicate measurements. Statistical significance of differences between groups is indicated with p-values (, P<0.05; **, P<0.01)*

5.4 Discussion

In the previous chapter it was shown that by using synthetic biology it was possible to "fix" the pantothenate pathway by introducing genes from a closely related species to make the species prototrophic for the production of pantothenate and therefore reducing the number of vitamins for the species to just two – biotin and thiamine. In this chapter, we have shown the viability of fixing biotin biosynthetic pathway in *Clostridium autoethanogenum*, along with pointing out some of the limitations of doing so.

Biotin is essential for all organisms, either by biosynthesis within the organism or by scavenging the vitamin from its environment. CoenzymeR is required for the production of biotin-dependent Acetyl-CoA carboxylase, which is involved

in the first step of the fatty acid synthesis in cells[160], which in turn are vital for the production of membrane components.

In Chapter three, it was shown that the production of biotin in the wild type *Clostridium autoethanogenum* was insufficient for autotrophic growth, and it was hypothesised that this was due to missing genes from the pathway. The genes for biosynthesis of biotin are not particularly prevelant in the clostridium genus. Many solventogenic species posess some of the required genes[176], but are missing key genes for parts of the pathway. Within the genus, most of the species which contain all of the genes for full biosynthesis from acetate are either recalcitrant organisms such as C. *thermocellulum* which grow at high temperatures or pathogens such as C. *botulinum*. It is possible that this is due to the excellent biotin scavenging pathway present in many clostridial species[175, 180] or that host organisms may use limitation of biotin as a method for control to mitigate against runaway over proliferation of certain species. Whilst many clostridial species contain parts of the biotin synthesis pathway, it has been shown that the intracellular synthesis at high cell densities is insufficient and supplementation of biotin in the media is required for normal cell growth and efficient solvent production[176]. Recent efforts by the Yang Group have shown that over expression of the genes *bioYDAB* have led to hyper producing strains for biotin which lead to an increased growth rate[176].

5.4.1 Determination of requirement for biotin by the Wild Type

In order to characterise requirement for biotin in C. *autoethanogneum* and to determine if biotin was a limiting factor in cell growth in the species, a serial dilution experiment was undertaken. Subsequent experiments showed that the decrease in biotin avaliable in the media from 100% to 10% does not lead to a

large decrease in growth of cells on plates, and the biotin avalable in the media is at least ten times higher than required for growth. However, there is a slightly slower growth rate in liquid cultures than cells growing in media containing 100% of the standard biotin addition. Results not shown here indicate that increase of biotin in the media to 200% does not lead to any increase in growth rate. This is possibly due to the diverse range of bacteria which use the B-vitamin solution mentioned and the concentration of biotin present should not limit growth in any condition. Growth is also possible in media containing only 1% of the biotin generally present in the media, although the growth is slower and the optical density at 8 days is lower (average 1.951 for 100% biotin vs 1.46 for 1% biotin) there is significant growth capability at 1%. This is probably due to the effectiveness of the biotin scavenging pathway which recycles biotin for the cell allowing a very small concentration of biotin to allow for significant growth[86].

5.4.2 Introduction of the *bioYDA* cluster of genes from *C. acetobutylicum*

Biotin biosynthesis is much more complicated than pantothenate synthesis as there are more genes involved in the biosynthesis of the vitamin, and whilst pantothenate and CoA production is highly conserved, there are a variety of potential routes to biotin synthesis. Furthermore, the pantothenate pathway is almost complete in *C. autoethanogenum* with the exception of three highly conserved genes, however, there are very few of the required genes for biotin synthesis within the wild type *C. autoethanogenum* genome.

Based on work published by the Yang group in 2016, efforts were made to "fix" biotin synthesis by introducing the genes *bioYDA* from *C. acetobutylicum*. In their work they found that the overexpression of the genes *bioYDA* and *B* led to an increase in the growth rate of cells[176]. They noted that no putative genes for

bio I, F or W were present, yet biotin synthesis was possible. Further investigation showed no putative genes for *bioH* or *bioC* were present in *C. acetobutylicum* either, and therefore, no complete currently understood pathway is present in the species. Due to the degree of relatedness of *autoethanogenum* and *acetobutylicum*, initial efforts were made to determine whether the introduction of the *bioYDA* genes, coupled with the already present *bioB* genes could result in prototrophy in *C. autoethanogenum*. Successful addition of the genes for *bioYDA* on plasmid in *C. autoethanogneum* resulted in no growth, however, the addition of the intermediate -pimelic acid, led to an optical density at 8 days for the transformed strain which rivalled the wild type strain growing with normal amounts of biotin, however, no growth was possbile for the transformed strain if pimelate was not present. It seems that whilst the addition of *bioYDA* confers the ability for *autoethanogenum* to convert pimelate into biotin, there is no capability of producing the pimelate moiety from acetate, and there must be a hitherto unknown genetic element in C. *acetobutylicum* which allows for the full biosynthesis of biotin which is not present in *C. autoethanogenum*.

5.4.3 Introduction of the genes *bioBDF* and *bioHCA* from *D. nigrificans*

As full biosynthetic capability without an intermediate was not possible with the addition of genes from *C. acetobutylicum*, and due to the recalcitrance of the organisms containing all the genes in the clostridial genus, efforts were made to obtain the missing genes from an organism as closely related as possible to *C. autoethanogenum* with genes in a user friendly format. *D. nigrificans* contained the genes *bioBDFHCA* in a convienient gene cluster of roughly 6000 base pairs, and the introduction of these genes by two plasmids (pMTL_FJAL21+22) was

effective, and prototrophic growth was possible in biotin free media at a comparable rate to the transformed strain and the wild type growing in media containing biotin. It seems that the introduction of the six genes from *D. nigrificans* is enough to confer prototrophy for biotin, with a reasonable growth rate.

However, the consequences of introduction of the genes by plasmid based amplification is that growth on plates, or in liquid media was not possible after two passages in media without biotin or antibiotics, and when replated on plates containing the antibiotic no growth was possible. This was probably due to the removal of the selection pressure, and the maintenance of two plasmids without selection pressure an unreasonable burden for the cell [155, 173, 181].

Due to the lack of ability for cells to maintain both plasmids without antibiotic selection, efforts were made to transform the genome with the six genes from *D. nigrificans* using the *pyrE* repair system. It was difficult to insert the six genes and many failed strategies for the introduction of the genes were attempted, however, eventually the plasmid pMTL_FJAL23 was capable of successful repair of the deleted *pyrE* gene and the import of the six genes under two promotors. Initially, there was reticence to the idea of inserting all of the genes in one plasmid as the plasmid was very large so there was a high likelyhood of failure[173], but eventually two successful colonies contained all six genes and a repaired *pyrE* gene with no SNP's in the region.

The characterisation of the +*biotin* strain showed growth on plates with biotin free media was comparable to the +*biotin* strain and the wild type growing with

biotin and the growth profile of the +*biotin* strain in biotin free media was similar to the wild type growing with standard biotin with a similar terminal OD.

Furthermore, the product profile of the transformed strains was comparable to that of the wild type growing in biotin containing media, so it seems there is not a significant hindrance to product production using the +*biotin* strain in biotin free media. Potentially some of the lack of acetate is due in part to the consumption of acetate for the production of biotin but the quanitities produced are likely to be so low that it is not very significant, and most of the difference in acetate production is likely due to the slightly lower optical density. Although the actual production of acetate and ethanol is very different for the two strains in +/- biotin conditions the ratio of acetate and ethanol is very similar. The inclusion of the *bioBDFHCA* genes from *D. nigrificans* confers autotrophy for biotin and allows for a growth profile and acetate and ethanol production which is very similar to the wild type growing without biotin. Similar results (with slightly different genes) in yeast have been found by Gasser[182] and led to a decrease in the cost of the media.

5.4.4 Double Prototrophy

In order to decrease the potential cost of the media as much as possible, and to determine if a double prototrophy was possible, a strain was developed containing the plasmid pMTL_FJAL3 containing the *panBCD* cluster and pMTL_FJAL24 containing the “big bio“ cluster. One plasmid contained erythromycin resistance and one contained thiamphenicol resistance. The transformation of both plasmids to create this strain was very difficult and there was very low efficiencey, and often colonies could only survive on plates supplemented with one antibiotic, but not both. However, a successful

transformant was able to grow with both antibiotics present and co ntained the two plasmids with bands which contained all of the desired genes. This strain was able to grow in media with both antibiotics and in media which was both biotin and pantothenate free and had a high optical density at 8 days when compared to the wild type strain growing with both vitamins in the media. The growth profile was somewhat slower but results from the Tyo et al seminal paper showed that growth dependent on plasmid based gene amplification places a large amount of extra metabolic burden on the cell and can result in a slower growth profile[181]. However, growth was possible in plates and in liquid media using CO as a carbon source with and without both vitamins so a double prototrophy is present.

The *+biotin +pantothenate* strain was capable of growing with all vitamins excluded except for thiamine which is the only true vitamin for this strain as biosynthesis is possible for all of the other pseudo-vitamins. Growth in only 10% of the standard addition of pantothenate yielded similar results to the *+pantothenate* strain growing in 10% pantothenate.

5.5 Chapter Summary

- *C. autoethanogenum* performs similarly in 10% of usual addition of biotin to media but capable of growth in only 1%
- Addition of the genes *bioYDA* from *C. acetobutylicum* does not confer prototrophy but with the addition of pimelic acid, growth is possible without biotin
- *bioBDFHCA* genes inserted from *D. nigrificans* confers prototrophy for biotin in *C. autoethanogenum*, but unstable on plasmid
- *bioBDFHCA* genes from *D. nigrificans* inserted into the genome by *pyrE* repair method confer prototrophy, high growth profile, and similar acetate and ethanol production in biotin free media to thewild type growing with standard vitamin supplementation
- *+pantothenate + biotin* strain is capable of autotrophic growth for all vitamins except thiamine.

6 A Study of the effects of Pantothenate Limitation on *Clostridium autoethanogenum* grown in a Continuous Stirred Tank Reactor.

6.1 Introduction

In industrial conditions, *C. autoethanogenum* is able to grow chemolithotrophically on CO and hydrogen in the form of syngas, and ferment these gases using the Wood-Ljungdahl pathway into acetate, 2,3-butanediol and ethanol[53]. With fast growth or carbon limitation, the ratio of products tends towards the creation of acetate in standard conditions with ambient temperatures, pressures and pH [99]. Wild Type *C. autoethanogenum* have been reported to produce a vast array of acetate to ethanol ratios from about 20:1 [89] to about 1:1 [114]. In an industrial setting, *C. autoethanogenum* can be grown in a large continuous reactors with an annual output of 300 million tonnes of ethanol[26]. Despite the scale up, the conditions are very similar to those which can be achieved in lab scale bioreactors with a reactor volume of 2L. Previous work shown in this thesis has demonstrated that pantothenate limitation may have an effect on product formation.

The focus of work in this chapter aimed to elucidate the effect of pantothenate limitation on *C. autoethanogenum* when growing in continuous culture which was as close to industrial conditions as possible, to lead to a better understanding of the organism and how it reacts to different conditions in order to optimise the generation of desirable products.

6.1.1 Batch cultures

In experiments carried out in previous chapters, the batch growth of cultures in 50ml serum bottles has always been used. Work by Hegner et al. demonstrated

that serum bottle conditions, although in many ways not comparable to continuous reactors, were a time and resource efficient method to screening different cultivation conditions[98] and information gathered from small scale batch processes could be used to elucidate predictable and transferable conditions from serum bottles to reactor vessels. Growth of *C. autoethanogenum* is simplest in batch culture using Hungate tubes or serum bottles. However, there are significant differences between growth in batch grown cultures and continuous cultures such as those grown in CSTR fermenters. A batch culture involves an original, and most importantly, a limited amount of medium, nutrients, carbon source and other major, minor and trace elements, and is a "closed system" in terms of nutrients and waste. If the system is closed, the growth rate will eventually reach zero, either due to limitation of a consumed nutrient or due to the levels of a product becoming intolerable for growth. Because of this limitation and inhibition, the culture is always in a transient state and can never reach "steady state" (defined later). The famous "S" shaped growth curve of batch cultures (lag phase, exponential phase, stationary phase and terminal phase) is created because of the differences in biomass, and the changes the differing biomass effects on its environment. Because of this movement between conditions, it is difficult to model the metabolism of the cells because it is constantly changing in batch culture. Furthermore, it is difficult to maintain a particular set of conditions to study a state (lag, exponential, stationary, death) as the system is closed. Often study is focused on the exponential phase when resources are plentiful, but many of the products downstream from pyruvate (lactate and 2, 3-butanediol) are produced in the stationary or late exponential phase when nutrient limitation is beginning to occur. The duration of the

exponential phase is entirely dependent on the availability of the growth limiting substrate, as sooner or later something will be limiting. The typical batch culture is exposed to growth with excess substrate followed by sudden starvation, and this has a huge effect on its growth rate, but of industrial importance, also its product formation.

6.1.2 Continuous culture

Maintaining a culture by continuously adding new media, and therefore, nutrients and removing potentially inhibiting waste, has been utilised as a method for more almost 60 years. A chemostat culture is an agitated culture with completely evenly distributed nutrients and biomass which is "fed" new media at a constant rate, and harvested at the same rate, to maintain a constant volume in the reactor. Continuous culturing allows biomass growth rate to vary without changing the environment, with the exception of a known variable (in this case pantothenate concentration)[183]. It also allows for the maintenance of a specific growth rate whilst the environmental conditions change, permitting for the distinction of whether the effects seen are due to a change in growth rate or environmental conditions. Furthermore, it also allows a culture to maintain a growth rate which is limited by a substrate, so its effects can be studied, whereas in a batch culture this stage is only present transiently, before a change in growth rate. The chemostat method simplifies a culture's system and allows maintenance of a desired state in order to enable the understanding of an organism's response to a change in conditions or limitation.

A further benefit of using the chemostat method of culture is that it allows for greater monitoring of conditions within the culture. The bioreactor system used in this study (described in the methods section of this chapter), continuously

measured pH, redox, temperature, and sampled the gas mixture entering and exiting the system, in order to ensure that the conditions inside the reactor were as stable as possible, and to allow us to generate steady state conditions.

6.1.3 Steady states definition

Within this study, the term “Steady State” denotes a period in which the acetate to ethanol ratio is within 10% +/- variation over a period of more than 4 days, which is equal to 3 reaction vessel volumes of media (4.5L) completely replaced. During this period we also expect there to be a less than 10%+/- variation in the daily optical density measurements, and a less than 15%+/- difference in the yields of acetate and ethanol (higher to account for variation in valerate concentrations in the HPLC samples). Within these periods there are likely to be outlier measurements, but these are possibly due to human error, and the average of the period is used to define the steady state. In many cases, the steady state period can be much longer than the requisite 4 days, but this is the defined minimum. When there are no changes of external conditions (dilution rate, changeover of media type, errors in pH etc.) and the acetate, ethanol production, acetate to ethanol ratio, and optical density are stable over this period then the system can be deemed “stable” and in a state in which it can be compared with the highest degree of accuracy to another state in which the conditions are the same with the exception of the pantothenate amount. In this study, there are two types of steady states. The first is with full pantothenate, the second is with 10% of the standard addition of pantothenate. Attempts were made to reach a steady state with 5% of the standard addition of pantothenate but this was never achieved. Due to the nature of processing the samples we do not know that steady

states have been achieved in real time and they have to be retroactively found from calculation of the HPLC data.

6.1.4 Product shifts

Non-carbon nutrient limitation has been previously linked to a product formation shift. Limitation of nutrients prompted an alternative product synthesis - Polyhydroxybutyrate in *Cupravidens necator* and *Rhodobacter sphaeroides*[30, 53, 89, 99]. Limitation of phosphates in the media yield a product shift in C. *acetobutylicum[114]*. Nutrient limitation experiments have often focused on the attempt to shift product formation to greater yields of ethanol, however, experiments were often not carried out in fully defined media with comparative steady states. Richter et al used *Clostridium ljungdahlii* with a two stage continuous fermenter with one 1L reactor for acidogenesis and one 4L reactor with cell recycling for solventogenesis[184]. The second stage fermenter was limited by sulphur, which resulted in a change from high acetate production in the first stage reactor to high ethanol production in the second reactor. Further experiments in a single stage chemostat altered gas transfer by change in agitation speed. Increased agitation led to increased mass transfer rates which increased cell density and increased ethanol: acetate ratio[114]. However, even at the higher agitation the reactor was still carbon limited. Increased ethanol ratios only appear to be possible when non-carbon limitation is in force, also known here as "non-growth conditions", and gas shift experiments have linked increased carbon availability to increased acetate production (Dr. A. Henstra, pre-published work, University of Nottingham).

6.1.5 Pantothenate limitation in bioreactor

As first mentioned in chapter three and detailed in chapter four, pantothenate is an essential vitamin for growth and viability in *C. autoethanogenum*. Pantothenate is a precursor for the creation of CoA which is essential in the functioning of the cell. Effects of the dilution of pantothenate present in the media to 10% of the standard addition have been shown to result in slower growth and fewer colonies on plates, and dilution to 1% showed almost no growth at all. However, the current results shown were elucidated in batch culture and as exemplified, continuous culture will allow us to gain a better understanding of the effects of limiting pantothenate on the organism.

Work in other groups has shown that acetogens favour the production of solvents over acetate in "low growth" conditions such as lower pH, strong reducing agents and nutrient limitation[99]. Groups have attempted to disrupt the natural product ratios by removing yeast extract and B-vitamin solutions, and despite the finding that the cell growth was reduced, they did see an increase in ethanol production[185]. Limitation of pantothenate may well reduce the organism's ability to perform reactions requiring CoA, affecting the amount of Acetyl-CoA produced, and the production of acetate could be affected. Other studies have demonstrated a shift to higher ethanol production levels at high biomass concentrations in order to maintain ATP homeostasis, but this leads to the depletion of Acetyl-CoA reserves[114]. It seems reasonable in this case that a decrease in available pantothenate may force this limitation of Acetyl-CoA to occur earlier.

In most of the previous work in the field[185-187], pantothenate may have been an accidental limiting factor for growth and production of products in this organism, however, the exact nature of what effects are due to pantothenate limitation have not been elucidated as in general all B-vitamins or yeast extract are reduced or removed. In this study we attempt to determine the exact effects of pantothenate limitation on optical density, product yields, metabolite change of growing cultures of *C. autoethanogenum*.

6.2 Methods and materials

6.2.1 Media recipes

6.2.1.1 Initial Bioreactor media

The continuous stirred fermentation tank can be a harsh environment for a newly inoculated culture, and in order to increase the likelihood of obtaining a successfully growing culture it is important to allow the inoculum to adapt to the new environment. In order to facilitate this, the initial media (recipe shown in section 2.5.1.5) within the bioreactor differs from the standard media used, and initially there is no dilution of the media until the culture reaches a high OD (around 4.0) at which point the dilution rate is slowly increased to roughly 0.6 (in 24 hours), and dilution occurs with standard media. 1.5L of this initial media is made and autoclaved inside the sealed reactor, and degassed with Nitrogen for 4 days before inoculation. The other reason for using this media and adding components afterwards is that this media does not precipitate during autoclaving due to the low pH, and the initial media needs to be autoclaved within the reactor. Prior to inoculation the media is supplemented with sodium acetate, fumarate, and yeast extract, and 30ml of vitamin solution.

6.2.1.2 Standard media for bioreactor

Once the culture has adapted to the media and the reactor conditions it is diluted with standard bioreactor media at a rate of approximately 0.6.

The media recipe listed in methods section 2.5.1.6, was used for the duration of the experiment with the single adaptation that in “low pantothenate” conditions a separate B vitamin solution was used which contained no pantothenate, and pantothenate was added separately in desired quantities. In “Standard conditions” (0.5mg/L) the usual amount of pantothenate in the B vitamin solution was added. Media was filter sterilised in 20L batches into autoclaved sterile 20L media bottles and oxygen was removed using nitrogen for a minimum of four days. The pH was balanced using 3M NaOH or 3M acetic acid to 3.6.

6.2.1.2.1 B vitamin solution

The B vitamin solution used in the bioreactors is listed in in methods section 2.5.3. To adapt the recipe to “low/ limited conditions” an identical bottle of vitamin solution was made with the exception of pantothenate from the contents. A separate bottle of concentrated pantothenate was made and this was added separately in the desired concentration (0.05mg/ml in the reactor). The solution was balanced to pH 7.4 in order to prevent precipitation. The solution was filter sterilised into an autoclaved 2L Duran and wrapped in aluminium foil and kept in the fridge to prevent degradation by light or heat, as many of the vitamins are light and heat labile.

6.2.2 Gas composition

A gas composition of ~15ml/minute of CO and 80ml/min of Nitrogen was added throughout the experiment.

6.2.3 Bioreactor operation

The bioreactor used in this project was a New Brunswick Scientific BioFlo 115 bioreactor system (Eppendorf) and BioCommand Batch Control software (Eppendorf). The total capacity of the bioreactor was 3L, and the *C. autoethanogenum* was grown in 1.5L of media. A diagram of the reactor set up is shown in figure 51. A constant temperature of 37ºC was maintained in the bioreactor by a water jacket. The continuously stirred tank reactor was fitted with Bioflo115 baffles, Rushton impellers and open-pipe sparger. An open pipe sparger was used and adapted from a standard BioFlo115 micro-sparger. Gases were supplied through thermal mass flow controllers (SmartTrak C100, Sierra Instruments). The liquid volume and position of Rushton impellers were determined following work previously undertaken by Dr. A. Henstra (University of Nottingham) and [188]. A standard BioFlo115 head plate with 13 ports to allow the addition of media, gases, antifoam, NaOH, Na_2S, and to allow the removal of waste gases and media, as well as access for sampling media, pH and redox probes and for the addition of supplements through a sterile port using needles and syringes. A peristaltic pump (Masterflex L/S precision, Cole-Parmer) fitted with two pump-heads and Marprene pump tubing (Watson-Marlow) was used to add growth medium to the culture at a constant rate and remove excess liquid (waste) from the culture. Sodium hydroxide, sodium sulfide and antifoam were added using program controlled pumps. The signal from the ORP probe was recorded through a separate analytical transmitter (M300, Mettler-Toledo) that was connected through an analogue input/output module (Eppendorf) with the BioCommand software. Exhaust gas was cooled through a condenser that was cooled to 10º C and an 80 cm water column served to generate

backpressure to direct exhaust gases to a gas chromatograph for analysis (see below).

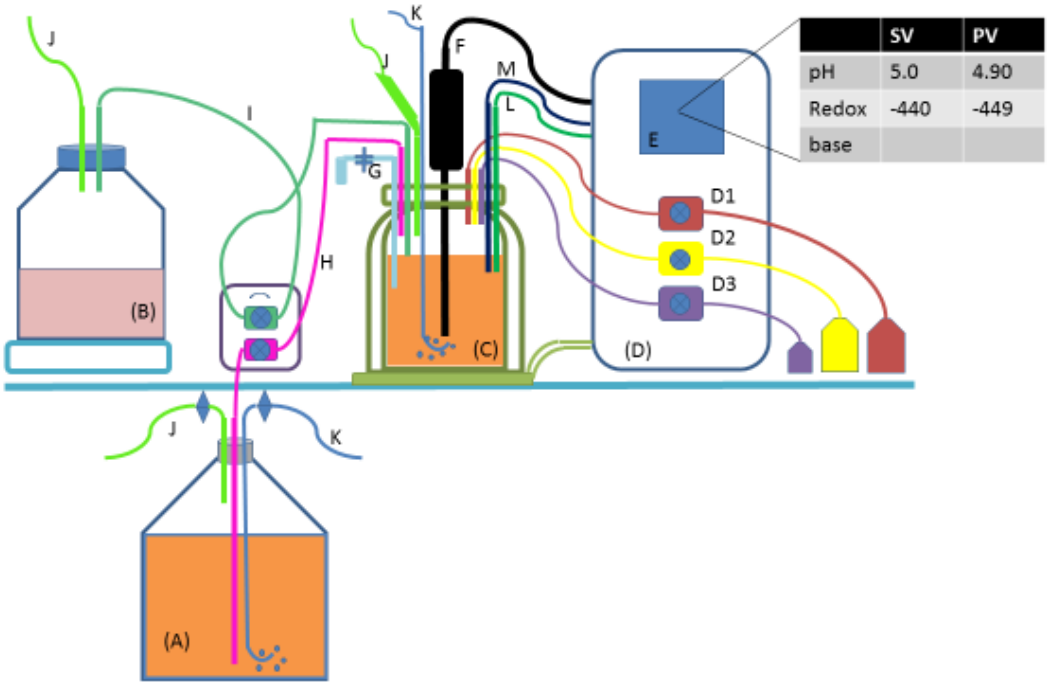


Figure 51 Diagram exemplifying bioreactor setup.
Orange indicates anaerobic media, pink indicated spent media. Blue indicated gases in, neon green represents waste gases. Black indicates agitator (F). Light green indicates fermentation vessel with water jacket (C). Red tubing (D1) indicates NaOH, yellow (D2) indicates Na2S and purple (D3) indicates antifoam. Hot pink indicates media addition (H) and the blue green (I) indicates waste media tubing. (J) Is for waste gas or gas outflow Purple (M) and green (L) lines indicate the pH and redox probes. Light blue (G) is the sampling port for the culture. The system is controlled by a BioFlo (D) computer and data recorded on a computer. (A) is the new media tank, (B) is the waste media tank, (E) is the control panel to determine pH levels, and control addition of NaOH, Na_2S and antifoam, (K) are "gas in" lines, note, the K entering (A) contains only nitrogen.

6.2.4 Inoculation

During the initial inoculation phase "initial bioreactor" media was used and sodium acetate was added to the media. CO was also added to the media and mixed with low agitation rates (200rpm) to achieve a low gas to liquid mass transfer, to avoid high partial pressures of CO as this can inhibit growth. 100ml of wild type *C. autoethanogenum* culture was inoculated into 1.5L of "initial" media from another stable reactor. The culture was grown with initial supplementation of yeast extract, sodium fumarate and sodium acetate and pH as

previously described. Once steady state was achieved, samples were taken. CO supply rates were decreased to 15 mL/min and kept constant through the rest of the experiment.

Once the pH has risen to approximately 5.7-6, (caused by the H_2S being stripped from the media and the consumption of acetate and CO by the inoculum and the production of hydrogen), the net acetic acid production lowers the pH. If the pH lowering is matched by an increase in optical density, the agitation rate is increased slowly to 600rpm, to increase gas to liquid mass transfer. Once an OD_{600} of ~3 was achieved, continuous culture began. Slowly increasing readings of hydrogen was an indication of a healthy culture.

6.2.5 Sampling methods

6.2.5.1 Gas Sampling

The composition of the gas entering bioreactors was analysed by gas chromatography (GC) using a micro-GC (Agilent, 490). Every six hours the gas composition entering the reactor was measured by the GC. On average every 30 minutes the gas composition exiting the reactor was measured. The data was collected by OpenLab EZchrom (Agilent).

6.2.5.2 Liquid media sampling

Sampling took place once per day. In order to take the sample, first 5ml was drawn off from the outlet tube and disposed of as waste and then a sample of 10ml was collected into a universal tube from the outlet tube. Back pressure from the reactor vessel allowed for collection, as media was pushed out of the unvalved outlet port when the tubing was unclamped.

Into three labelled Eppendorf microcentrifuge tubes, 1.5ml of the collected sample, was aliquoted, and centrifuged (at 4ºC, 15000xrpm,) and 900µl of the supernatant drawn off and put into a clean labelled Eppendorf and stored at -20ºC for HPLC analysis at a later date (and treated as mentioned in section 2.10.2 of methods).

500µl was aliquoted into a date labelled Eppendorf. The remaining supernatant was aspirated and disposed of and the dry pellet and separate 500µl sample were capped and flash frozen in liquid nitrogen and stored at -80ºC. LCMS samples were analysed by Laudina Safo (University of Nottingham). All metabolite data was analysed using Xcalibur (Thermo Fisher Scientific, Hemel Hempstead, UK).

