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Metabolic engineering of the non-sporulating, non-solventogenic *Clostridium acetobutylicum* strain M5 to produce butanol without acetone demonstrate the robustness of the acid-formation pathways and the importance of the electron balance

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ABSTRACT

The primary alcohol/aldehyde dehydrogenase (coded by the *aad* gene) is responsible for butanol formation in *Clostridium acetobutylicum*. We complemented the non-sporulating, non-solvent-producing *C. acetobutylicum* M5 strain (which has lost the pSOL1 megaplasmid containing *aad* and the acetone-formation genes) with *aad* expressed from the phosphotransbutyrylase promoter and restored butanol production to wild type levels. Because no acetone was produced, no acids (acetate or butyrate) were re-assimilated leading to high butyrate but especially acetate levels. To counter acetate production, we examined thiolase overexpression in order reduce the acetyl-CoA pool and enhance the butyryl-CoA pool. We combined thiolase overexpression with *aad* overexpression aiming to also enhance butanol formation. While limiting the formation of acetate and ethanol, the butanol titers were not improved. We also generated acetate kinase (AK) and butyrate kinase (BK) knockout (KO) mutants of M5 using a modified protocol to increase the antibiotic-resistance gene expression. These strains exhibited greater than 60% reduction in acetate or butyrate formation, respectively. We complemented the AKKO M5 strain with *aad* overexpression, but could not successfully transform the BKKO M5 strain. The AKKO M5 strain overexpressing *aad* produced less acetate, but also less butanol compared to the M5 *aad* overexpression strain. These data suggest that loss of the pSOL1 megaplasmid renders cells resistant to changes in the two acid-formation pathways, and especially so for butyrate formation. We argue that the difficulty in generating high butanol producers without acetone and acid production is hindered by the inability to control the electron flow, which appears to be affected by unknown pSOL1 genes.
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1. Introduction

Clostridium acetobutylicum is an anaerobic, spore-forming prokaryote that produces the solvents butanol, acetone, and ethanol. Increased interest in the development of biologically based chemicals and fuels has generated renewed attention in this industrially important strain. The desired product of the *C. acetobutylicum* fermentation is butanol, which has superior fuel characteristics to ethanol, such as higher energy content and lower water miscibility. The *C. acetobutylicum* genome has been sequenced and annotated (Nölling et al., 2001), and methods for genetic deletions (Harris et al., 2002a; Heap et al., 2007; Shao et al., 2007) and gene overexpression (Mermelstein and Papoutsakis, 1993) have been developed, making it an attractive organism for further strain development. Clostridia can also grow on a variety of substrates, from simple pentoses and hexoses to complex polysaccharides (Jones and Woods, 1986).

The metabolism of *C. acetobutylicum* is typically biphasic in batch culture: the cells first produce acetate and butyrate and later butanol, acetone, and ethanol. During growth, the production of acids lowers the pH of the culture, which combined with butyrate accumulation (Husemann and Papoutsakis, 1988) causes a shift in metabolism towards solvent production. As solvents are produced, the acids are typically re-assimilated and converted into solvents. With initiation of solvent formation, the cells commit to their sporulation program. In continuous culture or upon consecutive vegetative transfers, cells may degenerate whereby they become asporogenous and lose the capability to produce solvents. In this organism, the degeneration process is due to the loss of the pSOL1 megaplasmid (Cornillot et al., 1997), which carries the key solvent

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formation genes in the so-called *sol* locus made up of the *sol* operon (*aad–ctfA–ctfB*) (coding for the enzymes AAD and CoAT, Fig. 1) and the *adc* gene (coding for the enzyme AADC, Fig. 1). From the practical point of view, a solvent-producing, non-sporulating strain is most desirable because it is known that solventogenic clostridia produce solvents only during a rather narrow window of their sporulation program, namely during and/or near the stage of the characteristic, cigar-shaped, clostridial-cell form (Jones and Woods, 1986). The rest of the mixed-cell population does not likely contribute to solvent production, and this limits the specific cell productivity. Significantly, sporulating cells are not suitable for continuous or semicontinuous (fed-batch) fermentations due to the commitment to sporulation. Solvent-producing non-sporulating strains for other clostridia have been derived by random mutagenesis (Jain et al., 1993; Lemme and Frankiewiez, 1985). For *C. acetobutylicum*, chemical mutagenesis might not be an option, because it has been reported (Clark et al., 1989) to lead to strain degeneration (Cornillot et al., 1997). It has been shown however, that asporogenous strains (M5 and DG1) of *C. acetobutylicum* which have lost the pSOL1 megaplasmid can be complemented by plasmids carrying the *aad* gene (expressed from its autologous promoter) and this leads to butanol formation, albeit at relatively low levels (Cornillot et al., 1997; Nair and Papoutsakis, 1994), but no acetone production. Here, we examined whether such strains can be made to produce higher levels of butanol similar to those of the WT strain. Such strains would be very desirable for an additional important reason: they would not produce acetone, which is not a desirable product, thus significantly raising the selectivity of the process for butanol, and thus increasing its economic appeal.