6.2.5.3 Optical Density.

After the HPLC and LCMS sampling had occurred the universal tube containing the initial reactor sample was capped and shaken by hand and then 100ul was pipetted into a cuvette along with 900ul of ddH_20. The optical density at 600nm was then measured by spectrophotometer. This was carried out in triplicate as mentioned in 2.10.1.

6.2.5.4 Dilution Rate

The waste bottles for the reactors stood on weighing scales and this allowed us to calculate how many millilitres of waste media were eluted by the waste pipe per day. This weight allowed us to calculate the dilution rate (D). The dilution rate of a bioreactor was calculated by the following equation:

$$D = \frac{Medium\ Flow\ rate}{Culture\ Volume} = \frac{F}{V}$$

Equation 2 The equation for the dilution rate "D".
In steady state conditions, the specific growth rate of the micro-organism is equal to the dilution rate D. The dilution rate is medium Flow rate, F, over the volume, V, of culture in the bioreactor.

In steady state conditions, the aim was a turnover of the entire reactor volume (1.5L) in 24 hours occurred which led to a D= 0.6. If the Optical density was dropping due to the cells washing out, the dilution rate may have been lowered.

The dilution (D) of a reactor can impact the growth rate of cells in a reactor. The specific growth rate (μ) is inversely correlated to the doubling time of a culture. The doubling time (t_d) is calculated by the following equation:

$$\mu = \frac{\ln 2}{t_d}$$

When a culture is in steady state the t_d becomes a function of D in the following reaction:

$$t_d = \frac{\ln 2}{D}$$

6.2.5.5 Pilot experiment

A "pilot" pantothenate limitation reactor was run previous to this experiment. The results from this reactor were not low quality, and the reactor was run and sampled to a high standard and the experimental conditions were repeated, however, the GC data was not analysed due to a fault in the machine, furthermore, metabolomic data was not analysed for this reactor. Results for the HPLC

analysis of this reactor seem to lend weight to the conclusions seen in this reactor as the shift in product ratios is mirrored in the pilot reactor.

6.3 Results

6.3.1 Overview

The pantothenate limitation bioreactor was continuously operated at 37ºC. After the initial adaptation phase, and a suitable cell population was reached (determined by phase contrast microscopy and reaching an optical density of five) fresh "standard" media was added at a flow rate which gave a dilution rate (D) of 0.5. Which indicated a complete reactor volume (1.5L) change in 33 hours or a liquid flow rate of about 0.75ml/minute. Optical density and product formation was defined for the standard conditions. Agitation was set at 600rpm and a CO gas flow rate at 15 ml/min. The pH was set at 5.0 throughout the experiment, and maintained at 4.98 by the addition of NaOH. NaOH, was added by setting the pumps to "base" setting and the base was added until the reactor reached the appropriate pH. Agitation and liquid flow rate were lowered during "crash phases" in order to create gentler conditions in which the culture could recover.

6.3.2 Effects of Limiting Pantothenate Concentration

The reactor experiment was set up in order to elucidate the effects of pantothenate limitation on the reactor, by generating and comparing steady states from cultures growing with high amounts of pantothenate (0.5mg/ml) to those growing with low amounts of pantothenate (0.05mg/ml). Figure 52 shows the reactor conditions, optical density, HPLC data, dilution rate, and pantothenate concentration of the whole experiment with calculated steady states indicated by

the boxes. Steady state 1-4 (SS1, SS2, SS3, and SS4) were generated in conditions with a standard amount of pantothenate, and Steady state 5-8 (SS5, SS6, SS7, SS8) were produced in conditions with only 10% pantothenate. Although media was added with 5% (0.025mg/ml) pantothenate concentration, this was followed by a rapid rise in pH (an indicator of an imminent crash) and the pantothenate levels had to be returned to 10% to maintain stability in the reactor, once the reactor dropped below 10% pantothenate, the effects were seen and rectified in within hours, and so often are not seen on the graph which averages over 24 hours. Average results for different steady states are shown in table 21.

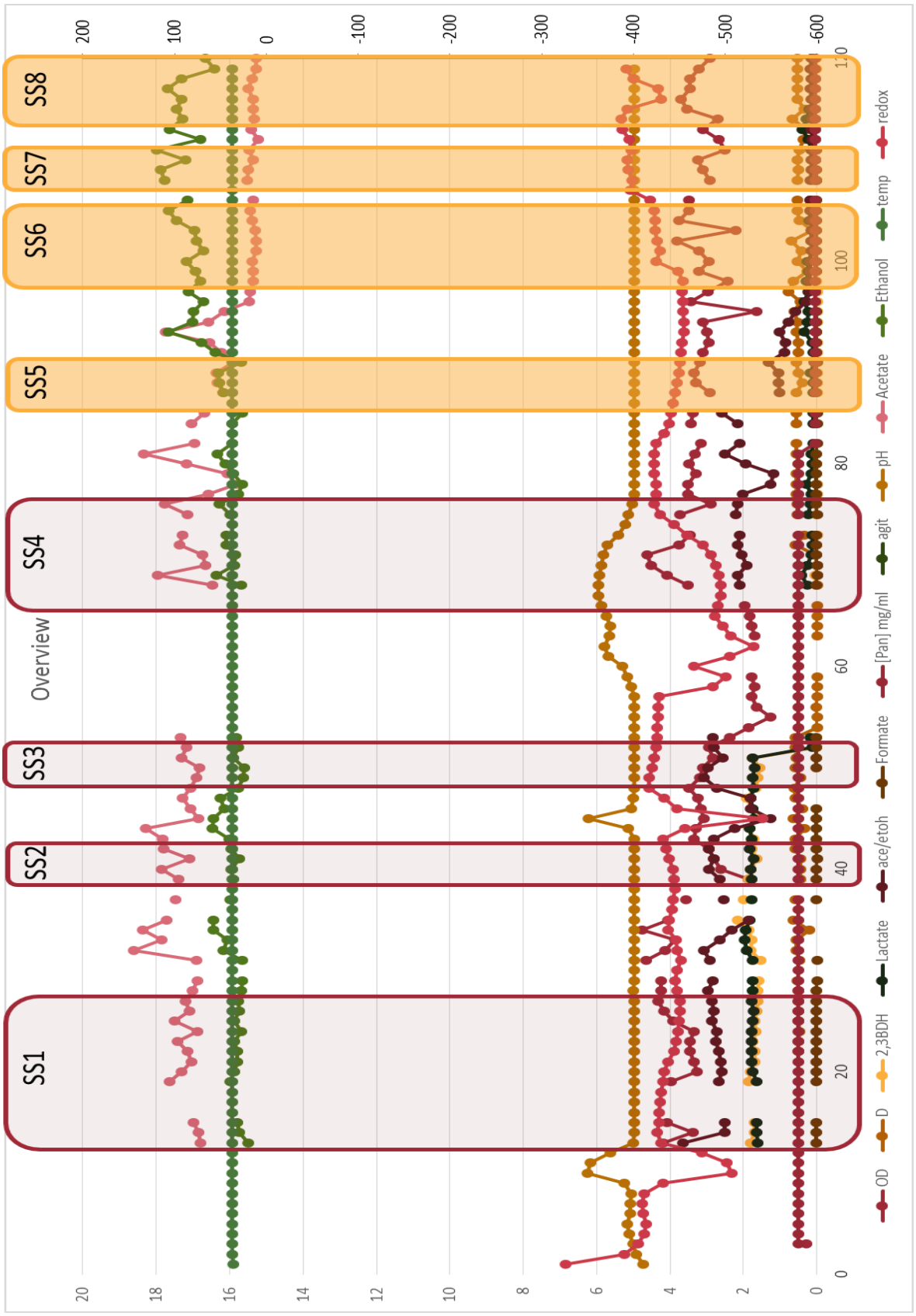


Figure 52Overview of total bioreactor data gathered over a period of 120 days.
Red boxes indicate high pantothenate steady states, yellow boxes indicate low pantothenate steady states. Left axis – OD, D, 2, 3-butanediol, formate, and ace: eth, [pan], pH, and lactate. Right axis- acetate, ethanol, temperature, redox.

	OD	D	Acetate (mM)	Ethanol (mM)	2,3BDO (mM)	Lactate (mM)	Acetate: ethanol	Formate (mM)	[Pan] mg/ml	redox
SS1	3.79	0.50	86.09	31.65	0.27	0.18	2.72	0.01	0.50	-439.16
SS2	2.93	0.52	105.74	36.74	0.21	0.18	2.87	0.01	0.50	-437.36
SS3	2.81	0.59	86.55	31.15	0.09	0.18	2.80	0.02	0.50	-423.16
SS4	3.79	0.29	84.44	40.05	0.01	0.22	2.10	0.01	0.50	-461.68
SS5	3.17	0.51	48.12	46.61	0.00	0.11	1.06	0.01	0.05	-448.93
SS6	3.27	0.45	13.96	84.82	0.06	0.18	0.16	0.01	0.05	-427.13
SS7	2.94	0.51	18.27	108.52	0.09	0.07	0.17	0.01	0.05	-395.81
SS8	3.38	0.50	13.67	82.80	0.06	0.13	0.17	-	0.05	-408.52

Table 21Averages of conditions in each steady state.
Steady states 1-4 have standard media, steady states 5-8 are in 10% pantothenate media

One of the most important aspects of this experiment was to determine the changes in the ratios of economically valuable products from the culture in different pantothenate concentration conditions in order to determine whether the drop in pantothenate concentration had a positive or negative impact on the product ratios seen when the culture is growing in different conditions over the course of the experiment as seen in figure 53, 54 and 55.

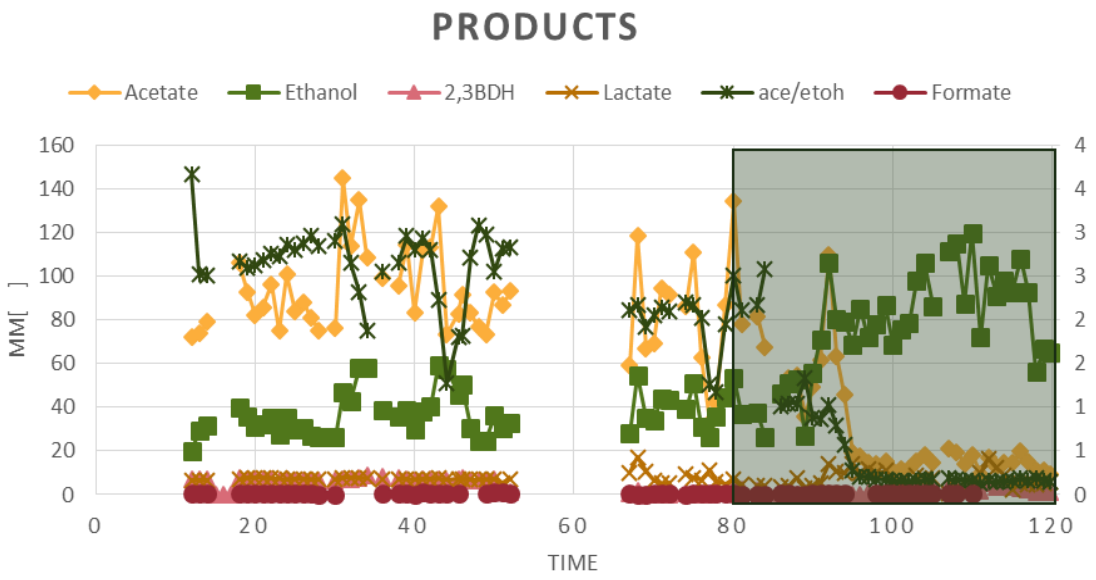


Figure 53 all products from bioreactor over time determined by HPLC. Green box indicates pantothenate limiting conditions. Acetate and ethanol on left axis, 2,3BDO, lactate, Formate and acetate: ethanol ratio on right axis. Values in mM

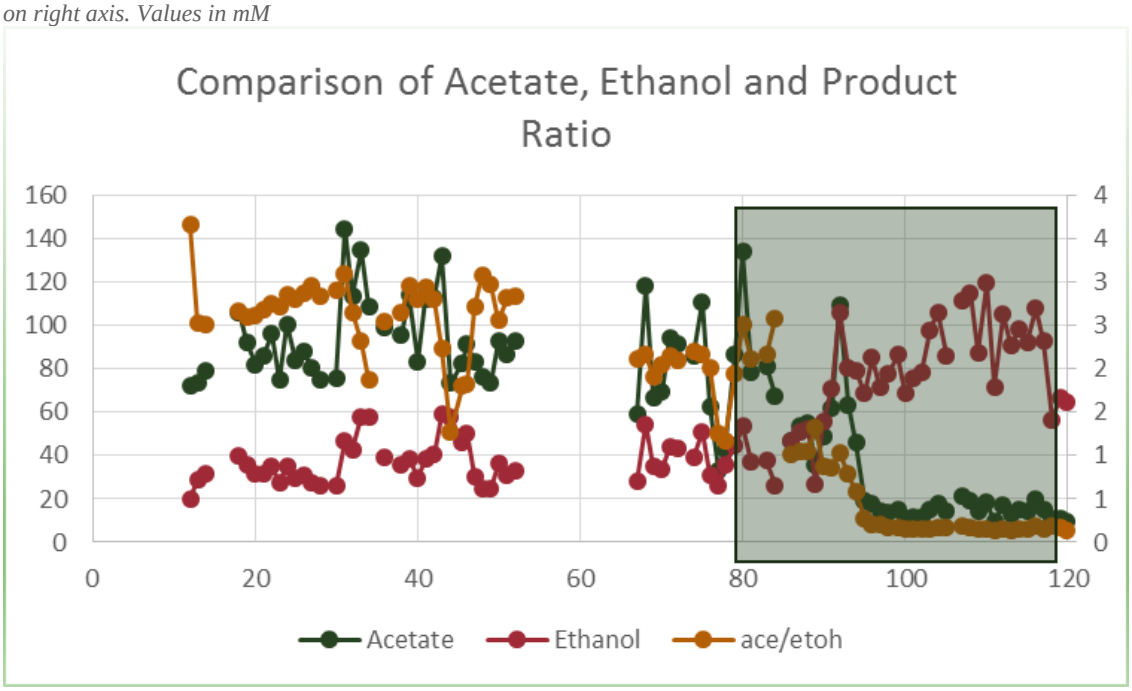


Figure 54Comparison of ethanol and acetate production and the relative ratio of products. The Green box indicates pantothenate limitation. No HPLC data gathered during major crash point between day 50 and day 65

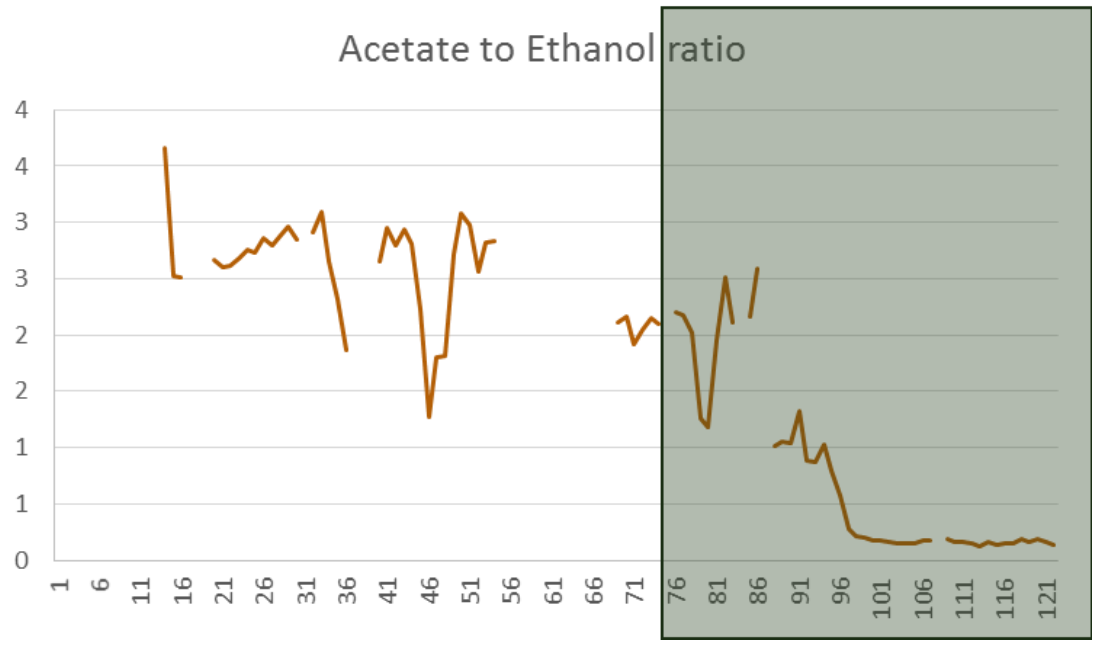


Figure 55 Acetate to ethanol ratio. Green box indicates pantothenate limitation

6.3.2.1 Gas consumption and crash indicators

Figure 56 and 57 shows the gas data from the bioreactor. Stability tends to be indicated by a high hydrogen production and CO consumption. When in steady state the gas mix entering the system was 15ml/minute of CO and 80ml/minute of nitrogen. The ingoing CO and hydrogen and the outgoing hydrogen and CO and CO_2 exiting the system were measured (figure 56). From this it was possible to calculate CO uptake (figure 57).

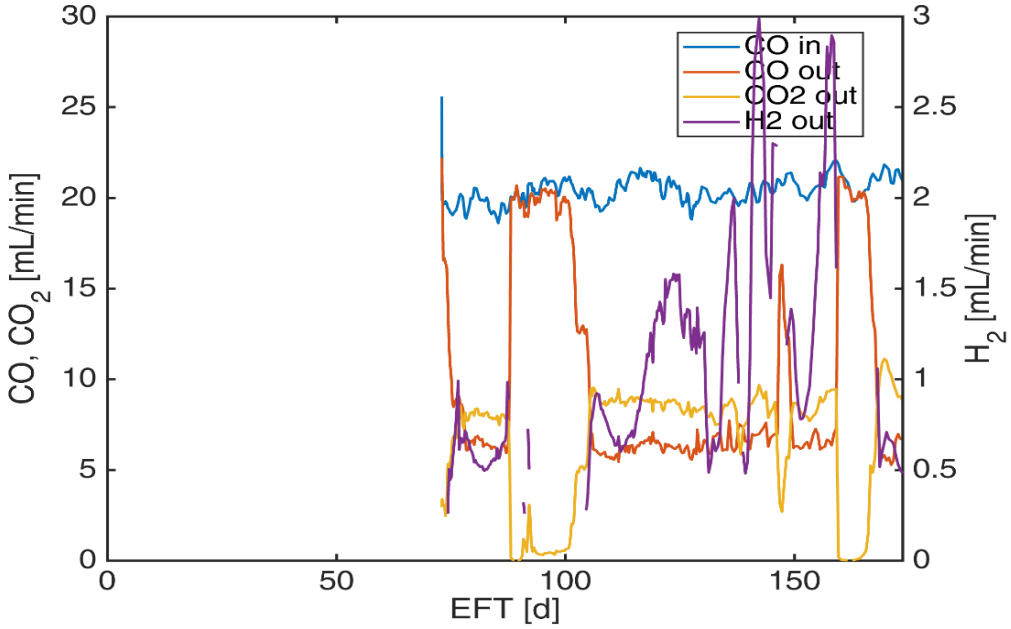


Figure 56GC analysis of Gas in and out of the bioreactor

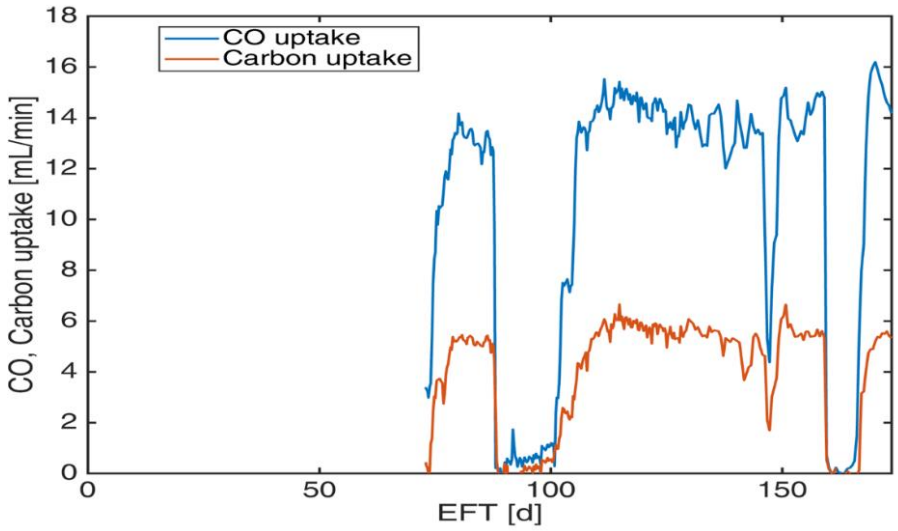


Figure 57 Calculations from GC data of CO up take and Carbon uptake from the bioreactor

6.3.3 Economically Valuable Product Changes

One of the most important aspects of this experiment was to determine the changes in the ratios of economically valuable products from the culture in different pantothenate concentration conditions in order to determine whether the drop in pantothenate concentration had a positive or negative impact on the product ratios seen when the culture is growing in the two different conditions.

Results were obtained by (close to) daily sampling and HPLC analysis. Two samples per day were analysed and averaged.

6.3.3.1 Comparison of Steady State changes

Figure 58 shows the average product concentration profile for acetate ethanol, 2,3BDO, lactate and formate, and optical densities. In steady states 1-4 with high amounts (0.5mg/ml) of pantothenate the production of acetate was high and ethanol was very low but this was reversed in steady states 6-8 in conditions with low pantothenate (0.05mg/ml), when high amounts of ethanol were produced and low amounts of acetate

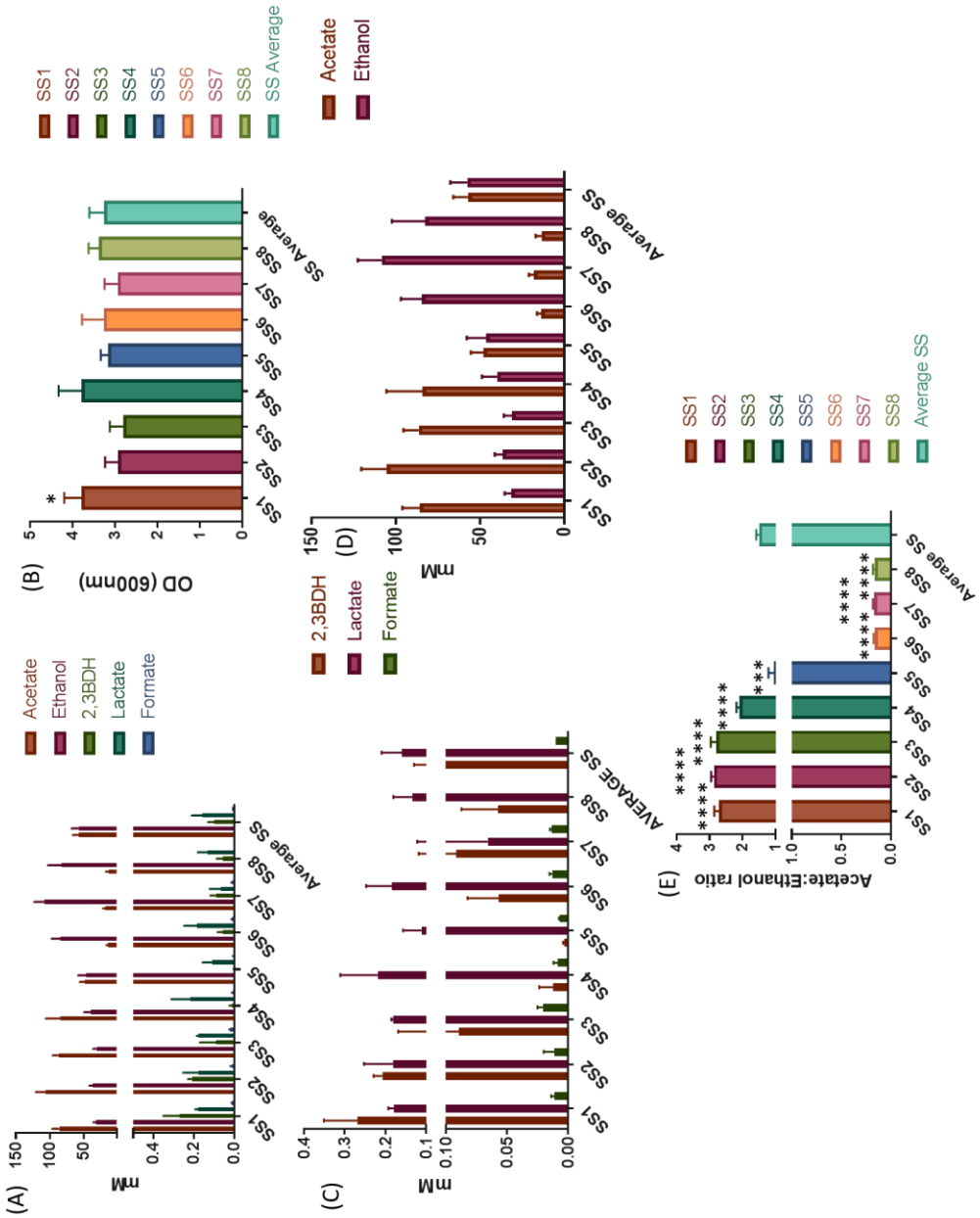


Figure 58 Comparisons of steady states.

Graph (A) shows all the different average HPLC product concentrations in each steady state. (B) Shows the optical density in each steady state. Statistical differences between the groups were calculated using the one tailed students paired t-test. There was a significant difference observed between the OD of SS1 and the average OD in Steady states with a confidence interval of > 95% (P value = 0.0148). Statistical analysis using a two tailed Students t- test shows that when compared to the overall average OD in steady states the only significantly different OD is in the first steady state, confidence intervals are 95% (C) minor economic products, for each steady state (D) shows the average acetate and ethanol in each steady state. Graph (E) shows the average acetate to ethanol ratio in each steady state. Statistical differences between the groups were calculated using the one tailed students paired t-test. There was a significant difference observed between the acetate: ethanol ratio in each steady state and the average acetate: ethanol ratio in all Steady states with a confidence interval of > 99.9% (P value <0.0001). Error bars represent standard deviation of the mean of the steady states. Statistical significance of differences between groups is indicated with p-values (, P<0.05; **, P<0.01, ***, P<0.001, ****, P<0.0001)*

Figure 59 shows the pooled averages of each of the steady states in high pantothenate conditions (SS1-4) and each of the steady states in Low Pantothenate conditions (6-8), and the comparison of the products and OD in the two conditions. Steady State 5 was excluded as the results were so dissimilar to other results in low pantothenate conditions.

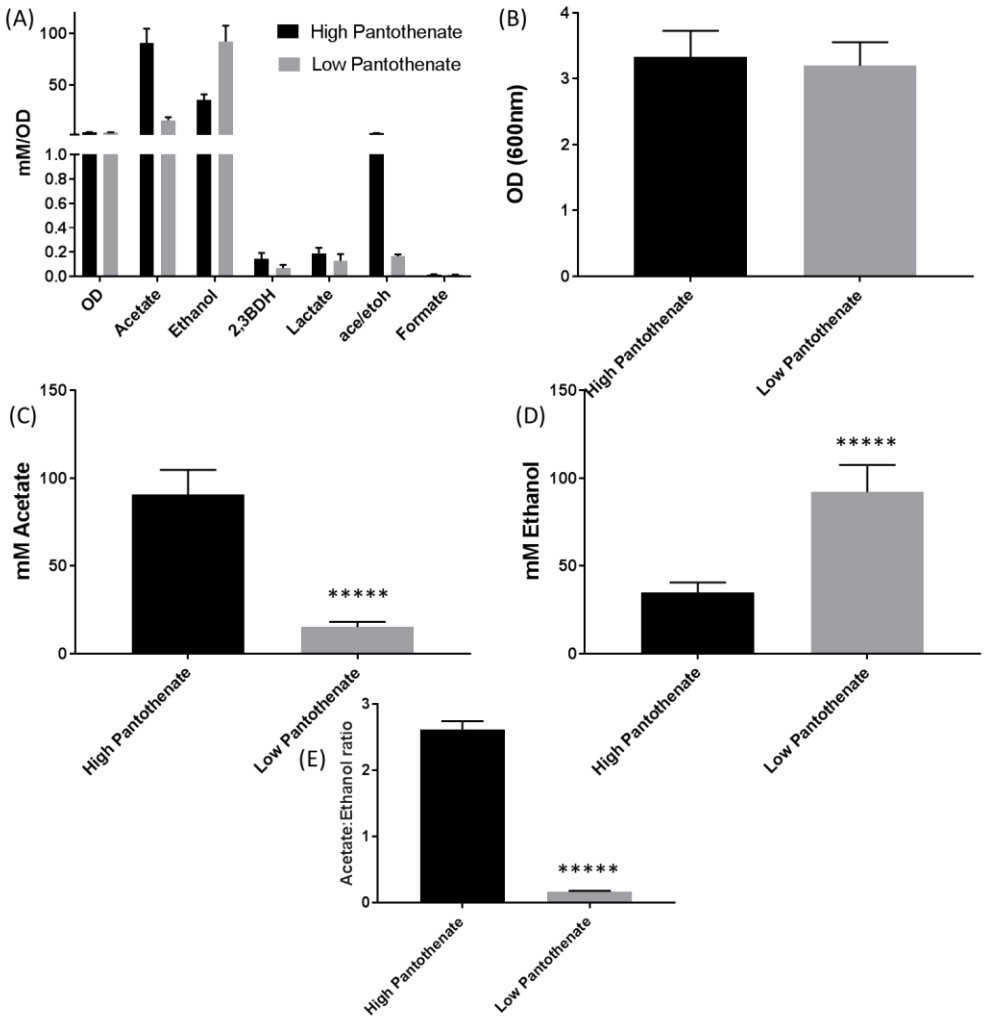


*Figure 59Pooled Steady state averages in high pantothenate conditions and low pantothenate conditions. (A) All HPLC products and optical density in the two separate conditions. (B) OD in high and low pantothenate conditions. Statistical analysis showed no significant differences between the optical densities in the two conditions. (C) Acetate production in high and low pantothenate conditions. Statistical analysis using an unpaired student's T- test showed a significant difference in acetate production in the two conditions P<0.0001. (D) Ethanol production in high and low pantothenate conditions. Statistical analysis using an unpaired student's T- test showed a significant difference in ethanol production in the two conditions P<0.0001 (E) Acetate: Ethanol ratio in high and low pantothenate conditions. Statistical analysis using an unpaired student's T- test showed a significant difference in the ratio of the two products in the two conditions P<0.0001. Statistical significance of differences between groups is indicated with p-values (****, P<0.0001)*

6.3.4 LC-MS

LC-MS analysis was carried out by Laudina Safo (University of Nottingham). Analysis was carried out with samples from four steady states: SS2 (HP1), SS4 (HP2), SS6 (LP1) and SS7 (LP2). The samples were assigned the terms HP1 and HP2 for high pantothenate (standard) addition and LP1 and LP2 for low pantothenate (10%) addition. Unfortunately, not all steady states could be analysed as samples were not always collected and steady states not determined until after the HPLC analysis was conducted. Samples were analysed in a targeted and untargeted approach.

6.3.4.1.1 Targeted LC-MS approach

The targeted approach uses quantified LC-MS results for 94 metabolites (those which could be quantified to a high degree of certainty. The targeted results also grouped well into separate steady states in PCA analysis as demonstrated by the 2D and 3D plot in figure 60). If there is any significantly reproducible effect of the pantothenate limitation then we should see a correlation of the results, and to some extent this is what we find.

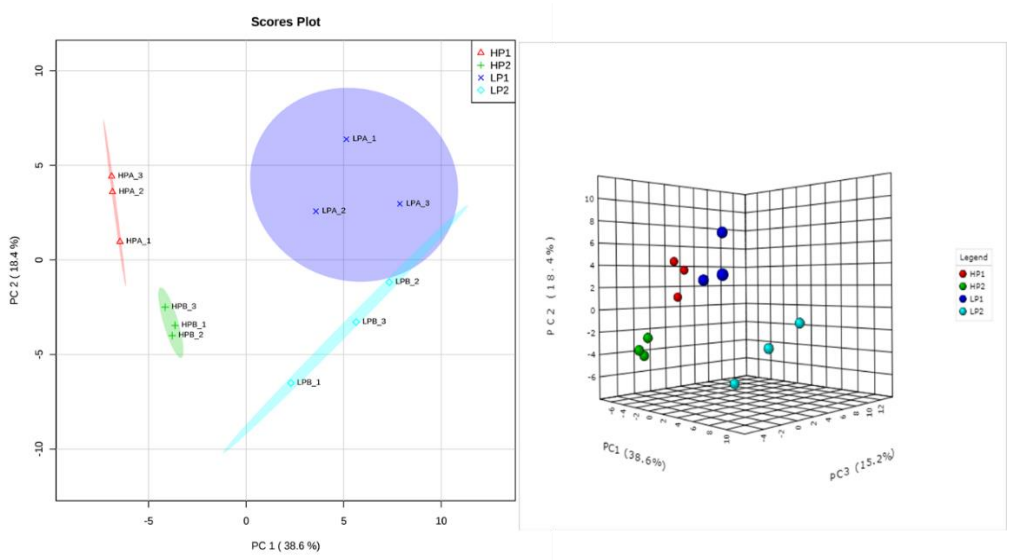


Figure 60 2D and 3D PCA of the four steady states of which LC-MS samples were run.

After the determination that the samples from each of the steady states clustered together, and there was a reasonable correlation between the two steady states from low pantothenate concentrations and the two from high concentrations, Metabaloanalyst was used to generate a clustered heat map of the changes to the 30 most variable metabolites shown in figure 61.

As we can see there is a very strong difference in the results from the high pantothenate condition and the low pantothenate condition. The overlap is particularly strong in the low pantothenate condition as one of the samples from LP2 has exchanged places with LP1 as the results were more in common with LP1 than LP2 and vice versa. Of particular interest is that in the low pantothenate condition there is much less Acetyl-CoA present in the cells than in high pantothenate conditions, possibly indicating that less is being produced due to the pantothenate limitation.

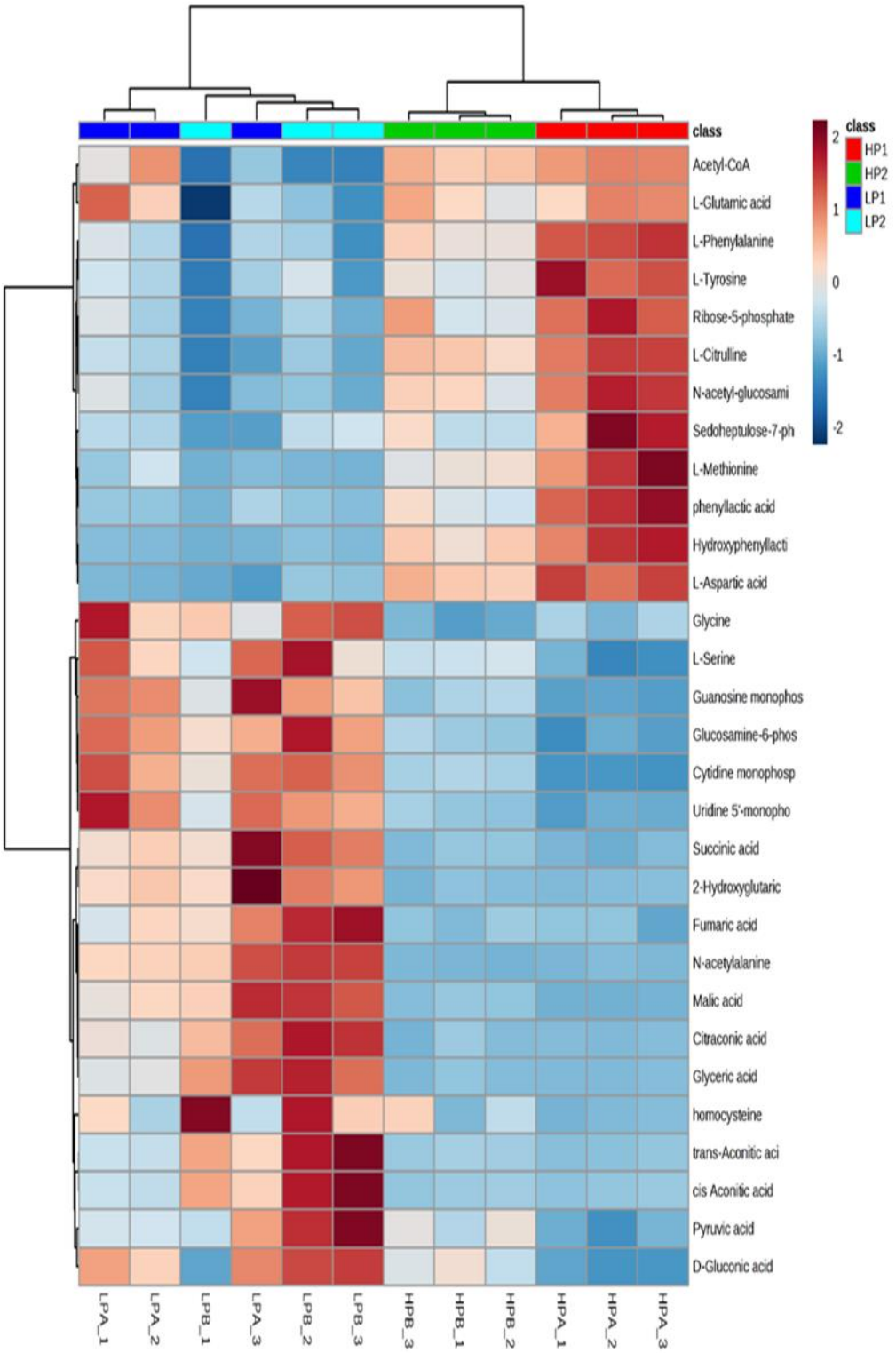


Figure 61 Clustered Heat Map analysis of 30 metabolites from C. autoethanogenum cultures in high (HP1 HP2) or low (LP1, LP2) cultures.

Furthermore, VIP targeted scores can be assigned to the 40 metabolites with the greatest changes in the cell between the two conditions as seen in figure 62, again in this we see a large change in the amount of Acetyl-CoA (10th most variation) in the two different conditions.

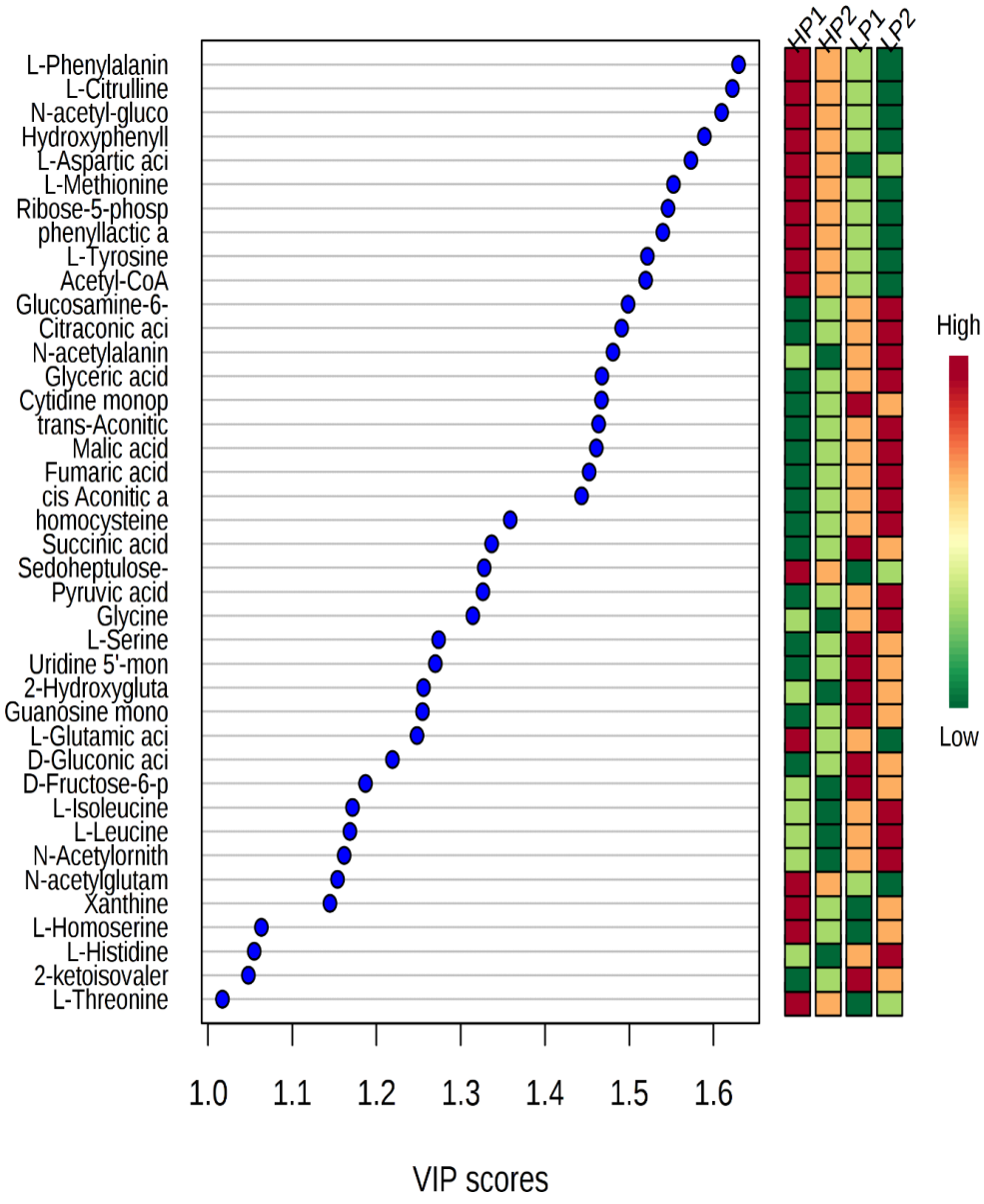


Figure 62 VIP scores of the 40 metabolites with the greatest change between the two different conditions.

6.3.5 Genomics

One prospective issue in long term experiments is the potential of strain evolution, and this probability is increased with the crashes and recovery bottlenecks seen in the experiment. This "issue" has two sides, on the one, the study was intended to ascertain the wild type response to these limiting conditions, on the other, a reactor adapted strain which is robust and tolerant to crashes, whilst also producing high levels of economic products is potentially beneficial and worthy of study. Full genome sequencing was carried out by MicrobesNG. However, despite sending samples for sequencing in April, 2017, sequences had not been returned at time of writing, due to an unforeseen backlog.

6.4 Discussion

Fermentation of carbon monoxide by *C. autoethanogenum* was successfully carried out over a period of 120 days in conditions with the standard addition of pantothenate in the media (.5mg/ml) and with only 10% of the standard addition of pantothenate in the media (0.05mg/ml). In chapter three we demonstrated a serial dilution experiment with pantothenate, in order to see at which percentage of standard addition growth was still possible. The results demonstrated that at at 10% standard addition of pantothenate, growth was still possible, at 1% although some growth occured, it was to a much lower degree than 100% or 10% pantothenate addition. During the experiment, eight steady states were achieved with four in each condition. Attempts were made to reduce the pantothenate concentration to 5% or .025mg/ml, however, this resulted in rapid increase of pH and in order to recover the culture, the bioreactor concentration of pantothenate had to be returned to .05mg/ml (10%).

6.4.1 Reactor conditions

The maintenance of a continuous culture grown for 120 days is complex and to allow for the generation of steady states, conditions must be kept as stringently as possible. The nature of the experiment was to test the response of a culture to nutrient limitation, therefore, crashes, or pre-crashes were common and certain characteristics could be recognised early to avoid a full–blown crash. Recovery from crashes was aided by reducing agitation and the addition of yeast extract, sodium acetate or sodium pyruvate. A large reduction in agitation exacerbated the crash as the change from CO partial pressures which were too high to too low was injurious to the culture. Pyruvate addition produced a spike in hydrogen production as the Wood-Ljungdahl pathway was bypassed and growth could occur by direct use of the supplemented carbon. Formate addition replaced the first step in the Wood-Ljungdahl pathway.

Reactor conditions are split into two categories –"independent" which can be altered by the experimenter such as agitiation, flow rate, gas inflow composition and rate, temperature, addition of reducing agent, antifoam, and pantothenate concentration in the media, and "dependent" conditions – cell density, pH (to some extent although this is limited by the experimenter), gas outflow composition. Due to the longevity of the experiment, during some periods, measurement of aspects of the reactor were not possible, and during the major crash periods HPLC data, and sometimes optical density were not gathered.

During the experiment the independent condtions and pH were kept as constant as possible, only changing in order to maintain a culture if it was failing. Because of the consistancy of conditions in the reactor it was possible to create steady

states where only one independent variable had changed- the pantothenate concentration.

6.4.2 Overview and steady states comparison

Figure 52 shows the results for all measured aspects of the bioreactor over the 120 day period. As shown, this resulted in a large amount of data, which was difficult to compare due to a large amount of "noise" in the reactor and variability in the measurements. In many categories such as the data for pH, or agitiation there were over 130,000 data points and around 1440 measurements per day. In order to generate data which could be compared these measurements were averaged over 24 hours. OD and HPLC samples were measured once per day. Pantothenate concentration was lowered to 10% after 76 days, and after 80 days the pantothenate concentration of the media in the reactor was judged to have been about 10%. To a large extent, the overall data for the economic products shows an increase in the ethanol produced by cells after the pantothenate is lowered and a decrease in acetate, resulting in a stark decrease in the ratio of the two products between the two different conditions, which has been shown a number of times on purpose and by accident. Formate, lactate and 2,3-butanediol is produced at a fairly constant rate thoughout the experiment. The production of hydrogen also increased in the 10% pantothenate condition but the consumption and uptake of CO remained fairly comparable. Whilst altering the concentration of pantothenate had a large impact on the product yields and ratios and on hydrogen production, there was very little impact on the optical density (proxy for rate of growth).

Steady states are defined in this study as periods in which the dependent variables are as comparable as possible, and there is a reasonable lack of variation in the

optical density, and the ethanol and acetate yields. This study had 8 independent steady states – four when pantothenate was in abundance and four when it was limited to 10%. The occurence of these states are shown on figure 52, and the comparison of the averages for different variables in this state are shown in figure 59.

The altering of the concentration of pantothenate had a large impact on the steady state product yields, ratios and hydrogen production but very little impact on the optical density (proxy for rate of growth). Steady states 1-4 show high acetate production (average 90.7 mM) and comparatively low ethanol production (average 34.9mM). This is in line with the expected values predicted by the literature[99] and reported values by industrial sponsors[89]. Steady states 6-8 which occured in low pantothenate conditions showed a much lower concentration of acetate (average 15.30mM) and a large increase in ethanol production (92.04 mM). Steady state 5, although occuring in the pantothenate limited phase of the experiment, shows different results with roughly equal amounts of acetate and ethanol (48.12 vs 46.61mM respectively). This is possibly due to the fact that whilst the surrounding media has only 10% pantothenate, the older cells still have enough residual pantothenate and CoA within them to produce high amounts of acetate and the cells are in a metabolically transitional stage. All steady states show a similar amount of CO in the outflow gases indicating a steady consumption, however, in steady states 5-8 the production of hydrogen is on average (average of steady state average 2-4 vs average of steady states average 5-8) twice as high. Optical density (and therefore growth rate) are also fairly constant throughout the steady states (although vary wildly accross the course of the experiment) indicating little change in cell density despite

pantothenate limitation. Serial dilution studies in chapter four implied that growth was possible in 10% of standard pantothenate addition cultures but it seems this limitation has a strong effect on hydrogen production and acetate:ethanol ratios.

Possible explanations for the results seen in the steady states with standard pantothenate production and with pantothenate limitation lie in the methods the cells are using to produce the economic products. As stated, the major differences between the two different conditions are a switch between high acetate production and low ethanol production in high pantothenate concentrations and low actetate, high ethanol production in low pantothenate concentrations with only a very slight change in the average optical density (and therefore, growth rate.) Acetogens have been shown to produce increased amounts of ethanol in "non carbon limiting" conditions caused by lowering B-vitamin concentrations or reduction of yeast extract in the media[185], here, we show the effects of limiting a single vitamin, pantothenate, resulting in similar reported results. *C. autoethanogenum* uses the Wood-Ljungdahl pathway in order to reduce CO and CO_2 to form Acetyl-CoA, which it uses to produce a range of downstream chemicals, notably, 2,3-butanediol, ethanol and acetate as well as utlising it for growth. The production of Acetyl-CoA is impossible in *C. autoethanogenum* without the presence of pantothenate.

The Wood-Ljungdahl pathway acts as an electron sink for a range of electron donors and is required for energy conservation in order to allow for growth and division of cells. CO is consumed via the Wood-Ljungdahl pathway, and converted into Acetyl-CoA which is used to produce acetate, ethanol, and allows cells to grow and produce CO_2 by the following reactions:

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2$$

CO_2 groups react with hydrogen and use it as an electron donor to produce acetate and ethanol, in order to reduce the levels of hydrogen present, and stabilise the reductive potential of the cells by the following reactions.

$$2CO_2 + 6H_2 \rightarrow CH_3CH_2OH + 3H_2O$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$$

The Wood-Ljungdahl pathway is used for growth, production of acetic acid and ATP in the CO-limiting phase where pantothenate is in abundance and produces ethanol and NAD(P) during the non-carbon limiting phase. After the production of Acetyl-CoA, the Acetyl-CoA is converted into acetate which can be reduced to acetaldehyde with reduced ferredoxin. This can be further reduced to ethanol using alcohol dehydrogenase. [89]

In normal conditions, ethanol formation from the Wood-Ljungdahl pathways is more exergonic than acetate production. Ethanol formation is not thermodynamically favourable but in non-carbon limited conditions with limited Acetyl-CoA production it is favourable to the cell to produce ethanol as it requires more hydrogen per mol produced than acetate production, so cells avoid acidogenesis. Cells can utilize either product in order to remove hydrogen produced by the Wood-Ljungdahl pathway. In conditions when pantothenate (and therefore, Acetyl-CoA) is abundant, it is more energetically favourable to produce larger amounts of acetate (as its production engenders ATP), however, if pantothenate is limiting then per mol of ethanol produced then the reducing potential is more effective.

It has been suggested that *C. ljungdahlii* shifts from acetate to ethanol production at high biomass concentrations in order to maintain ATP homeostasis, but this leads to the depletion of CoA[99]. *C. autoethanogenum*, as previously stated, is so closely related to *ljungdahlii* that it is likely that these conclusions could be drawn for both organisms. However, in our limitation phase, it is not high biomass depleting the CoA availability but the low pantothenate availability, producing the same results and changing the ratios of products accordingly.

2,3-butanediol is made by the cell following an enzyme cascade after the conversion of Acetyl-CoA into pyruvate and acetolactate. Acetyl-CoA produced from the Wood-Ljungdahl pathway, can be converted into pyruvate which leads to growth and production of biomass of the organism. The biomass (the proxy used here is optical density) production remains fairly constant throughout the experiment, regardless of condition and as most of the biomass is likely to be produced from pyruvate, the pyruvate flux is mostly geared towards biomass and products such as 2,3-butanediol cannot function as a sink for excess reducing power. Due to the lack of change in optical density and reduction in production of 2,3-butanediol it seems the conversion of Acetyl-CoA to pyruvate is unchanged but the amount of Acetyl-CoA converted into ethanol and acetate has a much greater change. This could be due to the limitation being only just on the verge of limiting as there is enough pantothenate to produce a consistent growth rate but not enough Acetyl-CoA for the cells to produce a normal amount of acetate and ethanol.

Although not seen in the results (due to averages taken over 24 hour periods) several attempts were made both intentionally on my part, and accidentally in an unfortunate mix-up of vitamin mixtures in other reactors, to drop the concentration of pantothenate to 5% of the standard addition. This resulted (once the concentration had dropped sufficiently due to wash out) in rapid rises of the pH in the culture and a rapid drop in hydrogen production and CO consumption. This phenotype was seen time and again as the pantothenate level dropped but just the addition of pantothenate to 10% would return the reactor to stability. Further addition to a "standard" amount of pantothenate would result in a rapid switch of product ratios back to high acetate production and low ethanol production until the pantothenate concentration returned to 10% at which point the acetate to ethanol ratio would revert to low acetate and high ethanol.

6.4.3 Redox Equivalents

Throughout the experiment as seen in figure 57, Carbon uptake is reasonably constant at both high and low pantothenate concentrations. CO must be converted to CO_2 for the first step of the western branch of the Wood Ljungdahl pathway, using the reaction:

$$CO+H_2O \rightarrow CO2 + 2[H]$$

Because the carbon monoxide uptake is reasonably constant, and the optical density (and to some extent biomass/ cell numbers) is fairly constant in the steady states of the two different conditions, in both high and low concentrations of pantothenate the same amount of reducing equivalents ([H]) are generated in either condition. Even in low pantothenate conditions large amounts of [H] are being produced as the carbon monoxide flows through the Wood-Ljungdahl

pathway, but there is a limited amount of Acetyl-CoA that the CO can be converted into (due to the pantothenate limitation). These generated reducing equivalents must be taken in to produce a reduced product from CO_2, such as acetate or ethanol.

The production of acetate from CO_2 requires 8 [H]

$$8[H] + 2CO_2 \rightarrow CH_3COOH + 2H_2O$$

And the production of Ethanol from CO_2 requires 12[H]

$$12[H] + 2CO_2 \rightarrow CH_3CH_2OH + 3H_2O.$$

As the production of ethanol requires more [H], it makes sense for the cell to produce more of this product if there is a potential of cell acidification, or a limited capacity to produce the two products, such as a limitation of the Acetyl-CoA.

6.4.4 Metabolic flux

Because acetate can be converted into ethanol by AOR an additional step can be affected by the metabolic flux.



Figure 63 Metabolic flux of CO_2 to ethanol via acetate

In figure 63 if A=0 neither product can be formed, if B=0, then only acetate can be formed. If the value of A is higher than B then more acetate than ethanol will be formed (less acetate will be converted into ethanol) and if the value of B is higher than A more ethanol will be formed (if A is not 0). Because more ethanol

than acetate is formed in low pantothenate conditions we can assume the value of B is higher than A and in high concentrations of pantothenate the value of A is higher than B. B should remain constant. It seems likely than pantothenate addition is affecting the value of A, as less acetate can be produced due to a decrease in the production of Acetyl-CoA when pantothenate is limited. As a decrease in pantothenate will decrease the flux through A, less acetate will be produced in low pantothenate concentrations if B remains the same.