In this study, we have used the non-sporulating, non-solventogenic strain M5 (Clark et al., 1989) as a platform in an effort to generate strains that produce primarily butanol. First, the strain was transformed with a plasmid to overexpress the *aad* gene from an alternate promoter that bypasses the endogenous solvent gene regulation. Efforts to further enhance butanol production were investigated using thiolase (*thl*, Fig. 1) overexpression, and inactivation of the acid-formation pathways, individually, by gene knockout (KO). From the fundamental point of view, this study also addresses the key question as to whether other pSOL1 genes are necessary for efficient cell growth and solvent production, and also if elimination of one of the acetate or butyrate formation pathways (Fig. 1) can be practically achieved in the M5 genetic background. The recently reported detailed transcriptional program at the full genome scale of this organism (Jones et al., 2008) has now elucidated much of the established physiology of this organism and provides a basis for more targeted metabolic engineering interventions (Papoutsakis, 2008) for producing solvent and other chemicals from this important industrial organism. This study adds substantial new information on the regulation of electron and carbon fluxes in this organism, and such information can now be exploited for useful applications in view of the aforementioned recent work. A key tool for pursuing these goals is the detailed metabolic flux analysis of the cell's primary metabolism (Fig. 1), as has been developed and extensively validated by our group over the years (Desai et al., 1999; Harris et al., 2000, 2001; Papoutsakis, 1984).

2. Materials and methods

2.1. Bacterial strains

The list of bacterial strains and plasmids are in Table 1.