6.4.5 Blackboard model Explanation for product ratio changes

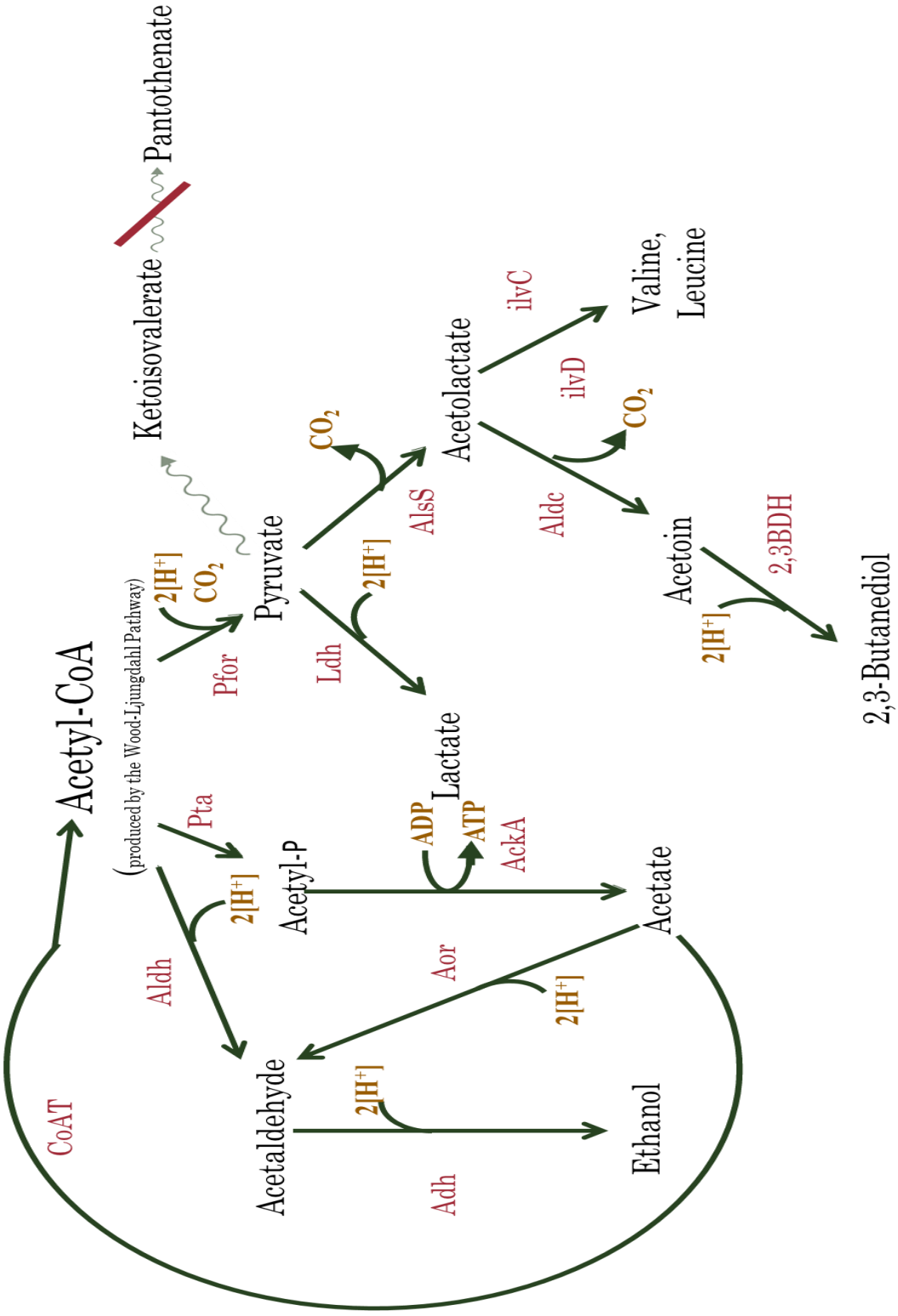


Figure 64 Blackboard model explanation of product ratio shifts.
2,3-BDH, 2,3-butanediol dehydrogenase; ACK, acetate kinase; ADH, alcohol dehydrogenase; ALDC, acetolactate decarboxylase; ALDH, aldehyde dehydrogenase; AOR, aldehyde oxidoreductase; CoAT, CoA transferase; IlvA, threonine deaminase; IlvC and D, Valine and Isoleucine biosynthesis; LDH, lactate dehydrogenase; PTA, phosphotransacetylase

Figure 64 is a simplified pathway of downstream products of Acetyl-CoA. In the pantothenate limitation experiment, the decreased access to pantothenate leads to a decrease in available CoA which leads to a decreased availability of Acetyl-CoA for use in the cell. Under limitation conditions the amount of acetate seen in the culture decreases, and the amount of ethanol increases (steady state 5 shows a partial crossover from one state to another). As previously stated, this could possibly be due to the fact that the production of ethanol avoids the production of acetic acid which can acidify the cell, interrupting pathways. When growth is abundant, as much acetate as possible can be generated because in doing so, ATP is generated and [H] is used up due to high production, however, when Acetyl-CoA is limited, the organism has to change which products it produces in order to prevent acidification. Conversion of acetate into ethanol saps an extra 4[H] from the cell so ethanol is a more effective electron sink than acetate. In this respect it is probable that there is not less acetate produced by the cell but more of it is being converted by AOR into ethanol, resulting in increased ethanol and decreased acetate. Growth and production of lactate and 2,3-butanediol remain fairly constant throughout the steady states and it is probable that the amount of Acetyl-CoA converted to pyruvate is not altered to these products. In particular the growth rate remains constant implying the conversion of Acetyl-CoA to pyruvate is not being altered by the limitation. The amount of Acetyl-CoA converted into ethanol and acetate, however, did drop between steady states 1-4 and steady states 5-8. In limiting steady states the overall production of acetate and ethanol was 104mM and in non-limiting conditions the total concentration was 125mM. If carbon monoxide usage is the same in both conditions, then

because ethanol production requires more reducing equivalents as shown in section 6.4.3, less ethanol can be made per mol of CO.

6.4.6 LCMS results

In addition to the key finding that there is a strong correlation between low pantothenate conditions and decreased availability of Acetyl-CoA, which had been previously suspected but confirmed by the LCMS analysis of samples in the two conditions, the data returned from the LCMS testing yielded several other interesting differences in the two conditions. Figure 62 shows the 30 metabolites with the largest changes between the two conditions. Most of the metabolites with the largest differences use CoA or Acetyl-CoA, or are involved in pathways which are dependent on one of the two chemicals. The amounts of Phenylalanine and tyrosine are much reduced in the low pantothenate results and this is possibly due to the fact that they can be broken down to form Acetyl-CoA, so if not much is present they may be broken down to rectify this to allow for more to be used for growth. Similarly hydroxyphenyllactic acid and phenyllactic acid are part of the tyrosine pathway, if conversion of these metabolites to CoA is not required then it makes sense for them to be present in higher concentrations in the high pantothenate conditions as they have not been converted to Acetyl-CoA. A further reason for high phenylalanine presence in high pantothenate conditions could be due to the involvement of Acetyl-CoA and CoA in phenylalanine metabolism when using phenylalanine N-acetyl-transferases, although it would make more sense if there were higher concentrations of a downstream product rather than phenylalanine as one would assume high amounts of pantothenate and therefore Acetyl-CoA availability could mean more could be converted. Glucosamine is present in very high concentrations in low pantothenate

conditions but very low in high pantothenate conditions because it the enzyme glucosamine N-acetyltransferase requires Acetyl-CoA in order to convert the glucosamine into N-acetyl-D-glucosamine which is seen in high concentrations in high pantothenate but low concentrations in low pantothenate because the Acetyl-CoA is not available for the reaction in such high amounts. Several components of the PPP pathway are present in high concentrations in the high pantothenate conditions it has been suggested that flow rate through the pathway is inhibited by high Acetyl-CoA presence, which could lead to a build-up of intermediate products such as sedoheptose-7-phosphate and ribulose-5-phosphate[189].

6.4.7 Conclusion

C. autoethanogenum is an industrially viable organism capable of producing ethanol, acetate, lactate and 2,3-butanediol in reasonable quantities in a chemostat culture. In non-carbon limiting conditions such as pantothenate limitation, the balance of reducing equivalents in the cell has a significant impact on results. Increased ethanol: acetate ratios are only shown in the literature during non-carbon limiting conditions. In all chemostat experiments run within the group we have seen that nutrient limitation (pantothenate, phosphate and nitrogen) significantly alters the product profile for the organism. Generally, the profile is shifted to more reduced, dissolved products. Maximum industrial reported yields are 6.5g of ethanol/L which is roughly 141mM of ethanol[99] in two stage- cell recycled CSTR. The maximum production of ethanol in a steady state within this experiment was 115mM, however, within a two stage cell recycled CSTR the yield could increase. Further limitation of pantothenate resulted in a crashing bioreactor so it is not prudent to lower concentrations of

pantothenate below 10%. From an industrial perspective, if conversion of acetate to ethanol could be forced, (resulting in low acetate yields due to almost full conversion to ethanol) it could have huge benefits to yield and cost effectiveness of the process. Here we have shown the potential exists to drastically increase yields of ethanol- a high value product from reactors and reduce the yield of acetate- a low value product without significantly altering growth rate or minor product production with the sole altering of a single variable – pantothenate concentration.

6.5 Chapter Summary

- A culture of wild type C. *autoethanogenum* growing in 10% of the standard pantothenate addition in a bioreactor produces more ethanol and less acetate than the same culture growing in 100% of the standard pantothenate addition
- Carbon monoxide consumption does not really vary between the two different conditions but hydrogen production does
- There is a change in the metabolites measured by LCMS by cultures in the two different conditions, particularly of note, there is less free Acetyl-CoA in low pantothenate conditions
- There is no significant change in the optical density between cultures in the two different phases
- There is little change in the formate, lactate or 2,3-Butanediol measured by HPLC

7 General Discussion

7.1 Introduction

For the last few decades there has been a clamouring amongst scientists, politicians, activist groups and random celebrities to "sort out the oil problem". Consequences of its extraction pollutes every environment on the planet, our usage is threatening huge climate change consequences and the demand and the price is only going to increase as our population expands. One of the major problems with oil, is that our current civilisation is so intrinsically bound to this finite, non-renewable resource. We rely on it and its derivatives for everything from medicines to transport to power production. Alternatives must be found, but we cannot cease usage, until sustainable substitutes to all of the derivatives are found. The global demand for oil currently exceeds 91 million barrels per day, and primary energy demand is expected to increase at a rate of 1.5% per annum[190]. The International Energy Association predicts globally we need to convert more than 30% of our global energy pool to zero carbon fuels in order to stabilise CO_2 levels in the atmosphere to 450 parts per million[190].

Acetate, ethanol and 2,3-butanediol production by *C. autoethanogenum* using gas fermentation offers a useful alternative to the industrial production from petroleum and is already being implemented in an industrial setting[156].

7.1.1 Ethanol

As Figure 65 shows, Global ethanol production in 2016 was 25.46 billion gallons (80 million tonnes)[191].

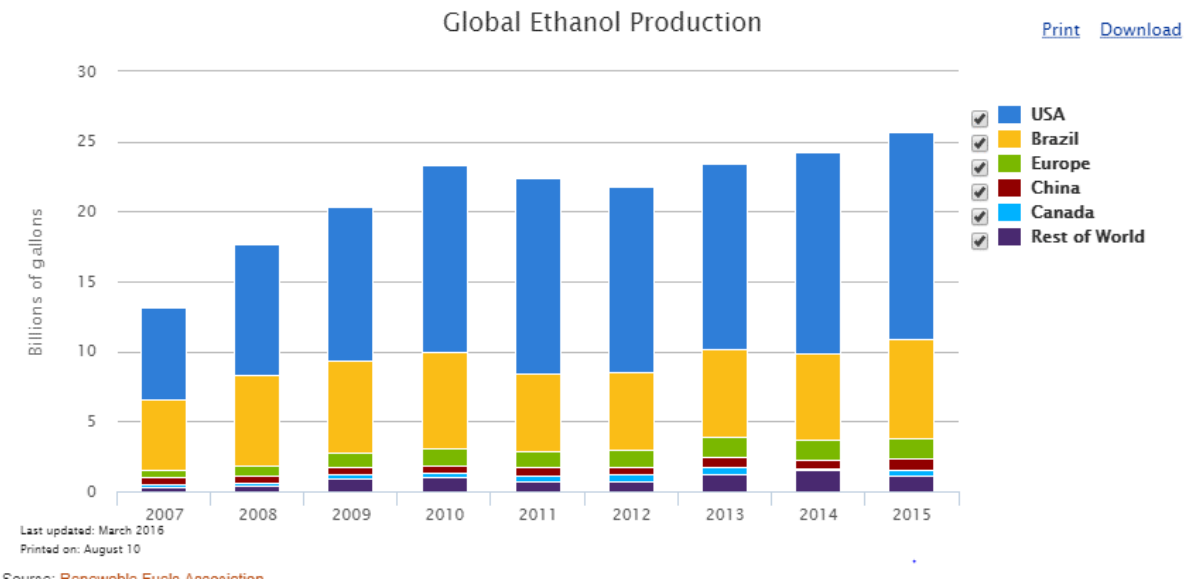


Figure 65Global ethanol production by country 2007-2016.
Brazil and the USA average 85% of global ethanol production per year. Source: Renewable Fuels Association

The largest single market for ethanol is as a fuel and fuel additive. The USA and Brazil are the world's largest producers of ethanol. In Brazil, by law their transportation fuels are required to have at least 25% ethanol by volume, however, in the US and Europe addition of ethanol to petrol is generally around 10%[190]. Options for using ethanol as a lightweight rocket fuel have been explored and a partnership between Virgin Airlines and LanzaTech has been brokered to attempt to build planes capable of flying using only ethanol produced by gas fermentation[23]. Ethanol has myriad markets as a platform chemical and for use in fuel cells, as a solvent, in paints, in personal hygiene products, as an antiseptic, for recreational purposes and it is involved in numerous industrial chemical reactions and the production of other organic compounds and downstream processes. Ethanol is produced in two key ways; either as a petrochemical by acid catalysed ethylene hydration or by fermentation by

microorganisms. Currently, the cheaper process is determined by the market price of oil or the feedstock price in fermentation (i.e. sugar).

If ethanol is produced industrially via the gas fermentation of syngas from steel mills per tonne of ethanol (in comparison to production by ethylene hydration); 1.8MT of CO is “averted from flare” and not released into the atmosphere, 2.9 kg of particulate matter, 10.7kg NO_x , 3.3kg SO_x and 5.2 barrels of petrol are displaced (net aversion)[21].

7.1.2 Acetate

The main market for acetate produced by gas fermentation is as acetic acid. Acetic acid can be produced synthetically or by fermentation by microorganisms. Roughly 75% of the acetic acid used industrially is created by the carbonylation of methanol, and the majority of the rest is produced by bacteria, particularly acetogens[192]. The largest markets for acetic acid are vinyl acetate monomer production (approximately 1.3 of the global acetic acid market), acetic anhydride production and ester production[193]. Acetic acid for vinegar production, though one of the most famous markets is relatively low. Acetic acid is also used as a solvent, in textiles, in adhesives and coatings, an antiseptic and is classed in the World Health Organisation as one of the most important medications required in a basic health system. The global acetic market is 16 million tonnes[194].

7.1.3 2,3-Butanediol.

2,3-Butanediol has a diverse range of downstream products,[195] which have a market of 32million tonnes per year and possibly $43 billion in sales. [89] Traditionally produced via the Reppe process in which formaldehyde is reacted with acetylene, [195] biological production of the diol was elucidated in 1906 by

Harden and Walpole[196]. However, the commercial demand began in earnest during World War II when 2,3-butanediol produced by biological fermentation could be used as an intermediate in the production of synthetic rubbers[197]. Due to the high octane value, the diol can be used as an octane booster for petrol or as high-grade aviation fuel[198], catalytically dehydrogenated into diacetyl (a food additive flavoured like butter) or Methyl ethyl ketone which is a solvent[199, 200]. The diol can be esterified to form a precursor of polyurethane foams which can be used for a variety of drugs and cosmetics and converted into different chemicals such as 1,3-butadiene, which is used in the manufacture of nylon. The diol can also be used in explosives, plasticisers, as a carrier for pharmaceuticals and printing inks as well as many other products[195]. Although biological production was reasonably efficient, it became cheaper to produce the diol via petroleum based routes, post-war[201]. Due to the increase in cost of crude oil, there has been a re-emergence in interest in the biological pathways to chemical compounds[202], and several companies are already implementing bacteriological based systems for production of 2,3-butanediol. LanzaTech has announced plans for a gas to 1,3-butadiene plant with Invista.

7.1.4 Production by *C. autoethanogenum*

Using *C. autoethanogenum* for production of these products offers the benefit of using a non- pathogenic strain, reducing the need for the high costs caused by implementing health and safety measures when using some potentially pathogenic organisms and also uses syngas fermentation, decoupling the production of these products from the usage of farmed or food sugars, but instead can be used to convert anthropogenic wastes into useful clean products. Further developments, by the optimisation of the process and in the organism will

hopefully provide a bright new future for the production of acetate, ethanol and 2,3-butanediol by *C. autoethanogenum.*

7.1.5 Aims of the project

The initial remit of this project which six PhD candidates embarked on in 2013, was to "Optimise industrial gas fermentation for commercial low-carbon fuel and chemical production through systems and synthetic biology approaches." Each candidate took this forward in a very different direction, but the approach shown in this work, was to attempt to optimise the media contents, and reduce feedstock costs, and hopefully improve yields of economic products by investigating the B-vitamin component of the media and their effects on the organism.

The following sections will address the objectives of this work and provide a summary of the research outcomes. In particular; the elucidation of which B-vitamins are "true" vitamins for the species and which vitamins exception do not affect viability, the generation of a pantothenate autotroph, a biotin autotroph, and a strain which only requires a single vitamin to grow by synthetic biology, and the performance of strains undergoing pantothenate limitation in a bioreactor. Finally, a range of experiments is proposed as future work to generate a robust *C. autoethanogenum* for industrial gas fermentation.

7.1.5.1 Elucidation of vitamin requirements

The work detailed in this project has demonstrated that despite popular addition of 10 B-"vitamins"-thiamine, riboflavin, niacin, pantothenate, pyridoxine, biotin, folic acid, PABA, cyanocobalamin and lipoic acid, only three are actually true vitamins and required for growth and viability of *C. autoethanogenum* and *C.*

ljungdahlii. These are: pantothenate, biotin and thiamine, and abundant growth was possible with the addition of just these three vitamins. Addition of other vitamins in many cases made the growth profile of the organism more comparable to growth without any vitamin deficiency, but was not required for viability. These tests were carried out in solid and liquid media and with carbon monoxide and fructose as carbon sources, at 37ºC. In all instances the vitamins required remained the same. Some initial growth occurred in media deficient in pantothenate, biotin and thiamine, however, these never entered a particularly rapid growth phase and it is possible that the initial growth was due in part to some carryover of vitamins which were not removed during the "washing" steps or due in part to the very low concentrations required within cells for viability. There was a dip in performance of product generation in the cultures deficient in cobalamin this could be in part due to the complexity of biosynthesis of the compound or its involvement in the Wood-Ljungdahl pathway, which accounts for the much greater effect seen when the cobalamin free cultures are growing using CO as a carbon source compared to fructose as a carbon source. Therefore, although the cobalamin is not required for growth, the exception could potentially have ramifications in an industrial setting if no cobalamin was present when growing using CO. The question remains of whether this effect would be translated when grown in an industrial setting and whether the slight drop in product would be offset in cost savings from not adding the unnecessary chemical.

7.1.5.2 Characterisation of Pantothenate Requirements and Generation of a Pantothenate Prototroph

In this section of the study it was determined that *C. autoethanogenum* and *C. ljungdahlii* were capable of abundant growth in media which contained only 10 % of the standard addition of pantothenate in the media but was capable of very limited growth with 1% of standard addition. Addition of the gene cluster *panBCD* from *C. acetobutylicum* whether conferred in the genome using the *ΔpyrE* system or by unintegrated replicative plasmids, resulted in the development of variant strains in *C. autoethanogenum* or *C. ljungdahlii* which allowed for the biosynthesis of pantothenate and permitted transformed cells to grow autotrophically in pantothenate free media, without interferring negatively with the production of valine or leucine. All three genes were essential for growth but the use of "provitamin" intermediates allowed biosynthesis if certain genes were present on plasmid – if *panB* and *C* were present, growth was possible if β-alanine was added, if *panC* and *panD* were introduced, growth was possible with pantolactate and if only *panC* was present growth was possible if pantolactone and β-alanine were present in the media. The prototrophy conferred for pantothenate by plasmid was stable over several passages without pantothenate in the media leading to the development of a selection system which did not require antibiotics.

7.1.5.3 Characterisation of Biotin Requirements and Generation of a Biotin Prototroph

C. autoethanogenum performed similarly in 10% of usual addition of biotin to media but was also capable of strong growth in only 1%. This was probably in large part due to the highly effective biotin scavenging pathway present in many

organisms including *C. autoethanogenum*. From the biotin synthetic pathway six genes were missing, *bioD, bioA, bioH, bioC, bioF*. Due to the large number of genes and lack of calcitrant close relatives containing the whole pathway, attempts to introduce the pathway were difficult. Based on information from the Yang et al paper, which produced a hyper-biotin producing strain by over expressing *bioYDA* and *B,* attempts to confer prototrophy for biotin by the addition of the genes *bioYDA* from *C. acetobutylicum* were made but unsuccessful in growing in biotin deficient media. However, the addition of pimelic acid as an intermediate (or pro-vitamin) mitigated this issue and, growth was possible without biotin, if the *bioYDA* cluster was introduced. The "big bio" cluster (*bioBDFHCA*) of genes inserted from *D. nigrificans* does confer prototrophy for biotin in *C. autoethanogenum*, when the genes are split on to two separate plasmids leading to abundant growth in biotin deficient media and the production of similar levels of economic products and growth rate, however, the plasmid is unstable and is easily lost without antibiotics acting as a selection pressure. The „big bio" cluster of genes was introduced into the genome by *pyrE* repair method and conferred prototrophy, high growth profile, and similar acetate and ethanol production in biotin free media to the wild type growing with standard vitamin supplementation, it seems that the use of a biotin prototroph and biotin free media does not significantly diminish the industrial viability of the organism.

7.1.5.4 Vitamin Prototrophs

A strain containing all the genes for both pantothenate and biotin prototrophy was developed which was capable of growing without any vitamins present in the media except thiamine. Although growth was possible a much lower viability

on plate was shown (although this exceeded 600 colonies) and a lower terminal optical density was seen at 8 days than the wild type when growing in media containing all vitamins. This is possibly due to the high cost to the cell of maintaining two large plasmids. However, the production of acetate and ethanol was very comparable to the wild type.

For all strains we provided a number of characterisation steps which demonstrate the unaltered physical properties of these strains with the exception that they are now capable of growth in media without the vitamin in question. Crucially, we demonstrated the variant strains possessed if not identical growth then comparable growth and viability to wild type strains growing with standard media addition and certainly growth which was more closely aligned to this condition than to wild type strains growing without one of the three "Key" vitamins. We provided evidence here of the viability of a strain which would grow in conditions with only a single vitamin added – thiamine.

7.1.5.5 Chemostat experiment

Finally in this project we determined the effects of limitation of an essential vitamin- pantothenate, on the growth of *C. autoethanogenum* in a CSTR bioreactor. The comparison of the steady states from abundant pantothenate to pantothenate limited conditions showed a marked difference in the production of acetate and ethanol but very little change in the production of 2,3-butanediol and lactate and no significant difference in the average optical density values. The carbon monoxide consumption was reasonably stable throughout the steady states, however, more hydrogen gas was produced in the pantothenate limiting

phase. It seems that the major difference between the two states is in the production of acetate and ethanol, and the switch in their ratios between high acetate and low ethanol in the pantothenate non- limiting state and high ethanol and low acetate in the pantothenate limited condition. This effect is commonly seen in "non-carbon" conditions, and has been seen when pH is lowered, when nitrogen is lowered, when phosphates are limited and when yeast extract or B-vitamin mixtures are reduced. In this experiment we show that these effects are seen in pantothenate limitation, and that although possibly not the only reason for the results seen in when B-vitamins and yeast are left out as seen in [185], is probably a major influence in these results. Generally this type of result is seen in limitation experiments when a key nutrient is limited (such as nitrogen or phosphates), and production of ethanol increases as the carbon monoxide avaliability increases along with biomass (up to 30ml/minute) as the limitation is not carbon based. In pantothenate limitation, the carbon monoxide is not limiting but the limitation effect comes from the cells inability to convert this gas to cellular carbon as Acetyl-CoA production is tied to pantothenate availability. A huge change in products was shown between the two conditions – an increase of 248% of ethanol concentration (minimum steady state value to maximum steady state value) with only the change in one variable, which could potentially both lower the costs of feedstock and increase the production of ethanol making the process more viable. Increased Ethanol production is important as a phenotype as it is a much higher value product than acetate with a much bigger market and is a precursor for many high value downstream products. Production of high levels of acetate are already possible but this easy change from one ratio to another by change of a single variable is both novel and exciting, and whilst

production ratios do not match industrial ones seen in experiments such as those shown in [184], potentially with further refinement higher titres could be reached.

7.1.6 Further work

As part of an effort to understand and characterise the requirements of *Clostridium autoethanogenum* for B-vitamins and to develop strains capable of autotrophic growth for vitamins, I believe this study contributes to the wider knowledge. In this study, pantothenate, biotin and thiamine were demonstrated to be the only essential B-vitamins for growth, and the effects of pantothenate limitation on a growing culture of *C. autoethanogenum* in a chemostat were investigated, whereas previously, all B-vitamins in general were limited. However, due to the time restraint of a PhD, several aspects of this study could be expanded. Further work could be done to try to expand the *+biotin* strain (which contained *bioBDFHCA* inserted by the *pyrE* method) to also include the *panBCD* genes on the chromosome. However, efforts to attempt this were unsuccessful and although in theory the *pyrE* could be deleted and complemented again to bring more genes in, this has not yet been successfully achieved in this organism, in this group.

Outstanding work by Peter Rowe (University of Nottingham) developing the CRISPRs methodology in *C. autoethanogenum* as his PhD project could yield interesting insights into the essential nature of other genes in the biosynthetic pathway by allowing for easy, rapid deletions. However, the developments came too late to be useful to my project as the protocol was still in its infancy. Of particular interest could be to determine if the deletion of the *ilvE* gene has any particular effects on product ratios, as potentially more panothenate could be made following work done by [167].

The obvious future direction this project could take was in trying to convey the missing thiamine genes into the organism and genome. However, the number of genes which need to be inserted was too high a challenge with current technology. Thiamine's active form is as thiamine diphosphate (ThDP) or Thiamine pyrophosphate (TPP) and it is used in the catabolism of sugars and amino acids and there is evidence Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. The genes involved in its production are: *thiFSGH*, *thiI*, and *dxs* which are required for thiazole synthesis, *thiC* for pyrimidine synthesis, *thiE* to link the thiazole and the pyrimadine, and four kinase genes *thiD, thiM, thiL, and pdxK*. *ThiS* -thiocarboxylate is the sulphur source[16]. In *Clostridium ljungdahlii* and *autoethanogenum*, the only genes encoded in the genome are *thiF, thiE, thiI, and thiM*, the rest of the entire biosynthetic pathway is not present in either species, and introduction of all of the missing genes would require the insertion of close to 10,000 base pairs.

The chemostat experiments undertaken in this work, both those shown here and ones worked on in conjunction with my colleagues, proved to be my favourite and in my opinion, the most interesting part of the project. Their blessing and their bane is in the generation of huge quantities of data, measuring myriad characteristics of a culture growing with a single variable, over such a long period of time. Sadly, part of their nature is to crash, and they often did, which made the data gathered less "clean" that it could have been. Had there been an unlimited amount of time, funding, access to equipment and the ability to control the cultures to a tighter degree, I would have liked to repeat the limitation switch (from low to high to low pantothenate conditions) as although we saw the rapid change in product ratios over the course of days during the addition pantothenate

in order to resuscitate a crashing culture. Unfortunately the agitation motor broke before this could be shown in a culture which had reached steady state. Furthermore, I would have liked to have conducted a similar experiment switching from a standard addition of vitamins to only adding pantothenate, biotin and thiamine in order to see what effect on the long term optical density and product ratios this reduction in pseudo vitamins would have in order to truly say whether the reduction could be economically viable. Also given enough time, I would have liked to have characterised the effects of biotin and thiamine limitation would have on the culture, although, I suspect the effects on the product ratios would not have been so stark, because as previously stated the majority of the reason for the product shift is because pantothenate limitation is a semi-proxy for Acetyl-CoA limitation. I would also have liked to have tried a gas shift experiment (increase carbon flow to 30ml/min) when the reactor was under pantothenate limitation to see whether an increase of carbon monoxide would have had any effect, however, the carbon monoxide consumption level was relatively stable throughout the experiment. Finally, it would have been interesting to characterise the +*pantothenate* strain in standard and pantothenate limiting conditions, as well as to see if it was possible in reactor conditions for the strain to grow with no pantothenate present. It could be possible to look at the expression levels of the different genes in the transformed strains when they are grown in different conditions by using qPCR or RNA-seq. In the realms of much further research, development of hyper production strains of vitamins, if they maintained their industrially favourable characteristics, could lead to the development into the market for synthetically produced vitamins as a value added product.