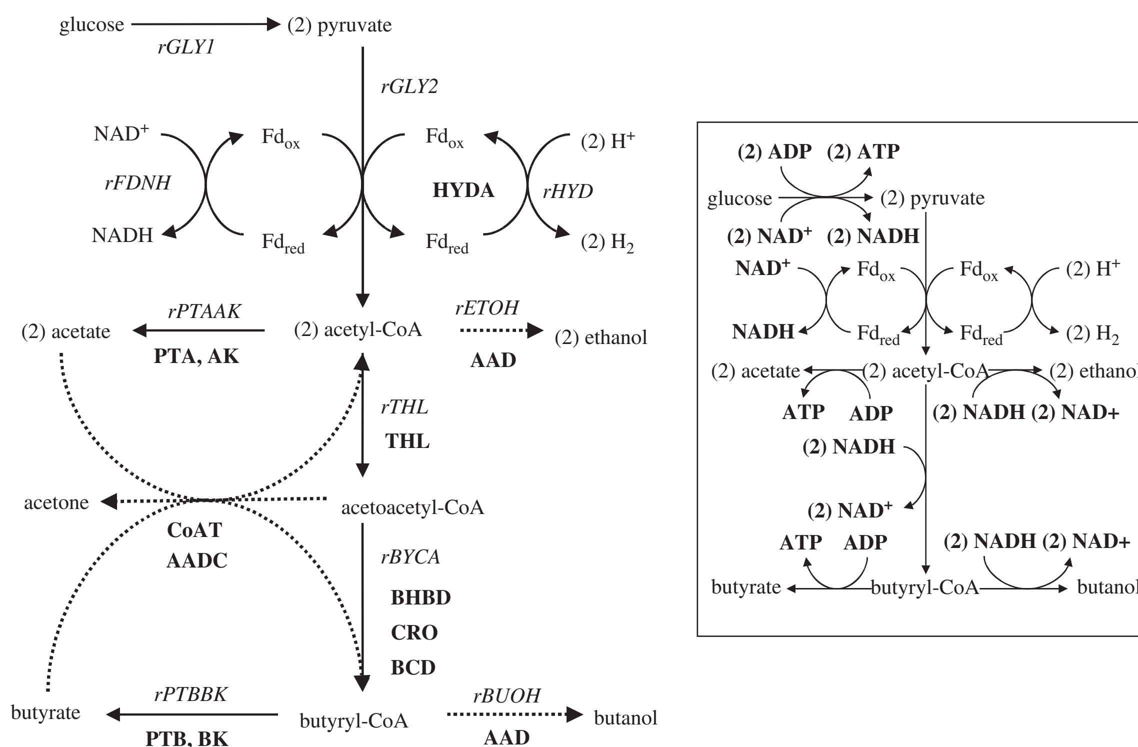


Fig. 1. Metabolic pathways in *C. acetobutylicum* and associated calculated *in vivo* fluxes. Selected enzymes are shown in bold and associated intracellular fluxes are shown in italics. Enzymes are abbreviated as follows: hydrogenase (HYDA); phosphotransacetylase (PTA); acetate kinase (AK); thiolase (THL); β-hydroxybutyryl dehydrogenase (BHBD); crotonase (CRO); butyryl-CoA dehydrogenase (BCD); CoA Transferase (CoAT); acetoacetate decarboxylase (AADC); butyrate kinase (BK); phophotransbutyrylase (PTB); alcohol/aldehyde dehydrogenase (AAD). *Note*: AAD is believed to be the primary enzyme for butanol and ethanol formation but additional genes exist that code for alcohol forming enzymes (*adhe2, bdhA, bdhB*, CAC3292, CAP0059). The pathways whose genes reside on the pSOL1 megaplasmid and are absent in M5 are shown as dotted lines. The boxed pathway shows the ATP generation and NADH production occurring during metabolism.

2.2. Culture conditions

Escherichia coli strains were grown aerobically at 37 °C and 200 rpm in liquid LB media or solid LB with agar (1.5%) media supplemented with the appropriate antibiotics (ampicillin at 50 μg/mL or chloramphenicol at 35 μg/mL). Frozen stocks were made from 1 mL overnight culture resuspended in LB containing 15% glycerol and stored at −85 °C. *C. acetobutylicum* strains were grown anaerobically at 37 °C in an anaerobic chamber (Thermo Forma, Waltham, MA). Cultures were grown in liquid CGM medium (Roos et al., 1985) (containing 0.75 g KH_2PO_4, 0.982 g K_2HPO_4, 1.0 g NaCl, 0.01 g $MnSO_4$, 0.004 g PABA, 0.348 g $MgSO_4$, 0.01 g $FeSO_4$, 2.0 g asparagine, 5.0 g yeast extract, 2.0 g $(NH_4)_2SO_4$, and 80 g glucose, all per liter) media or solid 2 × YTG pH 5.8 (containing 16 g Bacto tryptone, 10 g yeast extract, 4 g NaCl, and 5 g glucose, all per liter) plus agar (1.5%) supplemented with antibiotics as necessary (erythromycin at 100 μg/mL in liquid media and 40 μg/mL in solid media, thiamphenicol at 5 μg/mL). Frozen stocks were made from 10 mL of A_{600} 1.0 culture resuspended in 1 mL CGM containing 15% glycerol and stored at −85 °C.

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference[b]
Bacterial strains		
C. acetobutylicum		
M5	pSOL-	Clark et al. (1989)
M5 AKKO	pSOL-, *ack*-	This study
M5 BKKO	pSOL-, *buk*-	This study
E. coli		
Top10		Invitrogen
ER2275		New England Biolabs
Plasmids		
pAN1	Cm^r, Φ3T I gene, p15A origin	Mermelstein and Papoutsakis (1993)
pSOS94[c]	Acetone operon (*ptb* promoter)	Soucaille and Papoutsakis, unpublished
p94AAD3[c]	*aad* (*ptb* promoter)	This study
pTHLAAD[c]	*thl*, *aad* (*ptb* promoter)	This study
pLHKO[c]	Cm^r	Harris (2001)
pGW8-TOPO		Invitrogen
pSOS94-Cm[c]	Cm^r (*ptb* promoter)	This study
pAKKO	*ack* gene fragment, Cm^r (*ptb* promoter)	This study
pBKKO	*buk* gene fragment, Cm^r (*ptb* promoter)	This study

[a] Cm^r, chloramphenicol/thiamphenicol resistance gene; *ptb*, phosphotransbutyrylase gene; *aad*, alcohol/aldehyde dehydrogenase gene; *thl*, thiolase gene; *adc*, acetoacetate decarboxylase gene; *ack*, acetate kinase gene; and *buk*, butyrate kinase gene.
[b] ATCC, American Tissue Culture Collection, Rockville, MD.
[c] Contains the following: ampicillin resistance gene; macrolide, lincosimide, and streptogramin B resistance gene; *repL*, pIM13 Gram-positive origin of replication; ColE1 origin of replication.

2.3. Strain construction

The *aad* gene (CAP0162) was PCR amplified from *C. acetobutylicum* genomic DNA using primers (aad_fwd and aad_rev) to exclude the natural promoter. All primers and oligonucleotides used are shown in Table 2. The pSOS94 vector was digested with *Bam*HI and *Ehe*I and blunt ended, leaving the *ptb* promoter region (*ptb* codes for the first enzyme, PTB, of the butyrate formation from butyryl-CoA, Fig. 1). The *aad* PCR product and the linearized pSOS94 vector were ligated to create p94AAD3. The thiolase gene (*thl*) with its natural promoter was PCR amplified from genomic DNA (thl_fwd and thl_rev) with *Sal*I and *Eco*RI sites designed into the primers. The pIMP1 plasmid and the thiolase gene were digested with *Sal*I and *Eco*RI and ligated to create pTHL. Both pTHL and p94AAD3 were digested with *Sal*I to linearize pTHL and isolate the *aad* gene with the *ptb* promoter from p94AAD3. These fragments were ligated together to produce pTHLAAD.

Partial acetate kinase (*ack*; 341 bp) and butyrate kinase (*buk*: 446 bp) gene fragments were PCR amplified using the akko_fwd and akko_rev primers or the bkko_fwd and bkko_rev primers, respectively. Following PCR, the fragments were cloned into the pTOPO-GW8 cloning vector (Invitrogen, Carlsbad, CA) per manufacturer's directions. Following confirmation of positive clones, the vectors containing the *ack* or *buk* fragment were digested with *Dra*I or *Nsi*I, respectively. A modified chloramphenicol marker was generated by PCR amplifying a 687 bp region pLHKO with mod-CM/SDG-F and mod-CM/SDG-R primers. This fragment was then ligated into *Bam*HI and *Kas*I digested pSOS94. The new chloroamphenicol–thiamphenicol resistance gene was PCR amplified using primers pSOS94_fwd and pSOS94_rev. The fragment was ligated into the *Dra*I or *Nsi*I digested vectors described above to create pAKKO and pBKKO, respectively.

All plasmids were transformed into Top 10 chemically competent *E. coli* (Invitrogen). Plasmids were confirmed by sequencing. The plasmids were methylated using *E. coli* ER2275 (pAN1) to avoid the natural restriction system of *C. acetobutylicum* (Mermelstein and Papoutsakis, 1993). Once methylated, the plasmids were transformed by electroporating the *C. acetobutylicum* mutant M5 as described (Mermelstein et al., 1992).

Table 2
Primers used in plasmid construction

Primer name	Sequence (5′–3′)	Description
aad_fwd	TTAGAAAGAAGTGTATATTTAT	*aad* forward primer
aad_rev	AAACGACGGCCAGTGAAT	*aad* reverse primer
thl_fwd	CCATATGTCGACGGAAAGGCTTCA	*thl* forward primer
thl_rev	ACGCCTAGTACTGAATTCGCCTCA	*thl* reverse primer
mod-CM/SDG-F	CCGGATCCACTTGAATTTAAAAGGAGGGAACTTAGATGGTATTTGAAAAAATTGAT	Cm forward primer with modifed Shine-Dalgarno
mod-CM/SDG-R	CGGCGCCAGTTACAGACAAACCTGAAGT	Cm reverse primer
pSOS94_fwd	GGAATGGCGTGTGTGTTAGCCAAA	Cm and promoter forward primer
pSOS94_rev	TCACACAGGAAACAGCTATGACCA	Cm and promoter reverse primer
akko_fwd	ATATATGGCGCGCCAGGACACAGAATCGTTCATGGTGG	*ack* fragment forward primer
akko_rev	ATATATGGCGCGCCCCCTAACTTTAGAACCTTCTGCAC	*ack* fragment reverse primer
bkko_fwd	ATATATGGCGCGCCATCAATCCTGGCTCGACCTCAACT	*buk* fragment forward primer
bkko_rev	ATATATGGCGCGCCCGTTGTACGCAATTCCGCCTGTTA	*buk* fragment reverse primer