7.2 Concluding remarks

When competing with an established industry, any commercial endeavour has to offer a better product, be cheaper or easier to get hold of, or offer some kind of incentive in order to change the market. Gas Fermentation offers the production of chemicals from low grade syngas which can be generated by the pyrolysation of heterogeneous hydrocarbons from waste or pollutant sources, generating something useful out of something harmful. Optimisation of this process in any way will only serve to make this process more attractive, especially if it makes yields more efficient or feedstocks and media cheaper. In this project we have established the exact requirements for B-vitamins, for the organism which led to the characterisation of growth with just the three vitamins and the establishment that growth with just those three did not lead to a very drastic reduction in acetate or ethanol formation and a similar growth profile to the wild type growing with standard addition of vitamins. Despite much searching, quantification of the costs of vitamins added on an industrial scale were unable to be obtained so it was impossible to work out what the reduction in media costs are from only using three vitamins. On a lab scale growing the organism with just three vitamins reduces the cost of vitamins per litre of standard reactor media from 2.2p/L to .854p/litre (from the largest quantities available on Sigma Aldrich). From these three vitamins, for pantothenate and biotin, I established that the addition of only 10% of the standard addition led to abundant growth and established prototrophies by plasmid and in the genome for pantothenate and biotin and established a strain which was auxotrophic for only a single vitamin. Although growth was not as high in this strain, due in part to the high cost to the cell of the maintenance of two large plasmids, the cells grew and produced reasonable

quantities of ethanol and acetate. The (lab) cost of vitamins in standard media for a strain growing with only thiamine supplementation is only .1p/L which leads to a saving of 2.1p. Which although not much, could indicate a potentially significant reduction in cost in an industrial scale, if characterisation of the mutants show it as industrially viable.

It is clear from the conferences and the literature, that despite what certain politicians may espouse; there is no single solution on the horizon which will save us from the devastating impacts of climate change and energy crises. The only way forwards in our current capacity is to mitigate the effects as much as possible and convert as much chemical and energy generation to renewable sustainable sources, and to streamline and make processes such as gas fermentation as efficient and cheap as possible in order to be more competitive than chemical generation from non-renewable sources. Optimisation of the process, however small (even at .1p/L) is important in this effort.

8 Bibliography

1. Abrini, J., H. Naveau, and E.J. Nyns, *Clostridium autoethanogenum, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide.* Archives of Microbiology, 1994. **161**(4): p. 345-351.
2. Fulgoni, V.L., et al., *Foods, Fortificants, and Supplements: Where Do Americans Get Their Nutrients?* The Journal of Nutrition, 2011. **141**(10): p. 1847-1854.
3. Connor, J.M., *THE GREAT GLOBAL VITAMINS CONSPIRACY:SANCTIONS AND DETERRENCE.* Economists Unit Seminar, European Commission, Brussels, 2006.
4. Price, C., *The Vitamin Complex*. 2015: Oneworld Publications.
5. Organisation, U.W.W.B., *Levels and Trends in Child Malnutrition.* Joint Child Malnutrition reports, 2017.
6. Goldacre, M., N. Hall, and D.G.R. Yeates, *Hospitalisation for children with rickets in England: a historical perspective.* The Lancet. **383**(9917): p. 597-598.
7. Demain, A.L., *The Business of Biotechnology.* Industrial biotechnology, 2007. **3**(3): p. 269-283.
8. Graham, R.M., E. Deery, and M.J. Warren, *Vitamin B12: Biosynthesis of the corrin ring*, in *Tetrapyrroles*. 2009, Springer. p. 286-299.
9. De Baets, S., S. Vandedrinck, and E. Vandamme, *Vitamins and Related Biofactors, Microbial Production*, in *Encyclopedia of Microbiology, 2nd ed.;(Ed. J. Lederberg); Vol 4; Academic Press, USA, 2000*. 2000. p. 837-853.
10. Moore, Simon J. and Martin J. Warren, *The anaerobic biosynthesis of vitamin B₁₂*. Biochemical Society Transactions, 2012. **40**(3): p. 581-586.
11. Capozzi, V., et al., *Lactic acid bacteria producing B-group vitamins: a great potential for functional cereals products.* Applied Microbiology and Biotechnology, 2012. **96**(6): p. 1383-1394.
12. Voet, D. and J.G. Voet, *Biochemistry*. 2004: Wiley.
13. BALCH, W.E., et al., *Acetobacterium, a New Genus of Hydrogen-Oxidizing, Carbon Dioxide-Reducing, Anaerobic Bacteria.* International Journal of Systematic and Evolutionary Microbiology, 1977. **27**(4): p. 355-361.
14. ADNAN, O.H.H.a.N.A., *Coenzyme, Cofactor and Prosthetic Group*

Ambiguous Biochemical Jargon Biochemical education, 1994. **22**(2): p. 93-94.

15. Denessiouk, K.A., V.-V. Rantanen, and M.S. Johnson, *Adenine recognition: A motif present in ATP-, CoA-, NAD-, NADP-, and FAD-dependent proteins.* Proteins: Structure, Function, and Bioinformatics, 2001. **44**(3): p. 282-291.
16. Begley, T.P., et al., *Thiamin biosynthesis in prokaryotes.* Arch Microbiol, 1999. **171**(5): p. 293-300.
17. Administration, E.-U.E.I., *Annual Energy Outlook*, EIA, Editor. 2010.
18. Datar, R.P., et al., *Fermentation of biomass-generated producer gas to ethanol.* Biotechnology and Bioengineering, 2004. **86**(5): p. 587-594.
19. Ji, X.J., et al., *Engineering Klebsiella oxytoca for efficient 2, 3-butanediol production through insertional inactivation of acetaldehyde*

dehydrogenase gene. Applied Microbiology and Biotechnology, 2010. **85**(6): p. 1751-1758.
20. Munasinghe, P.C. and S.K. Khanal, *Biomass-derived syngas fermentation into biofuels: Opportunities and challenges.* Bioresource Technology, 2010. **101**(13): p. 5013-5022.
21. Lane, J. *LanzaTech: Biofuels Digest's 2015 5-minute guide*. 2015 [cited 2015 13th January]; Available from: http://www.biofuelsdigest.com/bdigest/2015/01/13/lanzatech-biofuels-digests-2015-5-minute-guide/.
22. Lane, J. *China Steel green-lights commercial-scale LanzaTech advanced biofuels project*. 2015; Available from: http://www.biofuelsdigest.com/bdigest/2015/04/22/china-steel-green-lights-46m-for-commercial-scale-lanzatech-advanced-biofuels-project/.
23. Quinn, J., *Planes will be made from wonder material graphene 'in 10 years'*. Th Telegraph, Business, 2017.
24. Burton, F. *LanzaTech and PNNL to Convert Syngas to Drop in Jet Fuel*. 2010 [cited 2014 02/03/2014].
25. CHEMREC. *The Magic of Syngas*. 2010 [cited 2014 05/04/2014].
26. Fung Min Liew, M.K.p.a.S.a.D.S., *Gas Fermentation for Commercial Biofuels Production*, Z. Fang, Editor. 2013: intech open.
27. Heiskanen, H., I. Virkajärvi, and L. Viikari, *The effect of syngas composition on the growth and product formation of Butyribacterium methylotrophicum.* Enzyme and Microbial Technology, 2007. **41**(3): p. 362-367.
28. Abubackar, H.N., et al., *Biological conversion of carbon monoxide: rich syngas or waste gases to bioethanol.* Biofuels, Bioproducts and Biorefining, 2011: p. 93-114.
29. Siedlecki, M., W. de Jong, and A.H.M. Verkooijen, *Fluidized bed gasification as a mature and reliable technology for the production of bio-syngas and applied in the production of liquid transportation fuels-A review.* Energies, 2011. **4**(3): p. 389-434.
30. Mohammadi, M., et al., *Bioconversion of synthesis gas to second generation biofuels: A review.* Renewable & Sustainable Energy Reviews, 2011. **15**(9): p. 4255-4273.
31. Wei, L., et al., *Co-gasification of hardwood chips and crude glycerol in a pilot scale downdraft gasifier.* Bioresource Technology, 2011. **102**(10): p. 6266-6272.
32. Dudynski, M., K. Kwiatkowski, and K. Bajer, *From feathers to syngas - Technologies and devices.* Waste Management, 2012. **32**(4): p. 685-691.
33. Klasson, K.T., et al., *Bioconversion of synthesis gas into liquid or gasesous fuels.* Enzyme and Microbial Technology, 1992. **14**(8): p. 602-608.
34. Sipma, J., et al., *Microbial CO conversions with applications in synthesis gas purification and bio-desulfurization.* Critical Reviews in Biotechnology, 2006. **26**(1): p. 41-65.
35. Klasson, K.T., et al., *Bioreactor design for synthesis gas fermentations.* Fuel, 1991. **70**(5): p. 605-614.
36. Köpke, M., S. Noack, and P. Dürre, *The past, present, and future of biofuels – Biobutanol as promising alternative*, M.A. dos Santos Bernades, Editor. 2011, InTech. p. 451-486.

37. Latif, H., et al., *Trash to treasure: production of biofuels and commodity chemicals via syngas fermenting microorganisms.* Current Opinion in Biotechnology, 2014. **27**: p. 79-87.
38. Bengelsdorf, F.R., M. Straub, and P. Dürre, *Bacterial synthesis gas (syngas) fermentation.* Environmental Technology, 2013. **34**(13-14): p. 1639-1651.
39. Dürre, P., *Clostridia*, in *Encyclopedia of Life Sciences*. 2007, John Wiley & Sons Ltd.
40. Dürre, P., *Handbook on Clostridia*, ed. P. Dürre. 2005: CRC Press Taylor & Francis Group.
41. Tracy, B.P., et al., *Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications.* Current Opinion in Biotechnology, 2011: p. 1-18.
42. Bahl, H. and P. Dürre, *Clostridia: Biotechnology and medical applications*. 2001, Veinheim: Wiley-VCH.
43. Lincke, T., et al., *Closthioamide: An unprecedented polythioamide antibiotic from the strictly anaerobic bacterium Clostridium cellulolyticum.* Angewandte Chemie-International Edition, 2010. **49**(11): p. 2011-2013.
44. Minton, N.P., *Clostridia in cancer therapy.* Nature Reviews Microbiology, 2003. **1**(3): p. 237-242.
45. Carere, C.R., et al., *Third generation biofuels via direct cellulose fermentation.* International Journal of Molecular Sciences, 2008. **9**(7): p. 1342-1360.
46. Desvaux, M., *Unravelling carbon metabolism in anaerobic cellulolytic bacteria.* Biotechnology Progress, 2006. **22**(5): p. 1229-1238.
47. Drake, H.L., A.S. Gössner, and S.L. Daniel, *Old acetogens, new light.* Annals of the New York Academy of Sciences, 2008. **1125**: p. 100-128.
48. Chen, J.S., J. Toth, and M. Kasap, *Nitrogen-fixation genes and nitrogenase activity in Clostridium acetobutylicum and Clostridium beijerinckii.* Journal of Industrial Microbiology & Biotechnology, 2001. **27**(5): p. 281-286.
49. Schuchmann, K. and V. Müller, *Autotrophy at the thermodynamic limit of life: A model for energy conservation in acetogenic bacteria.* Nature Reviews Microbiology, 2014. **12**(12): p. 809-821.
50. Köpke, M., et al., *Fermentative production of ethanol from carbon monoxide.* Current Opinion in Biotechnology, 2011. **22**(3): p. 320-325.
51. Müller, V., *Energy conservation in acetogenic bacteria.* Applied and Environmental Microbiology, 2003. **69**(11): p. 6345-6353.
52. Ragsdale, S.W. and E. Pierce, *Acetogenesis and the Wood–Ljungdahl pathway of CO2 fixation.* Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 2008. **1784**(12): p. 1873-1898.
53. Henstra, A.M., et al., *Microbiology of synthesis gas fermentation for biofuel production.* Current Opinion in Biotechnology, 2007. **18**(3): p. 200-206.
54. Martin, M., *Comparing ethanol production of carboxydotrophic clostridium strains during syngas fermentation with a two-stage continuous culture.* 2014.
55. Brown, S.D., et al., *Comparison of single-molecule sequencing and hybrid approaches for finishing the genome of Clostridium*

autoethanogenum and analysis of CRISPR systems in industrial relevant Clostridia. Biotechnology for Biofuels, 2014. **7**(40): p. 1-18.
56. Abrini, J., H. Naveau, and E.-J. Nyns, *Clostridium autoethanogenum, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide.* Archives of Microbiology, 1994. **161**(4): p. 345-351.
57. Tanner, R.S., L.M. Miller, and D. Yang, *Clostridium ljungdahlii sp. nov., an acetogenic species in clostridial rRNA homology group I.* Int J Syst Bacteriol, 1993. **43**(2): p. 232-6.
58. Drake, H.L., K. Küsel, and C. Matthies, *Acetogenic prokaryotes*, in *The Prokaryotes*, M. Dworkin, et al., Editors. 2006, Springer New York. p. 354-420.
59. Ragsdale, S.W., *The Eastern and Western branches of the Wood/Ljungdahl pathway: how the East and West were won.* BioFactors, 1997. **6**(1): p. 3-11.
60. Stupperich, E., et al., *Carbon monoxide fixation into the carboxyl group of acetyl coenzyme A during autotrophic growth of Methanobacterium.* FEBS Letters, 1983. **152**(1): p. 21-23.
61. Ragsdale, S.W., *Enzymology of the Wood-Ljungdahl pathway of acetogenesis.* Annals of the New York Academy of Sciences, 2008. **1125**: p. 129-136.
62. Jeoung, J.H., et al., *Carbon monoxide. Toxic gas and fuel for anaerobes and aerobes: carbon monoxide dehydrogenases.* Met Ions Life Sci, 2014. **14**: p. 37-69.
63. Ragsdale, S.W., *Life with carbon monoxide.* Critical Reviews in Biochemistry and Molecular Biology, 2004. **39**(3): p. 165-195.
64. Pezacka, E. and H.G. Wood, *The autotrophic pathway of acetogenic bacteria - Role of CO dehydrogenase disulfide reductase.* Journal of Biological Chemistry, 1986. **261**(4): p. 1609-1615.
65. Vignais, P.M. and A. Colbeau, *Molecular biology of microbial hydrogenases.* Current Issues in Molecular Biology, 2004. **6**: p. 159-188.
66. Calusinska, M., et al., *The surprising diversity of clostridial hydrogenases: a comparative genomic perspective.* Microbiology, 2010. **156**(Pt 6): p. 1575-88.
67. Vignais, P.M., *Hydrogenases and H^+-reduction in primary energy conservation.* Results and Problems in Cell Differentiation, 2007. **45**: p. 223-252.
68. Bertsch, J. and V. Muller, *Bioenergetic constraints for conversion of syngas to biofuels in acetogenic bacteria.* Biotechnol Biofuels, 2015. **8**: p. 210.
69. Hu, P., S.H. Bowen, and R.S. Lewis, *A thermodynamic analysis of electron production during syngas fermentation.* Bioresour Technol, 2011. **102**(17): p. 8071-6.
70. Thauer, R.K., K. Jungermann, and K. Decker, *Energy conservation in chemotrophic anerobic bacteria.* Bacteriological Reviews, 1977. **41**(1): p. 100-180.
71. Oelgeschläger, E. and M. Rother, *Carbon monoxide-dependent energy metabolism in anaerobic bacteria and archaea.* Archives of Microbiology, 2008. **190**(3): p. 257-69.

72. Chen, J., et al., *Infrared Studies of Carbon Monoxide Binding to Carbon Monoxide Dehydrogenase/Acetyl-CoA Synthase from Moorella thermoacetica.* Biochemistry, 2003. **42**(50): p. 14822-14830.
73. Drennan, C.L., T.I. Doukov, and S.W. Ragsdale, *The metalloclusters of carbon monoxide dehydrogenase/acetyl-CoA synthase: a story in pictures.* JBIC Journal of Biological Inorganic Chemistry, 2004. **9**(5): p. 511-515.
74. Ragsdale, S.W. and M. Kumar, *Nickel-Containing Carbon Monoxide Dehydrogenase/Acetyl-CoA Synthase.* Chemical Reviews, 1996. **96**(7): p. 2515-2540.
75. Ha, S.-W., et al., *Interaction of Potassium Cyanide with the [Ni-4Fe-5S] Active Site Cluster of CO Dehydrogenase from Carboxydothermus hydrogenoformans.* Journal of Biological Chemistry, 2007. **282**(14): p. 10639-10646.
76. Ragsdale, S.W., et al., *Properties of purified carbon monoxide dehydrogenase from Clostridium thermoaceticum, a nickel, iron-sulfur protein.* Journal of Biological Chemistry, 1983. **258**(4): p. 2364-2369.
77. Köpke, M., et al., *Clostridium ljungdahlii represents a microbial production platform based on syngas.* Proceedings of the National Academy of Sciences, 2010. **107**(29): p. 13087-13092.
78. !!! INVALID CITATION !!! {}.
79. Wang, S., et al., *NADP-specific electron-bifurcating [FeFe]-hydrogenase in a functional complex with formate dehydrogenase in Clostridium autoethanogenum grown on CO.* Journal of Bacteriology, 2013. **195**(19): p. 4373-4386.
80. Mock, J., et al., *Energy conservation associated with ethanol formation from H2 and CO2 in Clostridium autoethanogenum involving electron bifurcation.* Journal of Bacteriology, 2015. **Epub ahead of print**.
81. Tremblay, P., et al., *The Rnf complex of Clostridium ljungdahlii is a proton-translocating ferredoxin:NAD oxidoreductase essential for autotrophic growth.* mBio, 2012. **4**(1): p. 1-8.
82. Herrmann, G., et al., *Energy conservation via electron-transferring flavoprotein in anaerobic bacteria.* Journal of Bacteriology, 2008. **190**(3): p. 784-791.
83. Wang, S.N., et al., *NADP(+) reduction with reduced ferredoxin and NADP(+) reduction with NADH are coupled via an electron-bifurcating enzyme complex in Clostridium kluyveri.* Journal of Bacteriology, 2010. **192**(19): p. 5115-5123.
84. Köpke, M., et al., *Clostridium ljungdahlii represents a microbial production platform based on syngas.* PNAS USA, 2010. **107**(29): p. 13087-13092.
85. Miller, S.L. and G. Schlesinger, *Prebiotic syntheses of vitamin coenzymes: II. Pantoic acid, pantothenic acid, and the composition of coenzyme A.* J Mol Evol, 1993. **36**(4): p. 308-14.
86. Webb, M.E., et al., *Elucidating biosynthetic pathways for vitamins and cofactors.* Natural Product Reports, 2007. **24**(5): p. 988-1008.
87. Can, M., F.A. Armstrong, and S.W. Ragsdale, *Structure, Function, and Mechanism of the Nickel Metalloenzymes, CO Dehydrogenase, and Acetyl-CoA Synthase.* Chemical Reviews, 2014. **114**(8): p. 4149-4174.

88. Evans, D.J., *Chemistry relating to the nickel enzymes CODH and ACS.* Coordination Chemistry Reviews, 2005. **249**(15): p. 1582-1595.
89. Köpke, M., et al., *2,3-Butanediol Production by Acetogenic Bacteria, an Alternative Route to Chemical Synthesis, Using Industrial Waste Gas.* Applied and Environmental Microbiology, 2011. **77**(15): p. 5467-5475.
90. Furdui, C. and S.W. Ragsdale, *The roles of coenzyme A in the pyruvate:ferredoxin oxidoreductase reaction mechanism: rate enhancement of electron transfer from a radical intermediate to an iron-sulfur cluster.* Biochemistry, 2002. **41**(31): p. 9921-37.
91. Ragsdale, S.W., *Pyruvate Ferredoxin Oxidoreductase and Its Radical Intermediate.* Chemical Reviews, 2003. **103**(6): p. 2333-2346.
92. Alsaker, K.V. and E.T. Papoutsakis, *Transcriptional program of early sporulation and stationary-phase events in Clostridium acetobutylicum.* Journal of Bacteriology, 2005. **187**(20): p. 7103-7118.
93. Allen, T.D., et al., *Alkalibaculum bacchi gen. nov., sp. nov., a CO-oxidizing, ethanol-producing acetogen isolated from livestock-impacted soil.* International Journal of Systematic and Evolutionary Microbiology, 2010. **60**(Pt 10): p. 2483-2489.
94. Köpke, M., *Genetische Veränderung von Clostridium ljungdahlii zur produktion von 1-butanol aus synthesegas.* 2009, Ulm University.
95. Köpke, M., et al., *2,3-butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas.* Applied and Environmental Microbiology, 2011. **77**(15): p. 5467-5475.
96. Nölling, J., et al., *Genome sequence and comparative analysis of the solvent-producing bacterium Clostridium acetobutylicum.* Journal of Bacteriology, 2001. **183**(16): p. 4823-38.
97. Lee, S.Y., et al., *Fermentative butanol production by clostridia.* Biotechnology and Bioengineering, 2008. **101**(2): p. 209-228.
98. Hegner, R., et al., *Microbiome-based carboxylic acids production: from serum bottles to bioreactors.* RSC Advances, 2017. **7**(25): p. 15362-15371.
99. Mohammadi, M., et al., *Sustainable ethanol fermentation from synthesis gas by Clostridium ljungdahlii in a continuous stirred tank bioreactor.* Journal of Chemical Technology & Biotechnology, 2012. **87**(6): p. 837-843.
100. Vega, J.L., E.C. Clausen, and J.L. Gaddy, *Design of bioreactors for coal synthesis gas fermentations.* Resources, Conservation and Recycling, 1990. **3**(2-3): p. 149-160.
101. Haus, S., et al., *A systems biology approach to investigate the effect of pH-induced gene regulation on solvent production by Clostridium acetobutylicum in continuous culture.* BMC Systems Biology, 2011. **5**(1): p. 10-10.
102. Köpke, M. and P. Dürre, *Biochemical production of biobutanol*, R. Luque, J. Campelo, and J.H. Clark, Editors. 2011, Woodhead Publishing Ltd: Camebridge, UK. p. 221-257.
103. John R. Phillips, R.L.H.a.H.K.A., *Syngas Fermentation: A Microbial Conversion*

Process of Gaseous Substrates to Various Products. Fermentation, 2017.
104. White, H. and H. Simon, *Role of tungstate and/or molybdate in the formation of aldehyde oxidoreductase in Clostridium thermoaceticum*

and other acetogens; immunological distances of such. Archives of Microbiology, 1992: p. 81-84.
105. Simon, H., et al., *Reduction of 2-enoates and alkanoates with carbon monoxide or formate, viologens, and Clostridium thermoaceticum to saturated acids and unsaturated and saturated alcohols.* Angewandte Chemie-International Edition in English, 1987. **26**(8): p. 785-787.
106. Huber, C., et al., *Pterin cofactor, substrate specificity, and observations on the kinetics of the reversible tungsten-containing aldehyde oxidoreductase from Clostridium thermoaceticum - Preparative reductions of a series of carboxylates to alcohols.* Archives of Microbiology, 1995. **164**(2): p. 110-118.
107. Fast, A.G. and E.T. Papoutsakis, *Stoichiometric and energetic analyses of non-photosynthetic CO2-fixation pathways to support synthetic biology strategies for production of fuels and chemicals.* Current Opinion in Chemical Engineering, 2012. **1**(4): p. 380-395.
108. Basen, M., et al., *Single gene insertion drives bioalcohol production by a thermophilic archaeon.* PNAS USA, 2014. **111**(49): p. 17618-17623.
109. Tomas, C.A., J. Beamish, and E.T. Papoutsakis, *Transcriptional analysis of butanol stress and tolerance in Clostridium acetobutylicum.* Journal of Bacteriology, 2004. **186**(7): p. 2006-2018.
110. Gheshlaghi, R., et al., *Metabolic pathways of clostridia for producing butanol.* Biotechnology Advances, 2009. **27**(6): p. 764-81.
111. Huang, H., H. Liu, and Y.-R. Gan, *Genetic modification of critical enzymes and involved genes in butanol biosynthesis from biomass.* Biotechnology Advances, 2010. **28**(5): p. 651-657.
112. Köpke, M., et al., *Reconstruction of an acetogenic 2,3-butanediol pathway involving a novel NADPH-dependent primary-secondary alcohol dehydrogenase.* Applied and Environmental Microbiology, 2014. **80**(11): p. 3394-3303.
113. Richter, H., et al., *A Narrow pH Range Supports Butanol, Hexanol, and Octanol Production from Syngas in a Continuous Co-culture of Clostridium ljungdahlii and Clostridium kluyveri with In-Line Product Extraction.* Frontiers in Microbiology, 2016. **7**(1773).
114. Valgepea, K., et al., *Maintenance of ATP Homeostasis Triggers Metabolic Shifts in Gas-Fermenting Acetogens.* Cell Systems.
115. Bruant, G., et al., *Genomic analysis of carbon monoxide utilization and butanol production by Clostridium carboxidivorans strain P7.* PloS one, 2010. **5**(9): p. e13033.
116. Paul, D., et al., *Genome sequence of the solvent-producing bacterium Clostridium carboxidivorans strain P7T.* Journal of Bacteriology, 2010. **192**(20): p. 5554-5.
117. Seedorf, H., et al., *The genome of Clostridium kluyveri, a strict anaerobe with unique metabolic features.* PNAS USA, 2008. **105**(6): p. 2128-2133.
118. Hemme, C.L., et al., *Genome announcement - Sequencing of multiple clostridia genomes related to biomass conversion and biofuels production.* Journal of Bacteriology, 2010. **192**(24): p. 6494-6496.
119. Bruno-Barcena, J.M., M.S. Chinn, and A.M. Grunden, *Genome Sequence of the Autotrophic Acetogen Clostridium autoethanogenum JA1-1 Strain DSM 10061, a Producer of Ethanol from Carbon Monoxide.* Genome Announcements, 2013. **1**(4).