2.4. Genomic DNA isolation

In total, 50 mL of A_{600} 1.0 cells were collected by centrifugation at 4 °C, washed with lysis buffer and frozen at −20 °C. Cells were incubated with lysozyme and RNase A for 10 min at 37 °C. Cell debris was removed by centrifugation and the supernatant was extracted twice with phenol:chloroform:isoamyl alcohol, once with chloroform:isoamyl alcohol. DNA was precipitated with isopropanol and washed with 70% ethanol. DNA was then air dried and resuspended in TE buffer.

2.5. PCR

The PCR amplification of specific DNA sequences was performed according to manufacturer's instructions (Applied Biosystems). In a total reaction volume of 100 μL, the following components were added: 10 μL PCR buffer II, 10 μL $MgCl_2$ (10 mM), 6.6 μL dNTPs (10 mM), 1 μL each primer (100 mM), 500 ng chromosomal DNA template, 0.5 μL Amplitaq Polymerase Gold, and the remaining volume H_2O. For colony PCR colonies grown for 24–48 h were resuspended in 10 μL H_2O and 2 μL of suspension was used as the template.

2.6. Bioreactor experiments

Fermentations were carried out using a BioFlo 110 or BioFlo II (New Brunswick Scientific Co., Edison, NJ) bioreactor with 4.0 L working volumes. Fermentations used a 10% v/v inoculum of a pre-culture with A_{600} equal to 0.2. CGM media were supplemented with 0.10% (v/v) antifoam and 75 μg/mL clarithromycin. Fermentations were maintained at constant pH using 6 M NH_4OH. Anaerobic conditions were maintained through nitrogen sparging. Temperature was maintained at 37 °C and agitation at 200 rpm. Glucose was restored to the initial concentration (440 mM) in fermentations if glucose levels fell below 200 mM.

2.7. Analytical techniques

Cell density was measured at A_{600} using a Biomate3 spectrophotometer (Thermo Spectronic, Waltham, MA). Samples were diluted as necessary to keep absorbance below 0.40. Supernatant concentrations of glucose, acetone, acetate, acetoin, butyrate, butanol, and ethanol were determined using a high-pressure liquid chromatography system (HPLC) (Waters Corp., Milford, MA). The protocol from Buday et al. (1990) was modified adjusting the flow rate to 0.5 mL/min and a sample run time of 50 min.

2.8. Metabolic flux analysis

Metabolic flux analysis calculations were performed using a program developed by Desai et al. (1999). Product concentrations from bioreactor experiments were used to generate metabolic fluxes. Error associated with the calculated fluxes is typically less than 10%. The specific intracellular fluxes are detailed in Fig. 1.