120. Humphreys, C.M., et al., *Whole genome sequence and manual annotation of Clostridium autoethanogenum, an industrially relevant bacterium.* BMC Genomics, 2015. **16**: p. 1085.
121. Allcock, E.R., et al., *Autolytic activity of an autolysin-deficient mutant of Clostridium acetobutylicum.* Appllied Environmental Microbiology, 1981. **42**: p. 929-935.
122. Hermann, M., et al., *Isolation and characterization of butanol-resistant mutants of Clostridium acetobutylicum.* Applied Environmental Microbiology, 1985. **50**: p. 1238-1243.
123. Van Der Westhuizen, a., D.T. Jones, and D.R. Woods, *Autolytic Activity and Butanol Tolerance of Clostridium acetobutylicum.* Applied and environmental microbiology, 1982. **44**(6): p. 1277-81.
124. Oultram, J.D., et al., *Introduction of genes for leucine biosynthesis from Clostridium pasteurianum into Clostridium acetobutylicum by cointegrate conjugal transfer.* Molecular and General Genetics, 1988. **214**(1): p. 177-179.
125. Williams, D.R., D.I. Young, and M. Young, *Conjugative plasmid transfer from Escherichia coli to Clostridium acetobutylicum.* Journal of General Microbiology, 1990. **136**: p. 819-826.
126. Herbert, M., et al., *Gene transfer into Clostridium difficile CD630 and characterisation of its methylase genes.* Fems Microbiology Letters, 2003. **229**(1): p. 103-110.
127. Theys, J., et al., *Repeated cycles of Clostridium-directed enzyme prodrug therapy result in sustained antitumour effects in vivo.* British Journal of Cancer, 2006. **95**(9): p. 1212-1219.
128. Oultram, J.D., et al., *Introduction of plasmids into whole cells of Clostridium acetobutylicum by electroporation.* FEMS Microbiology Letters, 1988. **56**: p. 83-88.
129. Birrer, G.A., W.R. Chesbro, and R.M. Zsigray, *Electrotransformation of Clostridium beijerinckii NRRL B-592 with shuttle plasmid pHR106 and recombinant derivatives.* Applied Microbiology and Biotechnology, 1994. **41**(1): p. 32-38.
130. Mermelstein, L.D., et al., *Expression of cloned homologous fermentative genes in Clostridium acetobutylicum ATCC 824.* Bio-Technology, 1992. **10**(2): p. 190-195.
131. Allen, S.P. and H.P. Blaschek, *Electroporation induced transformation of intact cells of Clostridium perfringens.* Applied and Environmental Microbiology, 1988. **54**(9): p. 2322-2324.
132. Jennert, K.C.B., et al., *Gene transfer to Clostridium cellulolyticum ATCC 35319.* Microbiology-UK, 2000. **146**: p. 3071-3080.
133. Reysset, G., et al., *Transfection and transformation of Clostridium acetobutvlicum Strain N1-4081 protoplasts.* Biotechnology Techniques, 1988. **2**(3): p. 199-204.
134. Leang, C., et al., *A Genetic System for Clostridium ljungdahlii: A Chassis for Autotrophic Production of Biocommodities and a Model Homoacetogen.* Applied and Environmental Microbiology, 2012.
135. Pyne, M.E., et al., *Technical guide for genetic advancement of underdeveloped and intractable Clostridium.* Biotechnol Adv, 2014. **32**(3): p. 623-41.

136. Jones, D.T., et al., *Bacteriophage infections in the industrial acetone butanol (AB) fermentation process.* J Mol Microbiol Biotechnol, 2000. **2**(1): p. 21-6.
137. Williams, D.R., D.I. Young, and M. Young, *Conjugative plasmid transfer from Escherichia coli to Clostridium acetobutylicum.* Microbiology, 1990. **136**(5): p. 819-826.
138. Heap, J.T., et al., *A modular system for Clostridium shuttle plasmids.* J Microbiol Methods, 2009. **78**(1): p. 79-85.
139. Heap, J.T., et al., *Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker.* Nucleic Acids Res, 2012. **40**(8): p. e59.
140. Heap, J.T., et al., *The ClosTron: A universal gene knock-out system for the genus Clostridium.* Journal of Microbiological Methods, 2007. **70**(3): p. 452-464.
141. Wilkinson, S.R. and M. Young, *Targeted integration of genes into the Clostridium acetobutylicum chromosome.* Microbiology-Uk, 1994. **140**: p. 89-95.
142. Shimizu, T., et al., *The virr gene, a member of a class of 2-component response regulators, regulates the production of perfringolysin-O, collagenase, and hemagglutinin in Clostridium perfringens.* Journal of Bacteriology, 1994. **176**(6): p. 1616-1623.
143. Green, E.M., et al., *Genetic manipulation of acid formation pathways by gene inactivation in Clostridium acetobutylicum ATCC 824.* Microbiology-Uk, 1996. **142**: p. 2079-2086.
144. Green, E.M. and G.N. Bennett, *Inactivation of an aldehyde/alcohol dehydrogenase gene from Clostridium acetobutylicum ATCC 824.* Applied Biochemistry and Biotechnology, 1996. **57-58**: p. 213-221.
145. Liyanage, H., et al., *Clostridium beijerinckii and Clostridium difficile detoxify methylglyoxal by a novel mechanism involving glycerol dehydrogenase.* Applied and Environmental Microbiology, 2001. **67**(5): p. 2004-2010.
146. O'Connor, J.R., et al., *Construction and analysis of chromosomal Clostridium difficile mutants.* Molecular Microbiology, 2006. **61**(5): p. 1335-1351.
147. Wilkinson, S.R., et al., *Molecular genetics and the initiation of solventogenesis in Clostridium beijerinckii (formerly Clostridium acetobutylicum) NCIMB 8052.* Fems Microbiology Reviews, 1995. **17**(3): p. 275-285.
148. Tripathi, S.A., et al., *Development of pyrF-Based Genetic System for Targeted Gene Deletion in Clostridium thermocellum and Creation of a pta Mutant.* Applied and Environmental Microbiology, 2010. **76**(19): p. 6591-6599.
149. Ng, Y.K., et al., *Expanding the repertoire of gene tools for precise manipulation of the Clostridium difficile genome: allelic exchange using pyrE alleles.* PLoS One, 2013. **8**(2): p. e56051.
150. *Database Resources of the National Center for Biotechnology Information.* Nucleic Acids Res, 2017. **45**(D1): p. D12-d17.
151. Kanehisa, M., et al., *KEGG as a reference resource for gene and protein annotation.* Nucleic Acids Research, 2016. **44**(D1): p. D457-D462.

152. Finn, R.D., et al., *The Pfam protein families database: towards a more sustainable future.* Nucleic Acids Research, 2016. **44**(D1): p. D279-D285.
153. *UniProt: the universal protein knowledgebase.* Nucleic Acids Research, 2017. **45**(D1): p. D158-D169.
154. Li, W., et al., *The EMBL-EBI bioinformatics web and programmatic tools framework.* Nucleic acids research, 2015. **43**(W1): p. W580-4.
155. Seidman, C.E., et al., *Introduction of Plasmid DNA into Cells*, in *Current Protocols in Molecular Biology*. 2001, John Wiley & Sons, Inc.
156. Xiao, Z. and P. Xu, *Acetoin Metabolism in Bacteria.* Critical Reviews in Microbiology, 2007. **33**(2): p. 127-140.
157. Wan, P., S. Moat, and A. Anstey, *Pellagra: a review with emphasis on photosensitivity.* British Journal of Dermatology, 2011. **164**(6): p. 1188-1200.
158. Berger, F., M.a.H. Ramírez-Hernández, and M. Ziegler, *The new life of a centenarian: signalling functions of NAD(P).* Trends in Biochemical Sciences, 2004. **29**(3): p. 111-118.
159. Stancek, M., R. Schnell, and M. Rydén-Aulin, *Analysis of Escherichia coli nicotinate mononucleotide adenylyltransferase mutants in vivo and in vitro.* BMC Biochemistry, 2005. **6**: p. 16-16.
160. Cronan, J.E., *Biotin and Lipoic Acid: Synthesis, Attachment and Regulation.* EcoSal Plus, 2014. **6**(1): p. 10.1128/ecosalplus.ESP-0001-2012.
161. Wegkamp, A., et al., *Characterization of the Role of para-Aminobenzoic Acid Biosynthesis in Folate Production by Lactococcus lactis.* Applied and Environmental Microbiology, 2007. **73**(8): p. 2673-2681.
162. Holliday, G.L., et al., *Evolution of enzymes and pathways for the biosynthesis of cofactors.* Natural Product Reports, 2007. **24**(5): p. 972-987.
163. Williams, R.J., et al., *The biochemistry of B vitamins*. 1950: Reinhold Publ. Corp., New York. x + 741 pp.
164. Dusch, N., A. Pühler, and J. Kalinowski, *Expression of the Corynebacterium glutamicum panD Gene Encoding l-Aspartate-α-Decarboxylase Leads to Pantothenate Overproduction in Escherichia coli.* Applied and Environmental Microbiology, 1999. **65**(4): p. 1530-1539.
165. Hüser, A.T., et al., *Rational Design of a Corynebacterium glutamicum Pantothenate Production Strain and Its Characterization by Metabolic Flux Analysis and Genome-Wide Transcriptional Profiling.* Applied and Environmental Microbiology, 2005. **71**(6): p. 3255-3268.
166. Webb, M.E., A.G. Smith, and C. Abell, *Biosynthesis of pantothenate.* Natural Product Reports, 2004. **21**(6): p. 695-721.
167. Sahm, H. and L. Eggeling, *d-Pantothenate Synthesis inCorynebacterium glutamicum and Use of panBC and Genes Encoding l-Valine Synthesis ford-Pantothenate Overproduction.* Applied and Environmental Microbiology, 1999. **65**(5): p. 1973-1979.
168. Begley, T.P., C. Kinsland, and E. Strauss, *The biosynthesis of coenzyme a in bacteria.* Vitamins & Hormones, 2001. **61**: p. 157-171.
169. Leonardi, R. and S. Jackowski, *Biosynthesis of Pantothenic Acid and Coenzyme A.* EcoSal Plus, 2007. **2**(2): p. 10.1128/ecosalplus.3.6.3.4.

170. Cohen, S.N., et al., *Construction of Biologically Functional Bacterial Plasmids In Vitro.* Proceedings of the National Academy of Sciences, 1973. **70**(11): p. 3240-3244.
171. Oh, S., et al., *Optimizing conditions for the growth of Lactobacillus casei YIT 9018 in tryptone-yeast extract-glucose medium by using response surface methodology.* Applied and Environmental Microbiology, 1995. **61**(11): p. 3809-14.
172. Pansare, S.V. and R.P. Jain, *Enantioselective Synthesis of (S)-(+)-Pantolactone.* Organic Letters, 2000. **2**(2): p. 175-177.
173. Aune, T.E. and F.L. Aachmann, *Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed.* Appl Microbiol Biotechnol, 2010. **85**(5): p. 1301-13.
174. HARRIS, S.A., et al., *SYNTHETIC BIOTIN.* Science, 1943. **97**(2524): p. 447-448.
175. Streit, W.R. and P. Entcheva, *Biotin in microbes, the genes involved in its biosynthesis, its biochemical role and perspectives for biotechnological production.* Applied Microbiology and Biotechnology, 2003. **61**(1): p. 21-31.
176. Yang, Y., et al., *Improving the performance of solventogenic clostridia by reinforcing the biotin synthetic pathway.* Metabolic Engineering, 2016. **35**: p. 121-128.
177. Bower, S., et al., *Cloning, sequencing, and characterization of the Bacillus subtilis biotin biosynthetic operon.* Journal of Bacteriology, 1996. **178**(14): p. 4122-30.
178. Lin, S., R.E. Hanson, and J.E. Cronan, *Biotin synthesis begins by hijacking the fatty acid synthetic pathway.* Nat Chem Biol, 2010. **6**(9): p. 682-688.
179. Lin, S. and J.E. Cronan, *Closing in on complete pathways of biotin biosynthesis.* Molecular BioSystems, 2011. **7**(6): p. 1811-1821.
180. Hebbeln, P., et al., *Biotin uptake in prokaryotes by solute transporters with an optional ATP-binding cassette-containing module.* Proceedings of the National Academy of Sciences, 2007. **104**(8): p. 2909-2914.
181. Tyo, K.E.J., P.K. Ajikumar, and G. Stephanopoulos, *Stabilized gene duplication enables long-term selection-free heterologous pathway expression.* Nat Biotech, 2009. **27**(8): p. 760-765.
182. Gasser, B., M. Dragosits, and D. Mattanovich, *Engineering of biotin-prototrophy in Pichia pastoris for robust production processes.* Metabolic Engineering, 2010. **12**(6): p. 573-580.
183. Obom, K.M., A. Magno, and P.J. Cummings, *Operation of a Benchtop Bioreactor.* Journal of Visualized Experiments : JoVE, 2013(79): p. 50582.
184. Richter, H., M. Martin, and L. Angenent, *A Two-Stage Continuous Fermentation System for Conversion of Syngas into Ethanol.* Energies, 2013. **6**(8): p. 3987.
185. Phillips, J.R., E.C. Clausen, and J.L. Gaddy, *Synthesis gas as substrate for the biological production of fuels and chemicals.* Applied Biochemistry and Biotechnology, 1994. **45-46**(1): p. 145-157.
186. Abubackar, H.N., M.C. Veiga, and C. Kennes, *Biological conversion of carbon monoxide to ethanol: effect of pH, gas pressure, reducing agent and yeast extract.* Bioresour Technol, 2012. **114**: p. 518-22.

187. Klasson, K.T., et al., *Biological conversion of coal and coal-derived synthesis gas.* Fuel, 1993. **72**(12): p. 1673-1678.
188. Ungerman, A.J. and T.J. Heindel, *Carbon monoxide mass transfer for syngas fermentation in a stirred tank reactor with dual impeller configurations.* Biotechnol Prog, 2007. **23**(3): p. 613-20.
189. Wang, Y.-P., et al., *Regulation of G6PD acetylation by SIRT2 and KAT9 modulates NADPH homeostasis and cell survival during oxidative stress.* The EMBO Journal, 2014. **33**(12): p. 1304-1320.
190. Dudley, B., *BP Energy Outlook 2017 Edition.* 2017.
191. Association, R.F., *2016 Ethanol Industry Outlook: A High Octane Future.* Renewable Fuels association, 2016.
192. Cheung, H., R.S. Tanke, and G.P. Torrence, *Acetic Acid*, in *Ullmann's Encyclopedia of Industrial Chemistry*. 2000, Wiley-VCH Verlag GmbH & Co. KGaA.
193. Hermann, B.G. and M. Patel, *Today's and tomorrow's bio-based bulk chemicals from white biotechnology.* Applied Biochemistry and Biotechnology, 2007. **136**(3): p. 361-388.
194. Analysts, G.I., *Global Acetic Acid Industry.* Report ID: 2284890, 2014.
195. Garg, S.K. and A. Jain, *Fermentative production of 2,3-butanediol: A review.* Bioresource Technology, 1995. **51**(2–3): p. 103-109.
196. Harden, A. and G.S. Walpole, *Chemical Action of Bacillus lactis aerogenes (Escherich) on Glucose and Mannitol: Production of 2: 3-Butyleneglycol and Acetylmethylcarbinol.* Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character, 1906. **77**(519): p. 399-405.
197. Syu, M.J., *Biological production of 2,3-butanediol.* Applied Microbiology and Biotechnology, 2001. **55**(1): p. 10-18.
198. Zeng, A.-P. and W. Sabra, *Microbial production of diols as platform chemicals: Recent progresses.* Current Opinion in Biotechnology, 2011. **22**(6): p. 749-757.
199. Qin, J., et al., *Production of 2,3-Butanediol by Klebsiella Pneumoniae Using Glucose and Ammonium Phosphate.* Chinese Journal of Chemical Engineering, 2006. **14**(1): p. 132-136.
200. Xu, Y., et al., *Systematic metabolic engineering of Escherichia coli for high-yield production of fuel bio-chemical 2,3-butanediol.* Metabolic Engineering, 2014. **23**(0): p. 22-33.
201. Celińska, E. and W. Grajek, *Biotechnological production of 2,3-butanediol—Current state and prospects.* Biotechnology Advances, 2009. **27**(6): p. 715-725.
202. John, R.P., K.M. Nampoothiri, and A. Pandey, *Fermentative production of lactic acid from biomass: An overview on process developments and future perspectives.* Applied Microbiology and Biotechnology, 2007. **74**(3): p. 524-534.

9 9 Supplementary Material

1.1 Chapter Four

1.1.1 Sequencing comparison of insert into pMTL_FJAL3

Sequence comparison of *PanBCD* insert into the plasmid pMTL_FJAL3. Genes originally excised from *C. acetobutylicum.*

GGCCGCATGAAAAATACCACTGAAACATTTAAAAATTCTAAATTTAAAAAAGAGAAGCTCGTTATGTTAA
CAGCCTATGATTACTCAACTGCAAAGATAATTGATTCCTGCGACATCAATGGAATTCTCGTTGGAGATTCT
CTTGGCATGGTATGTCTCGGATACGAAAATACCTTAAGTGTAACAATGGAAGACATGATTCATCATACAA
AAGCTGTAGTACGTGGTGCAAAAAGCACCCTTATTGTAGCTGACTTACCTTTTATGTCATATCAAACTTCA
GTTTACGATGCTGTTTTTAACGCCGGTAGGCTTGTCAAAGAAGCTGGTGCTACTGCCATTAAACTTGAGGG
CGGTGCTTTAGTCTGCGATAGAATAAAAGCAATCGTGGATGCTCAAATTCCTGTAATGGGCCATATTGGT
CTTACTCCCCAATCTGTAAATGCCTTTGGCGGTTTTAAAATTCAGGGAAAAAATATCTCTAAAGCAAAAG
AACTAATTGAAGATGCTAAAAAGATAGAAGCAGCTGGAGCCTTTGCTATTACTTTAGAGGGTATTCCTGA
GAAACTTGCTAAAATTATAACTGAAAGCATTACAATACAACTATAGGAATTGGTGCTGGTAAACATTGTG
ATGGTCAAATACTTGTTTATCAAGATAGCTTGGAATGTTTTCTGATTTGGCGCCTAAATTTGTTAAAAGAT
ATGGTAATATAGGTGACGACATGAAAGAAGCTTTTAATAGTTATGCAAAAGAAGTACGAGAAGGCACAT
TTCCTGACGAAGCTCACTCATTTAAGATTGATCAGAGCATTATAGACGAAATTACCAAATAAGAAGGGAA
GTAAGATATTATGGAAATTTTGCATTCCATTAGTGATGTTAAAAAGTATATAAAGCAATGGAAAAAAGAA
GGACTGACTATAGGACTAGTTCCAACTATGGGTTACCTACATGATGGACACAAAAGTCTAATTGAAAGAG
CCTCAAAAGAAAACGATAAAGTTATAGTAAGTGACTTTGTAAATCCAATACAATTTGGACCTAATGAAGA
CCTTGACGTTTATCCTAGAGATTTAGATAGAGACGCTGAGGTTTGCACTAAAGCCGGAGCTTCAATTTTAT
TTAATCCAGAGCCTTCAGAAATGTACTTTGATGATGCGGTTACATTTGTAAATTCTTCTAAGATTACAGAC
ATACTTTGCGGAGCTAGACGCCCCGGACACTTTCGAGGAGTTTGTACCGTAGTTACAAAATTGTTTAACAT
AACCTGTCCTGATAGAGCTTATTTTGGTGAAAAAGACGCTCAACAAGTAGCTGTAATTAAAAGGATGGTT
AGGGACTTAAATTTTGATATTGAAATAGTAGCATGTCCTATAATTCGTGAAGAAGATGGTCTTGCAAAAA
GTTCTAGAAATTCTTATCTTTCTTCTGAAGAGAGAAAAGCTGCAACTATTCTCTCTAAGAGCTTAAACCTT
GCAAAAGAACTTCTCGATAACGGAGAGAAAAATGTATATAACATAAAAAAAGCTATAATTATTGAAATA
GGAAAAGAACCTCTTGCAAAAATAGATTACGTTGAAGTTGTAGATAGTCTTTCCCTTAAGTCTGTTTCTAA
AGTACAGCAATCGATTCTTGTTGCAATTGCTGTTTATATAGGAAAAATACGCCTTATAGATAATTTCACTT
GGAATATATAAATAAACTTTTGGAGGAATAAAATGCATTTAAATATGTTAAAATCTAAAATTCATAGAGC
AACAGTTGTTCAAGCTGATTTAAATTATGTTGGAAGCATAACTATTGATAGAAACCTTATGGATAAAGCC
AATATACTTGAATACGAAAAGGTAGAGATAGCAAATATAAATAATGGTGCTAGATTTGAAACCTACGTAA
TAGCTGGAGAAGCTGGAAGTGGAATAATATGCCTTAATGGAGCCGCTGCAAGATGTGCCCAAGCTGGCG
ATAAAGTAATAATTATGTGCTATTGCAGCTTAACACCTGAAGAAGCATCAGAACATAGACCAAAAGTCGT
ATTTGTAAATGATGACAATAGTATATCTAATGTTACAGAGTACGAAAAGCACGGGACAATTGGCTAAG

Figure 66 PanBCD genes amplified from C. acetobutylicum for insertion into pMTL_FJAL3. Green; panB, Blue; panC Purple; panD

1.1.2 Genes inserted into pMTL_FJAL4

Sequence of the insert into pMTL_FJAL4. The genes inserted are *panC* and *panD* from *C. acetobutylicum*.

GAAGGGAAGTAAGATATTATGGAAATTTTGCATTCCATTAGTGATGTTAAAAAGTATATAAAGCAAT
GGAAAAAAGAAGGACTGACTATAGGACTAGTTCCAACTATGGGTTACCTACATGATGGACACAAA
AGTCTAATTGAAAGAGCCTCAAAAGAAAACGATAAAGTTATAGTAAGTGACTTTGTAAATCCAATA
CAATTTGGACCTAATGAAGACCTTGACGTTTATCCTAGAGATTTAGATAGAGACGCTGAGGTTTGCA
CTAAAGCCGGAGCTTCAATTTTATTTAATCCAGAGCCTTCAGAAATTACTTTGATGATGCGGTTACAT
TTGTAAATTCTTCTAAGATTACAGACATACTTTGCGGAGCTAGACGCCCCGGACACTTTCGAGGAGT
TTGTACCGTAGTTACAAAATTGTTTAACATAACCTGTCCTGATAGAGCTTATTTTGGTGAAAAAGAC
GCTCAACAAGTAGCTGTAATTAAAAGGATGGTTAGGGACTTAAATTTTGATATTGAATAGTAGCATG
TCCTATAATTCGTGAAGAAGATGGTCTTGCAAAAAGTTCTAGAAATTCTTATCTTTCTTCTGAAGAGA
GAAAAGCTGCAACTATTCTCTCTAAGAGCTTAAACCTTGCAAAAGAACTTCTCGATAACGGAGAGA

AAAATGTATATAACATAAAAAAAGCTATAATTATTGAAATAGGAAAAGAACCTCTTGCAAAATAGA
TTACGTTGAAGTTGTAGATAGTCTTTCCCTTAAGTCTGTTTCTAAAGTACAGCAATCGATTCTTGTTGC
AATTGCTGTTTATATAGGAAAAATACGCCTTATAGATAATTTCACTTGGAATATATAAATAAACTTTT
GGAGGAATAAAATGCATTTAAATATGTTAAAATCTAAAATTCATAGAGCAACAGTTGTTCAAGCTG
ATTTAAATTATGTTGGAAGCATAACTATTGATAGAAACCTTATGGATAAAGCCAATTACTTGAATAC
GAAAAGGTAGAGATAGCAAATATAAATAATGGTGCTAGATTTGAAACCTACGTAATAGCTGGAGA
AGCTGGAAGTGGAATAATATGCCTTAATGGAGCCGCTGCAAGATGTGCCCAAGCTGGCGATAAAGT
AATAATTATGTGCTATTGCAGCTTAACACCTGAAGAAGCATCAGAACATAGACCAAAAGTCGTATTT
GTAAATGATGACAATAGTATATCTAATGTTACAGAGTACGAAAAGCACGGGACAATTGGCTAAG

Figure 67 PanCD fragment inserted into pMTL_FJAL4.
Blue; panC Purple; panD

1.1.3 Sequencing Comparison of insert into pMTL_FJAL5

Sequence of the insert into pMTL_FJAL5. The genes inserted are *panB* and *panC* from *C. acetobutylicum.*

GGCCGCATGAAAAATACCACTGAAACATTTAAAAATTCTAAATTTAAAAAAGAGAAGCTCGTTATG
TTAACAGCCTATGATTACTCAACTGCAAAGATAATTGATTCCTGCGACATCAATGGAATTCTCGTTG
GAGATTCTCTTGGCATGGTATGTCTCGGATACGAAAATACCTTAAGTGTAACAATGGAAGACATGAT
TCATCATACAAAAGCTGTAGTACGTGGTGCAAAAAGCACCCTTATTGTAGCTGACTTACCTTTTATGT
CATATCAAACTTCAGTTTACGATGCTGTTTTTAACGCCGGTAGGCTTGTCAAAGAAGCTGGTGCTACT
GCCATTAAACTTGAGGGCGGTGCTTTAGTCTGCGATAGAATAAAAGCAATCGTGGATGCTCAAATTC
CTGTAATGGGCCATATTGGTCTTACTCCCCAATCTGTAAATGCCTTTGGCGGTTTTAAAATTCAGGGA
AAAAATATCTCTAAAGCAAAAGAACTAATTGAAGATGCTAAAAAGATAGAAGCAGCTGGAGCCTT
TGCTATTACTTTAGAGGGTATTCCTGAGAAACTTGCTAAAATTATAACTGAAAGCATTACAATACCA
ACTATAGGAATTGGTGCTGGTAAACATTGTGATGGTCAAATACTTGTTTATCAAGATATGCTTGGAA
TGTTTTCTGATTTGGCGCCTAAATTTGTTAAAAGATATGGTAATATAGGTGACGACATGAAAGAAGC
TTTTAATAGTTATGCAAAAGAAGTACGAGAAGGCACATTTCCTGACGAAGCTCACTCATTTAAGATT
GATCAGAGCATTATAGACGAAATTACCAAATAAGAAGGGAAGTAAGATATTATGGAAATTTTGCAT
TCCATTAGTGATGTTAAAAAGTATATAAAGCAATGGAAAAAAGAAGGACTGACTATAGGACTAGTT
CCAACTATGGGTTACCTACATGATGGACACAAAAGTCTAATTGAAAGAGCCTCAAAAGAAAACGAT
AAAGTTATAGTAAGTGACTTTGTAAATCCAATACAATTTGGACCTAATGAAGACCTTGACGTTTATC
CTAGAGATTTAGATAGAGACGCTGAGGTTTGCACTAAAGCCGGAGCTTCAATTTTATTTAATCCAGA
GCCTTCAGAAATGTACTTTGATGATGCGGTTACATTTGTAAATTCTTCTAAGATTACAGACATACTTT
GCGGAGCTAGACGCCCCGGACACTTTCGAGGAGTTTGTACCGTAGTTACAAAATTGTTTAACATAAC
CTGTCCTGATAGAGCTTATTTTGGTGAAAAAGACGCTCAACAAGTAGCTGTAATTAAAAGGATGGTT
AGGGACTTAAATTTTGATATTGAAATAGTAGCATGTCCTATAATTCGTGAAGAAGATGGTCTTGCAA
AAAGTTCTAGAAATTCTTATCTTTCTTCTGAAGAGAGAAAAGCTGCAACTATTCTCTCTAAGAGCTTA
AACCTTGCAAAAGAACTTCTCGATAACGGAGAGAAAAATGTATATAACATAAAAAAAGCTATAATT
ATTGAAATAGGAAAAGAACCTCTTGCAAAAATAGATTACGTTGAAGTTGTAGATAGTCTTTCCCTTA
AGTCTGTTTCTAAAGTACAGCAATCGATTCTTGTTGCAATTGCTGTTTATATAGGAAAAATACGCCTT
ATAGATAATTTCACTTGGAATATATAA

Figure 68 PanB and PanC fragment inserted into pMTL_FJAL5.
Green; panB, Blue; panC

1.1.4 Insert sequence for pMTL_FJAL6

PanB gene from *C. acetobutylicum* as used in pMTL_FJAL6

ATGAAAAATACCACTGAAACATTTAAAAATTCTAAATTTAAAAAAGAGAAGCTCGTTATGTTAACA
GCCTATGATTACTCAACTGCAAAGATAATTGATTCCTGCGACATCAATGGAATTCTCGTTGGAGATT
CTCTTGGCATGGTATGTCTCGGATACGAAAATACCTTAAGTGTAACAATGGAAGACATGATTCATCA
TACAAAAGCTGTAGTACGTGGTGCAAAAAGCACCCTTATTGTAGCTGACTTACCTTTTATGTCATATC
AAACTTCAGTTTACGATGCTGTTTTTAACGCCGGTAGGCTTGTCAAAGAAGCTGGTGCTACTGCCATT
AAACTTGAGGGCGGTGCTTTAGTCTGCGATAGAATAAAAGCAATCGTGGATGCTCAAATTCCTGTAA
TGGGCCATATTGGTCTTACTCCCCAATCTGTAAATGCCTTTGGCGGTTTTAAAATTCAGGGAAAAAAT
ATCTCTAAAGCAAAAGAACTAATTGAAGATGCTAAAAAGATAGAAGCAGCTGGAGCCTTTGCTATT
ACTTTAGAGGGTATTCCTGAGAAACTTGCTAAAATTATAACTGAAAGCATTACAATACCAACTATAG
GAATTGGTGCTGGTAAACATTGTGATGGTCAAATACTTGTTTATCAAGATATGCTTGGAATGTTTTCT
GATTTGGCGCCTAAATTTGTTAAAAGATATGGTAATATAGGTGACGACATGAAAGAAGCTTTTAATA

GTTATGCAAAAGAAGTACGAGAAGGCACATTTCCTGACGAAGCTCACTCATTTAAGATTGATCAGA
GCATTATAGACGAAATTACCAAATAA

Figure 69 C. acetobutylicum panB gene sequence from FJAL6

Displayed above is the sequence of *panB*, incorporated into the pMTL-FJAL6 plasmid.

There was no difference between the expected and actual sequence.

1.1.5 Insert sequence for pMTL_FJAL7

PanC gene from C. *acetobutylicum* as used in pMTL_FJAL7

GAAGGGAAGTAAGATATTATGGAAATTTTGCATTCCATTAGTGATGTTAAAAAGTATATAAAGCAAT
GGAAAAAAGAAGGACTGACTATAGGACTAGTTCCAACTATGGGTTACCTACATGATGGACACAAA
AGTCTAATTGAAAGAGCCTCAAAAGAAAACGATAAAGTTATAGTAAGTGACTTTGTAAATCCAATA
CAATTTGGACCTAATGAAGACCTTGACGTTTATCCTAGAGATTTAGATAGAGACGCTGAGGTTTGCA
CTAAAGCCGGAGCTTCAATTTTATTTAATCCAGAGCCTTCAGAAATGTACTTTGATGATGCGGTTACA
TTTGTAAATTCTTCTAAGATTACAGACATACTTTGCGGAGCTAGACGCCCCGGACACTTTCGAGGAG
TTTGTACCGTAGTTACAAAATTGTTTAACATAACCTGTCCTGATAGAGCTTATTTTGGTGAAAAAGAC
GCTCAACAAGTAGCTGTAATTAAAAGGATGGTTAGGGACTTAAATTTTGATATTGAAATAGTAGCAT
GTCCTATAATTCGTGAAGAAGATGGTCTTGCAAAAAGTTCTAGAAATTCTTATCTTTCTTCTGAAGAG
AGAAAAGCTGCAACTATTCTCTCTAAGAGCTTAAACCTTGCAAAAGAACTTCTCGATAACGGAGAG
AAAAATGTATATAACATAAAAAAAGCTATAATTATTGAAATAGGAAAAGAACCTCTTGCAAAAATA
GATTACGTTGAAGTTGTAGATAGTCTTTCCCTTAAGTCTGTTTCTAAAGTACAGCAATCGATTCTTGTT
GCAATTGCTGTTTATATAGGAAAAATACGCCTTATAGATAATTTCACTTGGAATATATAA

Figure 70 C. acetobutylicum panC gene sequence from FJAL7.
The highlighted green section is not part of the panC gene and the highlighted blue section is the start of the panC gene.
Displayed above is the sequence of *panC*, incorporated into the pMTL-FJAL7 plasmid.

There was no difference in the expected and the sequencing results.

1.1.6 Insert Sequence for pMTL_FJAL8

PanD gene from C. *acetobutylicum* as used in pMTL_FJAL7

ATAAACTTTTGGAGGAATAAAATGCATTTAAATATGTTAAAATCTAAAATTCATAGAGCAACAGTTG
TTCAAGCTGATTTAAATTATGTTGGAAGCATAACTATTGATAGAAACCTTATGGATAAAGCCAATAT
ACTTGAATACGAAAAGGTAGAGATAGCAAATATAAATAATGGTGCTAGATTTGAAACCTACGTAAT
AGCTGGAGAAGCTGGAAGTGGAATAATATGCCTTAATGGAGCCGCTGCAAGATGTGCCCAAGCTGG
CGATAAAGTAATAATTATGTGCTATTGCAGCTTAACACCTGAAGAAGCATCAGAACATAGACCAAA
AGTCGTATTTGTAAATGATGACAATAGTATATCTAATGTTACAGAGTACGAAAAGCACGGGACAAT
TGGCTAAG

Figure 71 C. acetobutylicum panD gene sequence from FJAL8.
The Green Highlighted sequence indicates the part of the sequence not part of the panD gene and the blue highlighted region indicates the start of the panD gene
Displayed above is the sequence of *panD*, incorporated into the pMTL-FJAL8 plasmid.

There was no difference in the expected and the sequencing results.

1.1.7 *pyrE* deletion

```
EMBOSS_001         1 AGTTGGAATAGGTGGAATAGTAAGCTTTCAAGATGCGATAGAATTTATAA      50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         1 AGTTGGAATAGGTGGAATAGTAAGCTTTCAAGATGCGATAGAATTTATAA      50
```

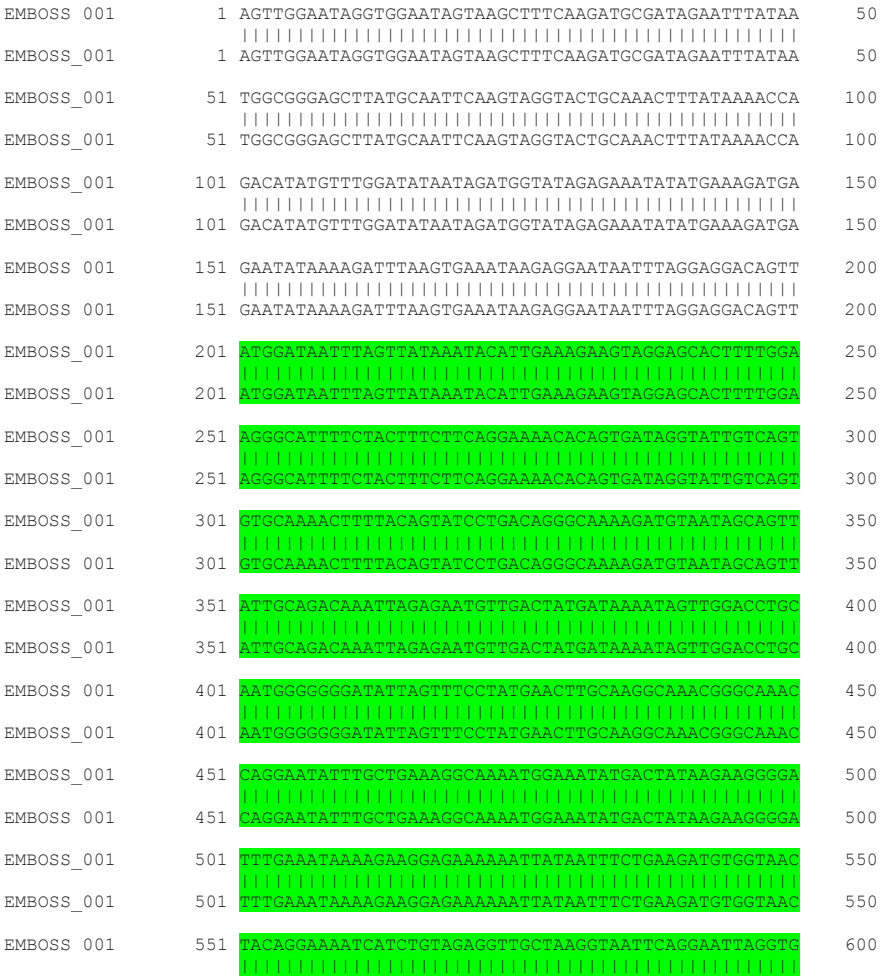




Figure 72 pairwise sequence analysis of the ΔpyrE strain of C. autoethanogenum, when compared to the wild type.
The ΔpyrE strain is on the bottom, the Wild type on the top. Yellow denotes the homology of the Long Homology arm downstream of the deletion (full sequence not shown due to length) , Green; is the expected sequence of pyrE, the bottom (ΔpyrE) strain shows the truncation of pyrE from the 5' end by 227bp, and the insertion of the lacZα gene shown in red.

1.1.8 *C. autoethanogenum +pantothenate* strain insertion at *pyrE* locus

Conformation of insertion of *panBCD* genes with P_{thl} promotor at *pyrE* locus with *pyrE* repair. Sequencing is identical for *C. ljungdahlii. pyrE repair and the first 700bp of panBCD insert are shown here, but the whole sequence is not shown due to length.*



```
EMBOSS_001      551 TACAGGAAAATCATCTGTAGAGGTTGCTAAGGTAATTCAGGAATTAGGTG    600
EMBOSS_001      601 GAGAGGTTGTAGGCATATGTTGCATAGTAGACAGAAGAGCAGAAGGTGTC    650
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      601 GAGAGGTTGTAGGCATATGTTGCATAGTAGACAGAAGAGCAGAAGGTGTC    650

EMBOSS_001      651 AAAATAGAATATCCAATTTATAGTGCAGTAAAACTTAATATAAACACTTA    700
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      651 AAAATAGAATATCCAATTTATAGTGCAGTAAAACTTAATATAAACACTTA    700

EMBOSS_001      701 TGATAAGGAAAATTGTCCTATGTGTAAGCAAGGACAAGAATATGTAAAGC    750
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      701 TGATAAGGAAAATTGTCCTATGTGTAAGCAAGGACAAGAATATGTAAAGC    750

EMBOSS_001      751 CTGGAAGTAGAGTATTCAAATAA----------------------------    773
                    |||||||||||||||||||||||
EMBOSS_001      751 CTGGAAGTAGAGTATTCAAATAAGCGGCCGCACTAGCCCGCTTTTTAACA    800

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001      801 AAATATATTGATAAAAATAATAATAGTGGGTATAATTAAGTTGTTAGAGA    850

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001      851 AAACGTATAAATTAGGGATAAACTATGGAACTTATGAAATAGATTGAAAT    900

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001      901 GGTTTATCTGTTACCCCACAAATAGCTTTAAGGAGGATTATTAATGAAAA    950

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001      951 ATACCACTGAAACATTTAAAAATTCTAAATTTAAAAAAGAGAAGCTCGTT   1000

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1001 ATGTTAACAGCCTATGATTACTCAACTGCAAAGATAATTGATTCCTGCGA   1050

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1051 CATCAATGGAATTCTCGTTGGAGATTCTCTTGGCATGGTATGTCTCGGAT   1100

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1101 ACGAAAATACCTTAAGTGTAACAATGGAAGACATGATTCATCATACAAAA   1150

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1151 GCTGTAGTACGTGGTGCAAAAAGCACCCTTATTGTAGCTGACTTACCTTT   1200

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1201 TATGTCATATCAAACTTCAGTTTACGATGCTGTTTTTAACGCCGGTAGGC   1250

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1251 TTGTCAAAGAAGCTGGTGCTACTGCCATTAAACTTGAGGGCGGTGCTTTA   1300

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1301 GTCTGCGATAGAATAAAAGCAATCGTGGATGCTCAAATTCCTGTAATGGG   1350

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1351 CCATATTGGTCTTACTCCCCAATCTGTAAATGCCTTTGGCGGTTTTAAAA   1400

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1401 TTCAGGGAAAAAATATCTCTAAAGCAAAAGAACTAATTGAAGATGCTAAA   1450

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1451 AAGATAGAAGCAGCTGGAGCCTTTGCTATTACTTTAGAGGGTATTCCTGA   1500

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1501 GAAACTTGCTAAAATTATAACTGAAAGCATTACAATACCAACTATAGGAA   1550

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1551 TTGGTGCTGGTAAACATTGTGATGGTCAAATACTTGTTTATCAAGATATG   1600

EMBOSS_001      774 ----------------------------------------------------    773

EMBOSS_001     1601 CTTGGAATGTTTTCTGATTTGGCGCCTAAATTTGTTAAAAGATATGGTAA   1650

EMBOSS_001      774 ----------------------------------------------------    773
```

```
EMBOSS_001      1651
```

Figure 73 pairwise sequence analysis of the transformed strain of C. autoethanogenum "+pantothenate" when compared to the wild type.
+pantothenate strain is on the bottom, the Wild type on the top. Green highlighted region denotes the repaired pyrE sequence, Pink; the P_{thl} promotor, Blue; the inserted panBCD genes. The first 700 bases of the panBCD insertion are shown here but the whole insertion is not shown here due to length.

1.1.9 *C. autoethanogenum weak pantothenate* strain insertion at *pyrE* locus

Conformation of insertion of *panBCD* genes with P_{sac_fdx} promotor at *pyrE* locus with *pyrE* repair. Repair of *pyrE* is shown and the first 300bp of the *panBCD* insert is shown but the rest of the alignment is omitted due to length.

```
EMBOSS_001         1 AGTTGGAATAGGTGGAATAGTAAGCTTTCAAGATGCGATAGAATTTATAA     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         1 AGTTGGAATAGGTGGAATAGTAAGCTTTCAAGATGCGATAGAATTTATAA     50

EMBOSS_001        51 TGGCGGGAGCTTATGCAATTCAAGTAGGTACTGCAAACTTTATAAAACCA    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        51 TGGCGGGAGCTTATGCAATTCAAGTAGGTACTGCAAACTTTATAAAACCA    100

EMBOSS_001       101 GACATATGTTTGGATATAATAGATGGTATAGAGAAATATATGAAAGATGA    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       101 GACATATGTTTGGATATAATAGATGGTATAGAGAAATATATGAAAGATGA    150

EMBOSS_001       151 GAATATAAAAGATTTAAGTGAAATAAGAGGAATAATTTAGGAGGACAGTT    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       151 GAATATAAAAGATTTAAGTGAAATAAGAGGAATAATTTAGGAGGACAGTT    200

EMBOSS_001       201 ATGGATAATTTAGTTATAAATACATTGAAAGAAGTAGGAGCACTTTTGGA    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       201 ATGGATAATTTAGTTATAAATACATTGAAAGAAGTAGGAGCACTTTTGGA    250

EMBOSS_001       251 AGGGCATTTTCTACTTTCTTCAGGAAAACACAGTGATAGGTATTGTCAGT    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       251 AGGGCATTTTCTACTTTCTTCAGGAAAACACAGTGATAGGTATTGTCAGT    300

EMBOSS_001       301 GTGCAAAACTTTTACAGTATCCTGACAGGGCAAAAGATGTAATAGCAGTT    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       301 GTGCAAAACTTTTACAGTATCCTGACAGGGCAAAAGATGTAATAGCAGTT    350

EMBOSS_001       351 ATTGCAGACAAATTAGAGAATGTTGACTATGATAAAATAGTTGGACCTGC    400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       351 ATTGCAGACAAATTAGAGAATGTTGACTATGATAAAATAGTTGGACCTGC    400

EMBOSS_001       401 AATGGGGGGGATATTAGTTTCCTATGAACTTGCAAGGCAAACGGGCAAAC    450
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       401 AATGGGGGGGATATTAGTTTCCTATGAACTTGCAAGGCAAACGGGCAAAC    450

EMBOSS_001       451 CAGGAATATTTGCTGAAAGGCAAAATGGAAATATGACTATAAGAAGGGGA    500
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       451 CAGGAATATTTGCTGAAAGGCAAAATGGAAATATGACTATAAGAAGGGGA    500

EMBOSS_001       501 TTTGAAATAAAAGAAGGAGAAAAAATTATAATTTCTGAAGATGTGGTAAC    550
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       501 TTTGAAATAAAAGAAGGAGAAAAAATTATAATTTCTGAAGATGTGGTAAC    550

EMBOSS_001       551 TACAGGAAAATCATCTGTAGAGGTTGCTAAGGTAATTCAGGAATTAGGTG    600
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       551 TACAGGAAAATCATCTGTAGAGGTTGCTAAGGTAATTCAGGAATTAGGTG    600

EMBOSS_001       601 GAGAGGTTGTAGGCATATGTTGCATAGTAGACAGAAGAGCAGAAGGTGTC    650
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       601 GAGAGGTTGTAGGCATATGTTGCATAGTAGACAGAAGAGCAGAAGGTGTC    650

EMBOSS_001       651 AAAATAGAATATCCAATTTATAGTGCAGTAAAACTTAATATAAACACTTA    700
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       651 AAAATAGAATATCCAATTTATAGTGCAGTAAAACTTAATATAAACACTTA    700

EMBOSS_001       701 TGATAAGGAAAATTGTCCTATGTGTAAGCAAGGACAAGAATATGTAAAGC    750
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       701 TGATAAGGAAAATTGTCCTATGTGTAAGCAAGGACAAGAATATGTAAAGC    750

EMBOSS_001       751 CTGGAAGTAGAGTATTCAAATAA--------------------------     773
                     |||||||||||||||||||||||
EMBOSS_001       751 CTGGAAGTAGAGTATTCAAATAAGCGGCCGCACTAGCAAATATAGTGGTT    800

EMBOSS_001       774 -------------------------------------------------     773
```

```
EMBOSS_001      801 TTATAATTGAAAATTATATAATTATATTAATACATAACTTAATATTTCTT    850
EMBOSS_001      774 --------------------------------------------------    773
EMBOSS_001      851 AGGAAATAATAAGACAAATAGCTTTAAGGAGGATTATTAATGAAAAATAC    900
EMBOSS_001      774 --------------------------------------------------    773
EMBOSS_001      901 CACTGAAACATTTAAAAATTCTAAATTTAAAAAAGAGAAGCTCGTTATGT    950
EMBOSS_001      774 --------------------------------------------------    773
EMBOSS_001      951 TAACAGCCTATGATTACTCAACTGCAAAGATAATTGATTCCTGCGACATC   1000
EMBOSS_001      774 --------------------------------------------------    773
EMBOSS_001     1001 AATGGAATTCTCGTTGGAGATTCTCTTGGCATGGTATGTCTCGGATACGA   1050
EMBOSS_001      774 --------------------------------------------------    773
EMBOSS_001     1051 AAATACCTTAAGTGTAACAATGGAAGACATGATTCATCATACAAAAGCTG   1100
EMBOSS_001      774 --------------------------------------------------    773
EMBOSS_001     1101 TAGTACGTGGTGCAAAAAGCACCCTTATTGTAGCTGACTTACCTTTTATG   1150
EMBOSS_001      774 --------------------------------------------------    773
EMBOSS_001     1151 TCATATCAAACTTCAGTTTACGATGCTGTTTTTAACGCCGGTAGGCTTGT   1200
```

Figure 74 pairwise sequence analysis of the transformed strain of C. autoethanogenum "+pantothenate" when compared to the wild type.
+pantothenate strain is on the bottom, the Wild type on the top. Due to the length of the sequence, only the beginning of the insertion is shown, but intermediate primers proved the whole panBCD fragment had been correctly inserted. Green highlighted region denotes the repaired pyrE sequence, Pink; the $P_{sac_fdx\ promotor}$, Blue; the inserted panBCD genes (regions not part of the gene not highlighted but expected)

1.2 Chapter Five Sequencing

1.2.1 Genes inserted into pMTL_FJAL20

Sequence of the insert into pMTL_FJAL20. The genes inserted are *BioYDA* from *C. acetobutylicum*.

ATGATTATGGTTTCTATTTTTGCAGCATTAACTGCAGTGGGGGCATTTATAAAAATACCAATACCATT
TGTTCCATTTACACTTCAATGGCTATTTTGTGCATTAAGTGGCATTATTCTTGGAGCTAAGTTAGGAG
CATTATCTCAAATAATTTATGTTGGAATGGGGCTTATGGGAGCTCCAATTTTTACGGAAGGTGGTGG
GATATATTACATATTCAAACCTACATTTGGATATCTTATGGGATTTATAGTTGCATCCTTTGTGATAG
GAAAATTGTCATCAAAAATAAAGAAAACAAATTTTATAAAATCCTTAATGTGTGTTTTAAGTGGTTT
AACTTTTGTGTATTTATTTGGGGTAGTTTATTTGTATGTAATTTATAACCTATACATGGGTGATAAAAA
AACTTTTTCATGGGCAATATTTTATGGATGTATTATTTGTATAGGGGGCGATATTTTGATAAGTATAA
TTGCTGCAATACTATCTATGAAGCTTAGAAGTATAAAAAATGTTAGTGAATTTGGAGGATTATAAAT
GAGAGGTATATATTTAGTTGGAACCGATACGGAAATTGGTAAGACAGTAATATCGGCGGCACTTGTT
TATATTTTATGTAAAGCAGGCTACAAAACAGCTTATTTTAAAGCAGCTTTAAGTGATGCTGAAGAAC
TTAACGGAAAGTTGATTCCTGGAGATACCGAATTTGTGTGCTCTCTTTCAGGAATACAGGAAGATTA
TGAAAAACTAACTCCGTACATATATAAAACGGCAGTATCTCCATACTTAGCATCAAAAATAGAAAA
TAAGCCGATTGATATACAAGTGATTAAGGACAAGCTTACTAAATTAGAAAATGAGTATGGCTATGT
ACTTTGTGAGGGGAGTGGAGGAGTAATTTGTCCAATTACTGATGTGGATAATAAGATATACACCTTG
GGTAATTTGATAAAAGATATGAAAATGGATGTTATCTTAGTTTGTAGAGCAGGTCTTGGAACTATAA
ATCATACTGTTTTAACTGTTAGTTACTTAAAAAATAATGGAATTGAGGTTAAAGGGATTATAGTAAA
TCAATATGAGGAAAATAGGCTTTGCAAAGATAATATAAATATGATTAAAAAGATGACAGGGATCCC
CATATTAGGGGTAATGCCTTATATTAATGAAAATAAAAAAAACTTTATAGAAGAATTAAAATATAA
TGCTTGTAATATATTCAAAGCAGATGAAATTGCAGGCTTAATGAAAGAAGTATTGAACTTGTTTGGTG
GTGGAAGATTATGAAAGATATTGAAAGAGATGATTTGAAATATATATGGCATCCTTGTACACAAAT
GAAGGATTATGAGAAGATGCCACCAATAGTAATCGATAAAGGTGAAGGCTGTTATCTATATGATGT
TAATGGAAATTCATATTTAGATTGCATAAGTTCTTGGTGGACAAATATTTTCGGGCATAGTAATAAG
AGAATTAATAAGGCAATTGAAAAGCAGTTAAATGAAATTGAACATGTTATTTTTGCACATTTTTCAA
ATAAGCCTGCTGTAGAATTTTCTAAAAGTCTTATTCAGATTGCCCCAGAGGGATTAGACAAGATATT
TTTTTCGGATAATGGATCTTCAGCTGTAGAGATAGCTCTAAAAATGAGTTTTCAATATTATCAGCAAA
CAGGAAAAGTTAAAAAGACTAAATTTGCAGCTTTAACTAATGCTTATCATGGAGAAACTATAGGGC
CCTTATCCGTTGGGGATTTAGACTTATATAGCAAAATTTATAAACCACTTCTTATAGATACCTTAAGA

ATCCAAGGGCCAGATTGCTATAGATGTAAATACAATAAGGAAAGACATAGCTGTGAAGCTGAATGT
TTTGAGGAAATGGAGAAAAGTCTTAAGAAACATAATAGTGAATTATGTGCTATTATTATTGAGCCTA
TGATTCAAGGGGCGGCAGGAATGAAAATGTATTCTCCTAAGTACTTAAAGAAGCTTAGATATGCAT
GTGATTATTATGATATAAGCGTTATATTTGATGAAATAGCTGTGGGGTTTGGAAGAACAGGAAAAAT
GTTTGCCTGCGAAAATGCTGACATAAGCCCTGATATAATGTGCCTCTCAAAGGGAATTAGTGCAGGC
TATATGCCTATGTCAGTTGTTTTAACTACTAGTAAAATATACAACGCATTTTATGATGATTATGTAAA
TTTAAAGGCTTTCATACACAGCCATACGTATGCTGGAAATGCTTTAGCTTGTGCGGCAGCAATTGAA
AGTCTTAAAATATTTAAAGAAGAGAGTATAATAGAAAAGAATATAGGGAAATCCAAGCTTATTGAG
AAGCTTACAAAAAATAGAGCAAGGAATAATAAGTTTATTGGTGATATTCGCAGTCTCGGTATGGTG
ACAGCAATAGAACTGGTTAAAGATAAAGATACAAAGGAGAGCTTTGACTGGAAAAGTAGAGTAGG
ATATGAAATATATAAAATAGCATTAAAAAAAGGCCTTCTTTTAAGACCTATAGGTGATGTGCTTTAT
TTTATGCCTCCTTATAGTATTGGAGAAAAAGATATAGAATTCATGGTGGAGAAATGTTTTGAAAGTA
TAGAGCAGTATCTTGAATAA

Figure 75 bioYDA fragment inserted into pMTL_FJAL20.
Green; bioY, Blue; bioD; Purple; bioA

1.2.2 Genes inserted into pMTL_FJAL21

Sequence of the insert into pMTL_FJAL21. The genes inserted are *bioBDF* from *D. nigrificans.*

ATGAGCAATATTTTGAAACATATCGAGAATAAACTGCTAAATGGGAAGTTAATTGACTTTGCAGAA
GCGCTGGAACTGTCTGAGGATAGGATTAAAAGTGAGGAATTATTTAACCTGGCAGATCAGATATGC
AAAAAATATATGGGGAATAAGGTTGATCTGTGCTCCATTATCAATGCAAAGTCGGGAGAGTGTTCTG
AGAACTGCAAATACTGTGCCCAGTCCGGACATTACAACACCGGGGTTCCAAGTTATCCGCTCATCC
ATATCCGGGAAGTATTGGCTTTAGCTAAAGAGAACGAAAGAAACGGCGTTCACAGGTTTTCCATTGT
AACCAGTGGAAAAAGCCTGACCAACAATGATTTTGAAAAAGTTTTAGAAATGATTGCAGCGTTGCG
GGTGGAGACAAATCTAAAGCTGTGTGCGTCTCTTGGCAGTATCTCCTTTGAACGGGCGTTACGTCTA
AAAGAGGCAGGTCTTTCAATGTACCACCACAATATTGAAACATGCAAGGAATACTATGCAAACATC
TGTGACACCCATACCTAGATGACAGAATACATACCATCAAAAATGCAAAAAAGGCAGGGCTTGAA
GTATGCTGCGGCGGTATTATTGGGATGGGCGAAAGTATGGAACAAAGGATAAAAATGGCCTTTGAA
ATCAGGGAGCTGGGTATTACTTCGATTCCTATCAATATCCTGAATCCCATTAAAGGGACACCCTTGG
AACATGTTGAAAAAATTGAGCCGATGGAAATAGTAAGGACCGTTTCATTGTTCCGGTTAATTATACC
CTATGCTTCTATTAGGTATGCCGGTGGAAGAAGTGCATTGGGAGAACTCCAGCAAAAGAGTTTCATC
GCTGGCCTCAATGCATTGATGGTCGGGAACTACCTGACAACGGTAGGGAATACAATTCCGGATGAC
GTAAAAATGATACAGGAAATTGGTCTGGAGGTATAGGCTTGGCAAGGGAAATATTTGTACTGGGTA
CAGATACAGATGTCGGGAAAACGGTGATATCGGCAGGCATCATGTACAGGCTTCTGTTAAAAGGGT
ATAAGGCCTGCTATTTTAAGCCTGTTTCCAGTGGTGGAGCCTACATTGGGGGAGACTTCTATTCCTAT
GATGTATCCTTTGTAAAAAACGTTTCGGGATTCCCTGAAGTGGAGGACAATATAAATCCCTTTCGAT
TTAGAACGCCGGTTTCACCACACCTGGCGTCCTCGCTTGAAAACAGGAAGATAAATATCAACGTGA
TATTGGATACATACAAAGAATTAAAAAAGAAATACGATTTCATCTTGGTGGAAGGGTGTGGAGGTC
TTGCTGTTCCCTTAACAGATGAAGGATACATGCAGTACCAGCTGATCAAGGAACTTGGCCTGGGGTG
TGTTTTGGTTTCTAAAACGACTCTAGGCACCATAAACCATACACTTTTGACATTAAAGTTTGCTGAAA
ACATTGGCATAAAGATCGAAGGTATACTTTTTAACGGCTACAATGGAAGCCGCTGGGAGGAAGACA
ATATCAAAACAATAAGAAAACTGGCGGATGTTGCTGTCCTCGGCATCATACCCAAAATGGAAGGAA
TTCATGTTGAAAACCTAAAAGCAGGAAATTTGAAAGCTGTTTTTGATGAATTAACATTTATCGAGGA
TTTAATTGCCGGAACAAAGGATTGAAAGCAAATGTACAAGTTTGCGGAAGCTTTGGAATCGATAAA
GGAAAAAGGTCTTTATCGAGAAATGTTGTATCTTGATGCTGCGCCAGGACCGCATACGATGGTTGGG
GGCAAACAGATGCTTCTGCTGTCATCGAACAACTATTTGGGTTTATGTAACGATGATCGGCTTAAGG
AAGCGGCCATTGCAACCATAAGAGAGTTCGGAGTGGGATCGGGTGGCTCAAGACTTACCACAGGA
AGCTACAGGTTGCACAGAGAACTGGAAGAAAGGCTTGCCCAATTTAAGGGAACGGAAGCCTGTCTG
GTTTTCAGTACGGGATATGCAGCAAATTTAGGGACAATCGCGGGTCTTGCGGACAAGGGCTGGGTC
ATCTTTTGCGACAGGCTGAATCATGCCAGCATCATTGACGGCTGCCGCTTGAGCGGAGCAAAGCTTG
TAATATACAAGCATTGTGACATGAACGATCTTGAGAAAAAGATTAAAAGGTATCATACAGGTAAAG
GTTTGATTGTGAGCGACGGGGTGTTCAGTATGGACGGAGATATAGCACCCCTTGCCGAAATCGTAAA
ACTAGCGAAAAAATATTCTATAATGACAATGGTTGACGATGCTCATGCCACAGGTGTTCTGGGACCG
AACGGGGGAGGTTCCGTCGATTACTTTGGCTTACAAAATGAAGTTGATATTCAAATGGGCACCTTGA
GCAAGGCTTTCGCCGGCGAAGGAGGATATATCGCCGGGAAGCAGAGCTTGATAGACTACTTGAGAC
ACAAGGCAAAAAGTTTTATCTACTCGACGGCCCTGGCACCGCATAATATAGCGGTTTCGTTAAAAGG
ACTGGAGCTCATTCAAAAAGAGCCTGAAAGAAGAAAAAGCCTCCTTGAGAAATCTCGGTGGTTCAG
AGGCGAATTGGTCAAAAATGGTTTTGACGTACCAATGAACAACACGCCGATTATACCGTTAATGGTT
GGTGAAGCAGACAAGGCGGTTACATTCAGCAGGATGCTATATGATGAGGGAGTCTATGTTCCTGCC
ATCAGGCCGCCGACGGTTCCCAATGGGTCCAGCCGGCTGCGTTTTTCCATCATGGCGACGCATACCC
ATGAGGATTTGGCGATGGCGCTTCAAAAGACTATTGTGATTGGAAAAGAACTGGGATTGATTTAA

Figure 76 bioBDF fragment inserted into pMTL_FJAL21
. Green; bioB, Blue; bioD; Purple; bioF

1.2.3 Genes inserted into pMTL_FJAL22

Sequence of the insert into pMTL_FJAL22. The genes inserted are *bioHCA* from *D. nigrificans.*

ATGGAAAAACAGAGATTAATCCTGCTGCCCGGATGGGGTATGGATGCCAAGGTGTGGAGCCCGGTT
ACAGAGGTTTTATCGAAATATTTTGTTCTTATTTATTGTGACTGGTACCGGGTGAAGTCGGTTGAAGA
GTATACCGCACGGGTTTCCCGTGTAATCGATAAAAACGTTGACGGGTGTTTTTCACTGCTTGGGTGGT
CTTTAGGCAGTCTATTGGCCATTGAAGCCGCATGTCTGTACAAAGATAGGATAAACAAGTTAATACT
TGTCAGTGGAACCAGCCGGTTTATCAGGGATAAAAAAAGCGGATATAGGTGCGGTTGGCCGCGGAA
TGTTGTTGAAAAGATGAAATCTGCGCTGCTCCATGATCGACAAAAAACGCTCGATTCCTTCTGTAAG
GCCATGTTTTCAGAAGAAGAAATACAACAAGGGTGGCCTGAGACGTTCACGGGAAAACCTGACCG
AAACAGAGGAACTTATGAGACACAGGAATTGTTGGCCGGCCTGGATTTTTTAATGCAAGCTGACTA
CCGGCAAAAGCTGCATGAAATCGAGGCGCCTGTGCTGATGATCCATGGCGAAAAAGACACCATTTG
TCCTGTTTCCGCCTCAGAATATATTTCGTCCCAATTGAAAGGGGATGTCTTCCTCAAGATTATGAAAG
GGGTTGGACATATTCCTTTCCATACGGGAATGGAAGAATTTAATTACCACTTAAAGGGGTTTATTAC
CAGAGGAAAAGCATGAATGATAGACAAGAACGTGCTTAAAAAAAATTTCAGCAGGAATGCCGTCA
ACTATGATACATACGCAGTCATTCAAAAGAAGATGGCCCATCAGTTGTTAAAGACAATTTCTTTTGA
ACACAAGGATACTCATGACAGTATTAACATTCTGGATATTGGATGTGGTACAGGATACTTCACAGAA
CAACTGGCACGTTGCTATCCAAATGCCAACATAACGGCTGTGGATATTGCACCGGGGATGATTGAA
TACGCCAGGAAAAAGTTAAGAAGCAAAAAAATTGAATTTCTATGTGCTGATATTGAGGAAGTAGAA
ATAAACAGGAAGTATGACCTGATTGTTTCCAATGCAACTTTTCAATGGTTCAACCAATTGGAACAGA
CCATCATAAAGCTTGATGCTATGTTGAAGAACAGTGGAATGCTTGCCTTCTCAACCTTTGGCCCAAT
GACTTTTAATGAGTTGCACCGGTCCTATAAAATGGCCGCAGATAAACTAAACCTGGTGATTGATTCA
CTGCCGGGACAGAAATTTTTTAGCTGCGAAGATCTTTTGAATATCTGTCAGAGAAGCTTGGCAGACA
CAAGCTTTTCGTATAAGGTTGCCGGTTTAGAAAAATATGAATATGAATACTTCGATACAGTAAGGGA
TTTTTTGAAATCTGTTAAAAAAATCGGTGCAAACAACAGTAATTGTGACCGTAAAACAATTCCGGCG
CTGATAAAAGAAATGATAAAGATATACGAAACTACATTTACAGAGGGCAACAGGATTAAAGTGAC
CTACCATTGCATTTTTTTGACGGTAGAAAAACGAAAGACAGATCCGGTATAAGGAGAAGCTAATGA
ATTTACATGAATTGCAAAAGAAAGATTTAAACTACATCTGGCACCCGTGTTCCCAGATGAAAGACT
ATGAAGAATTTCCGCCCATTGTTGTGGAGAGAGGAGAGGGTGCCTATCTCTTTGACGTTTCCGGTAA
CCGATACCTGGATGCAATTTCTTCGTGGTGGGTAAATCTTTTCGGCCATTGTAATCACAGAATTAACA
ATGCGGTCAAAAAGCAGCTTGATCAATTGGAACACGTCATATTTGCGAATTTTTCCCACCGACCGGC
CATCGAACTTGCGGAGAAAATTGTTGAAATAACTCCCGCTGGATTGAACAAAGTATTTTTTGCTGAT
AACGGTTCTTCGGCAGTTGAAGTTGCCTTAAAGATGAGTTTTCAATACCACCAGCAAACAGGAAGCT
GCCGAAAGAAAAGATTTATTGCTGTTTCAGACGCCTATCATGGTGAAACGATCGGGGCTTTATCAGT
TGGAGACATAGATCTTTACAGCAAAATCTATAAGCCCTTGTTATTGGATACCATCAAAGTACCCGGT
CCCGATTGTTACCGTTGTATCTATGGCCAGGAACGTCAAAGCTGCCAGACCGAATGTTTTCATCAGA
TGGAAAATGTTATCGAGCAAAAGCATAATGAGGTATGTGCAGTAATTATTGAACCAATGGTACAGG
CGGCAGCAGGAATGAAAATGTACCCTGCACAATACCTGACGAAACTAAGAGAGATCTGTTCTCAAT
ATAATATCCATCTTATCGCGGATGAAATCGCCGTGGGGTTTGGCCGAACCGGAAAGATGTTTGCCTG
TGAGCATGCATCTGTTTCCCCTGACATCATCTGCCTGTCAAAAGGACTTACAGCAGGCTACCTTCCG
CTTTCACTGGTGGTTGTAACCGATAATATATATAATGCCTTTTATAGTGAATACACCGAGCAAAAAG
CCTTTCTGCACAGCCACAGCTATACCGGAAATCCTTTGGCATGTTCTGTGGCTCTGGAATCATTAAAC
ATTTTCAAAGAAGACAATGTTTTAGAGCGAAACAAATCAAAGGCTGAACAAATTAACAAAATAAC
CAAAAACCTGGCAAACAAACATCCATATATCGGAGAGTTCAGACAGATAGGTATGATAGGCGCTTT
GGAGCTTGTTGAAGATAAAGTCACTAAACAAGCCTTTGCTTGGGAAAAACGGGTGGGGTACAATAT
CTGCAAACTAGCTTTAAAAGAGGGGTTGCTTTTAAGACCCTTAGGTAATGTTTTATACTTTATGCCTC
CTTATATTATCGAAGAAAAAGATATTGAACGGATGGTGACGGCGACTTTTAAA
GCAATTAATAACTATTTTGGAATAGATTAA

Figure 77 bioHCA fragment inserted into pMTL_FJAL22 . Red; bioH, Olive; bioC; Grey; bioA

1.2.4 *C. autoethanogenum* +*Biotin* strain insertion at *pyrE* locus

Conformation of insertion of *bioBDFHCA* genes with P_{thl} and P_{Fdx}promotor at *pyrE* locus with *pyrE* repair. Due to length, only conformation of *pyrE* repair and insertion of *bioB* is shown.

```
EMBOSS_001         1 AGTTGGAATAGGTGGAATAGTAAGCTTTCAAGATGCGATAGAATTTATAA      50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         1 AGTTGGAATAGGTGGAATAGTAAGCTTTCAAGATGCGATAGAATTTATAA      50

EMBOSS_001        51 TGGCGGGAGCTTATGCAATTCAAGTAGGTACTGCAAACTTTATAAAACCA     100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        51 TGGCGGGAGCTTATGCAATTCAAGTAGGTACTGCAAACTTTATAAAACCA     100
```


EMBOSS_001 101 GACATATGTTTGGATATAATAGATGGTATAGAGAAATATATGAAAGATGA 150
EMBOSS_001 101 GACATATGTTTGGATATAATAGATGGTATAGAGAAATATATGAAAGATGA 150
EMBOSS_001 151 GAATATAAAAGATTTAAGTGAAATAAGAGGAATAATTTAGGAGGACAGTT 200
EMBOSS_001 151 GAATATAAAAGATTTAAGTGAAATAAGAGGAATAATTTAGGAGGACAGTT 200
EMBOSS_001 201 ATGGATAATTTAGTTATAAATACATTGAAAGAAGTAGGAGCACTTTTGGA 250
EMBOSS_001 201 ATGGATAATTTAGTTATAAATACATTGAAAGAAGTAGGAGCACTTTTGGA 250
EMBOSS_001 251 AGGGCATTTTCTACTTTCTTCAGGAAAACACAGTGATAGGTATTGTCAGT 300
EMBOSS_001 251 AGGGCATTTTCTACTTTCTTCAGGAAAACACAGTGATAGGTATTGTCAGT 300
EMBOSS_001 301 GTGCAAAACTTTTACAGTATCCTGACAGGGCAAAAGATGTAATAGCAGTT 350
EMBOSS_001 301 GTGCAAAACTTTTACAGTATCCTGACAGGGCAAAAGATGTAATAGCAGTT 350
EMBOSS_001 351 ATTGCAGACAAATTAGAGAATGTTGACTATGATAAAATAGTTGGACCTGC 400
EMBOSS_001 351 ATTGCAGACAAATTAGAGAATGTTGACTATGATAAAATAGTTGGACCTGC 400
EMBOSS_001 401 AATGGGGGGGGATATTAGTTTCCTATGAACTTGCAAGGCAAACGGGCAAAC 450
EMBOSS_001 401 AATGGGGGGGGATATTAGTTTCCTATGAACTTGCAAGGCAAACGGGCAAAC 450
EMBOSS_001 451 CAGGAATATTTGCTGAAAGGCAAAATGGAAATATGACTATAAGAAGGGGA 500
EMBOSS_001 451 CAGGAATATTTGCTGAAAGGCAAAATGGAAATATGACTATAAGAAGGGGA 500
EMBOSS_001 501 TTTGAAATAAAAGAAGGAGAAAAAATTATAATTTCTGAAGATGTGGTAAC 550
EMBOSS_001 501 TTTGAAATAAAAGAAGGAGAAAAAATTATAATTTCTGAAGATGTGGTAAC 550
EMBOSS_001 551 TACAGGAAAATCATCTGTAGAGGTTGCTAAGGTAATTCAGGAATTAGGTG 600
EMBOSS_001 551 TACAGGAAAATCATCTGTAGAGGTTGCTAAGGTAATTCAGGAATTAGGTG 600
EMBOSS_001 601 GAGAGGTTGTAGGCATATGTTGCATAGTAGACAGAAGAGCAGAAGGTGTC 650
EMBOSS_001 601 GAGAGGTTGTAGGCATATGTTGCATAGTAGACAGAAGAGCAGAAGGTGTC 650
EMBOSS_001 651 AAAATAGAATATCCAATTTATAGTGCAGTAAAACTTAATATAAACACTTA 700
EMBOSS_001 651 AAAATAGAATATCCAATTTATAGTGCAGTAAAACTTAATATAAACACTTA 700
EMBOSS_001 701 TGATAAGGAAAATTGTCCTATGTGTAAGCAAGGACAAGAATATGTAAAGC 750
EMBOSS_001 701 TGATAAGGAAAATTGTCCTATGTGTAAGCAAGGACAAGAATATGTAAAGC 750
EMBOSS_001 751 CTGGAAGTAGAGTATTCAAATAAGCGGCCGCACTAGCCCGCTTTTTAACA 800
EMBOSS_001 751 CTGGAAGTAGAGTATTCAAATAA---------- 773
EMBOSS_001 801 AAATATATTGATAAAAATAATAATAGTGGGTATAATTAAGTTGTTAGAGA 850
EMBOSS_001 775 ---------- 774
EMBOSS_001 851 AAACGTATAAATTAGGGATAAACTATGGAACTTATGAAATAGATTGAAT 900
EMBOSS_001 775 ----------- 774
EMBOSS_001 901 CGTTTATCTGTTACCCCAATGAGCAATATTTTGAAACATATCGAGAATAA 950
EMBOSS_001 775 ---------- 774
EMBOSS_001 951 ACTGCTAAATGGGAAGTTAATTGACTTTGCAGAAGCGCTGGAACTGTCTG 1000
EMBOSS_001 775 ---------- 774
EMBOSS_001 1001 AGGATAGGATTAAAAGTGAGGAATTATTTAACCTGGCAGATCAGATATGC 1050
EMBOSS_001 775 ---------- 774

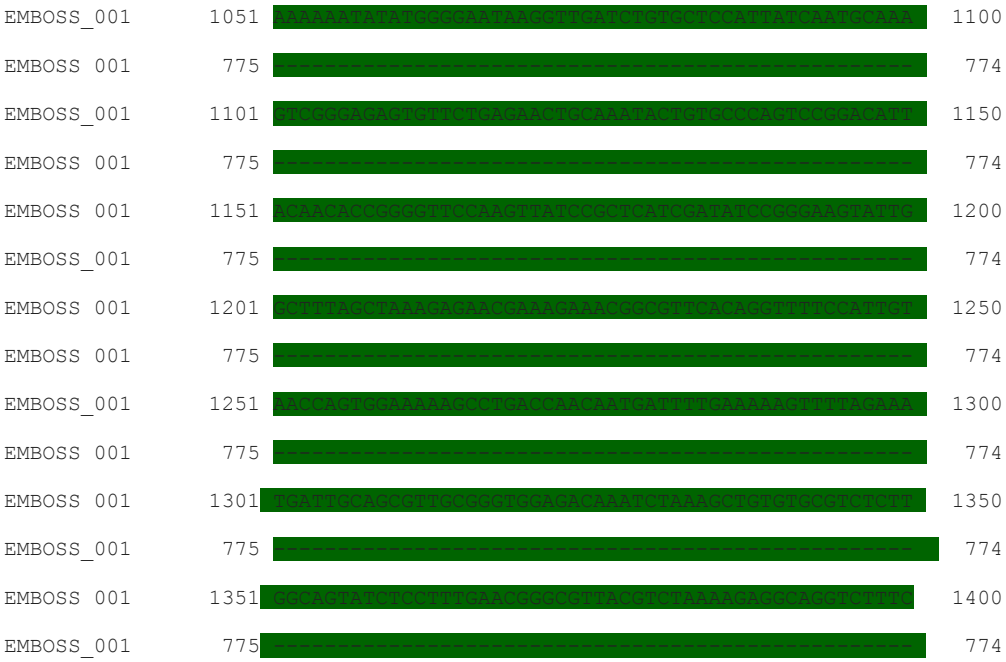


Figure 78 pairwise sequence analysis of the transformed strain of C. autoethanogenum "+biotin" when compared to the wild type.
+biotin is on the top, the Wild type on the bottom. Limited sequence shown here, the full sequence was roughly ten pages long. Light Green highlighted region denotes the repaired pyrE sequence, Pink; the P_{thl} and P_{fxd} promotor, dark Green; bioB,

1.2.5 "Big-biotin" Fragment insert for pMTL_FJAL24

CCGCTTTTTAACAAAATATATTGATAAAAATAATAATAGTGG
GTATAATTAAGTTGTTAGAGAAAACGTATAAATTAGGGATAAACTATGGAACTTATGAAATAGA
TTGAAATGGTTTATCTGTTACCCCAATGAGCAATATTTTGAAACATATCGAGAATAAACTGCTA
AATGGGAAGTTAATTGACTTTGCAGAAGCGCTGGAACTGTCTGAGGATAGGATTAAAAGT
GAGGAATTATTTAACCTGGCAGATCAGATATGCAAAAAATATATGGGGAATAAGGTTGATCTGT
GCTCCATTATCAATGCAAAGTCGGGAGAGTGTTCTGAGAACTGCAAATACTGTGCCCAGTCCGG
ACATTACAACACCGGGGTTCCAAGTTATCCGCTCATCGATATCCGGGAAGTATTGGCTTTAGCT
AAAGAGAACGAAAGAAACGGCGTTCACAGGTTTTCCATTGTAACCAGTGGAAAAAGCCTGACCA
ACAATGATTTTGAAAAAGTTTTAGAAATGATTGCAGCGTTGCGGGTGGAGACAAATCTAAAGCT
GTGTGCGTCTCTTGGCAGTATCTCCTTTGAACGGGCGTTACGTCTAAAAGAGGCAGGTCTTTCA
ATGTACCACCACAATATTGAAACATGCAAGGAATACTATGCAAACATCTGTGACACCCATACCT
ATGATGACAGAATACATACCATCAAAAATGCAAAAAAGGCAGGGCTTGAAGTATGCTGCGGCGG
TATTATTGGGATGGGCGAAAGTATGGAACAAAGGATAAAAATGGCCTTTGAAATCAGGGAGCTG
GGTATTACTTCGATTCCTATCAATATCCTGAATCCCATTAAAGGGACACCCTTGGAACATGTTG
AAAAAATTGAGCCGATGGAAATAGTAAGGACCGTTTCATTGTTCCGGTTAATTATACCCTATGC
TTCTATTAGGTATGCCGGTGGAAGAAGTGCATTGGGAGAACTCCAGCAAAAGAGTTTCATGGCT
GGCCTCAATGCATTGATGGTCGGGAACTACCTGACAACGGTAGGGAATACAATTCCGGATGACG
TAAAAATGATACAGGAAATTGGTCTGGAGGTATAGGCTTGGCAAGGGAAATATTTGTACTGGGT
ACAGATACAGATGTCGGGAAAACGGTGATATCGGCAGGCATCATGTACAGGCTTCTGTTAAAAG
GGTATAAGGCCTGCTATTTTAAGCCTGTTTCCAGTGGTGGAGCCTACATTGGGGGAGACTTCTA
TTCCTATGATGTATCCTTTGTAAAAAACGTTTCGGGATTCCCTGAAGTGGAGGACAATATAAAT
CCCTTTCGATTTAGAACGCCGGTTTCACCACACCTGGCGTCCTCGCTTGAAAACAGGAAGATAA
ATATCAACGTGATATTGGATACATACAAAGAATTAAAAAAGAAATACGATTTCATCTTGGTGGA
AGGGTGTGGAGGTCTTGCTGTTCCCTTAACAGATGAAGGATACATGCAGTACCAGCTGATCAAG
GAACTTGGCCTGGGGTGTGTTTTGGTTTCTAAAACGACTCTAGGCACCATAAACCATACACTTT
TGACATTAAAGTTTGCTGAAAACATTGGCATAAAGATCGAAGGTATACTTTTTAACGGCTACAA
TGGAAGCCGCTGGGAGGAAGACAATATCAAAACAATAAGAAAACTGGCGGATGTTGCTGTCCTC
GGCATCATACCCAAAATGGAAGGAATTCATGTTGAAAACCTAAAAGCAGGAAATTTGAAAGCTG
TTTTTGATGAATTAACATTTATCGAGGATTTAATTGCCGGAACAAAGGATTGAAAGCAAATGTA
CAAGTTTGCGGAAGCTTTGGAATCGATAAAGGAAAAAGGTCTTTATCGAGAAATGTTGTATCTT
GATGCTGCGCCAGGACCGCATACGATGGTTGGGGGCAAACAGATGCTTCTGCTGTCATCGAACA
ACTATTTGGGTTTATGTAACGATGATCGGCTTAAGGAAGCGGCCATTGCAACCATAAGAGAGTT
CGGAGTGGGATCGGGTGGCTCAAGACTTACCACAGGAAGCTACAGGTTGCACAGAGAACTGGAA
GAAAGGCTTGCCCAATTTAAGGGAACGGAAGCCTGTCTGGTTTTCAGTACGGGATATGCAGCAA
ATTTAGGGACAATCGCGGGTCTTGCGGACAAGGGCTGGGTCATCTTTTGCGACAGGCTGAATCA
TGCCAGCATCATTGACGGCTGCCGCTTGAGCGGAGCAAAGCTTGTAATATACAAGCATTGTGAC
ATGAACGATCTTGAGAAAAAGATTAAAAGGTATCATACAGGTAAAGGTTTGATTGTGAGCGACG
GGGTGTTCAGTATGGACGGAGATATAGCACCCCTTGCCGAAATCGTAAAACTAGCGAAAAAATA
TTCTATAATGACAATGGTTGACGATGCTCATGCCACAGGTGTTCTGGGACCGAACGGGGGAGGT

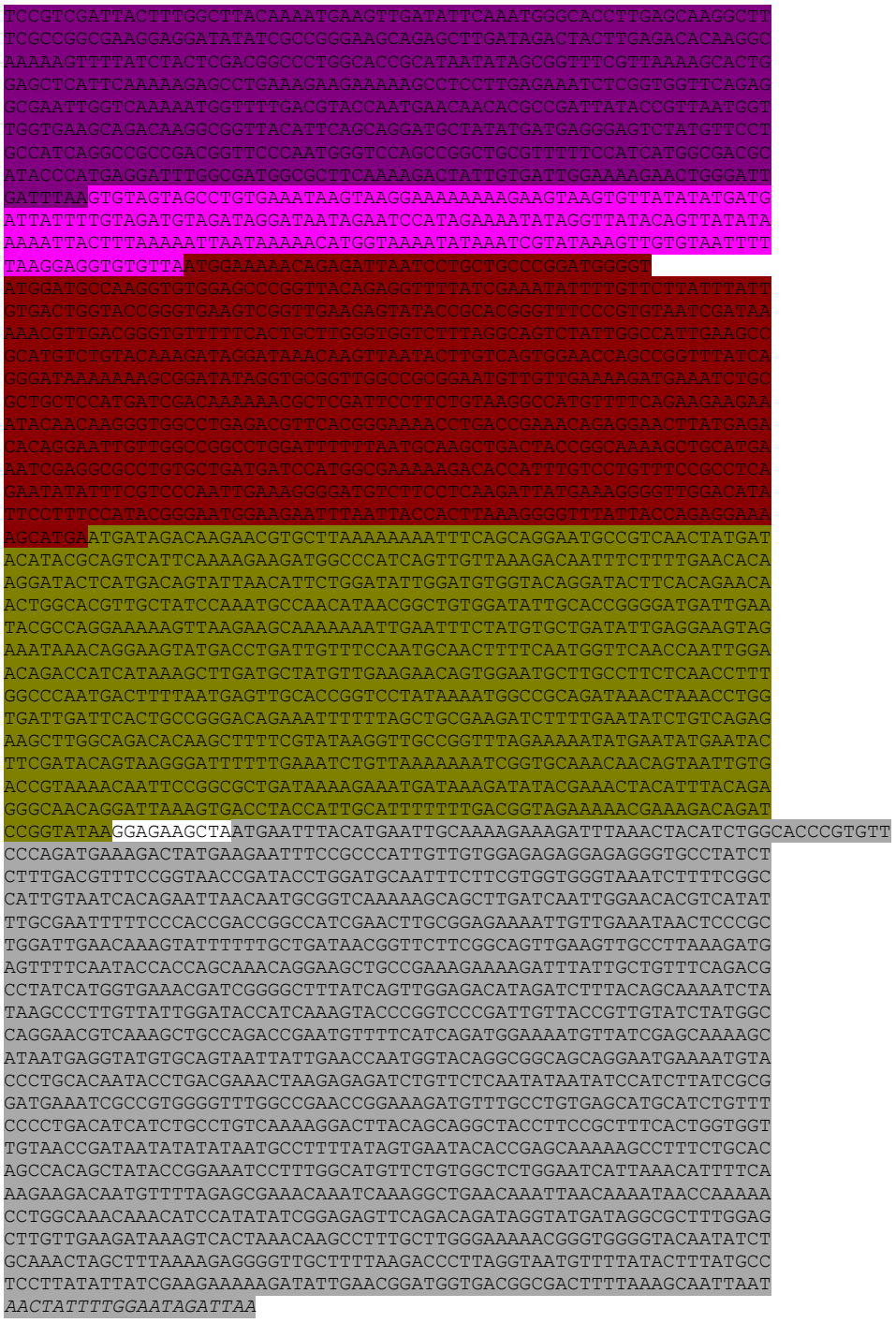


Figure 79 "big-biotin" fragment inserted into pMTL_FJAL24
Containing the genes bioBDFHCA from D. nigrificans under the control of the promotors P_{thl} and P_{fdx}.
Pink; the Pthl and Pfxd promotor, Green; bioB, Blue; bioD; Purple; bioF Red; bioH, Olive; bioC; Grey; bioA .