3. Results

3.1. Expression of aad using the ptb promoter for increased butanol production in strain M5

The asporogenous, non-solventogenic strain M5 strain (Clark et al., 1989) has lost the megaplasmid pSOL1 that contains all the genes required for solvent production (Cornillot et al., 1997). The megaplasmid contains two independent bifunctional aldehyde/alcohol dehydrogenases (AADHs) capable of forming butanol from butyryl-CoA (Nölling et al., 2001). The primary AADH gene, *aad*, is induced during batch cultures at low pH (Nair et al., 1994), while the second AADH gene, *adhE2*, is induced in alcohologenic cultures at near neutral pH (Fontaine et al., 2002). Both genes were expressed in pSOL1-deficient backgrounds (Fontaine et al., 2002; Nair and Papoutsakis, 1994): both strains were only able to produce about half the levels of butanol that is typically produced by the WT strain. *aad* expression in the pSOL1 deficient background relied on the expression from the endogenous promoter. Expression of *adhE2* utilized the *thl* promoter, which has high, constitutive-like expression (Tummala et al., 1999), but was unable to achieve high butanol titers. As solvent formation and the sporulation process are co-regulated in the WT strain by a common transcriptional regulator, Spo0A (Harris et al., 2002b), it was thought that the natural promoter of *aad* may not be sufficiently induced in the asporogenous strain M5. Thus, the natural promoter of *aad* was replaced with the *ptb* promoter of the *ptb*–*buk* operon (Fig. 1), which is expressed highly especially in the early part of the fermentation (Tummala et al., 1999). Additionally, butyrate is the primary product of the pSOL1 deficient strain M5 and any promoter titration effects would be beneficial for the reduction of butyrate formation, whose accumulation leads to premature growth inhibition of the M5 strain. Strain M5(p94AAD3) was constructed to overexpress *aad* from the *ptb* promoter. Butanol formation in test tube cultures confirmed the proper expression and function of the AAD protein (M5 forms no butanol at all). Controlled pH 5.0 fermentations were carried out to characterize the strain (Table 3). At this pH, M5(p94AAD3) produced 92 mM butanol which is less than typical levels of ca. 160 mM produced in WT fermentations, but higher than the 84 mM produced by M5(pCAAD), which expresses *aad* from its endogenous promoter. Acid production was also altered. Butyrate formation was reduced from 99 mM in M5(pCAAD) to 72 mM in M5(p94AAD3), while acetate levels increased to 159 mM from 101 mM. The decreased butyrate formation could be due to either the titration effect of addition plasmid copies of the *ptb* promoter or from increased competition for butyryl-CoA from AAD. The increased acetate formation may reflect an effort by the cells to compensate for the loss of ATP generation from the decreased butyrate formation. In view of the fact that the *ptb* promoter and generally both butyrate and acetate formation are affected by pH levels (Bahl et al., 1982; Monot et al., 1984), we next tested the impact of culture pH on product formation.

3.2. Culture pH profoundly impacts solvent production in M5(p94AAD3) with maximal butanol titers at pH 5.75

We investigated the impact of culture pH in controlled pH fermentations carried out at pH 5.5, 5.75, and 6.0 (Fig. 2 and Table 3). Butanol titers at pH 5.5, 5.75, and 6.0 were 140, 150, and 138 mM, respectively, significantly higher than the 92 mM reached at pH 5.0. Interestingly, butanol production was slightly decreased at pH 6.0, combined with dramatic increases in the levels of acids produced. Acetate levels increased with increasing pH, reaching a maximum value of 256 mM at pH 6.0, compared with the 159 mM produced at pH 5.0. Butyrate levels were fairly similar between cultures maintained at pH 5.0–5.75, but nearly doubled to 144 mM at pH 6.0. In order to obtain insight into how pH had such profound effects on cell metabolism, we carried out metabolic flux analysis, which we discuss next.

3.3. Metabolic flux analysis of M5(p94AAD3) to assess the impact of culture pH

At pH 5.0, glucose utilization, rGLY1, and the conversion of pyruvate to acetyl-CoA, rGLY2, in M5(p94AAD3) began very high,

Table 3
Final product concentrations in pH controlled batch fermentations of *C. acetobutylicum* M5 and recombinant strains

Strains	Fermentation characteristics[a]						
	pH	Max A_{600}	Doubling time	Butanol	Ethanol	Acetate	Butyrate
M5[b]	5.0	–	1.15	0	6	107	169
M5(pCAAD)[b]	5.0	–	1.59	84	8	101	99
M5(p94AAD3)	5.0	11.64	2.19	92	20	159	72
M5(p94AAD3)	5.5	12.81	1.82	140	36	222	73
M5(p94AAD3)	5.75	10.81	2.08	150	44	248	87
M5(p94AAD3)	6.0	9.23	1.98	138	34	256	144
M5(pTHLAAD)	5.5	8.90	1.86	108	19	172	84
M5(pTHLAAD)	6.0	8.25	1.88	77	17	148	196
M5 AKKO(p94AAD3)	5.5	7.58	2.25	92	22	180	75
M5 AKKO(p94AAD3)	6.0	9.00	2.65	51	21	179	287
M5[c]	–	5.29	1.20	0	6	23	73
M5 AKKO[c]	–	3.81	1.48	0	7	9	75
M5 BKKO[c]	–	1.42	2.93	0	3	17	26

[a] Doubling time (hours), product concentrations are shown in mM.
[b] Results from M5 and M5(pCAAD) are from a previous study (Nair and Papoutsakis, 1994).
[c] Results are from static flask experiments without pH control.

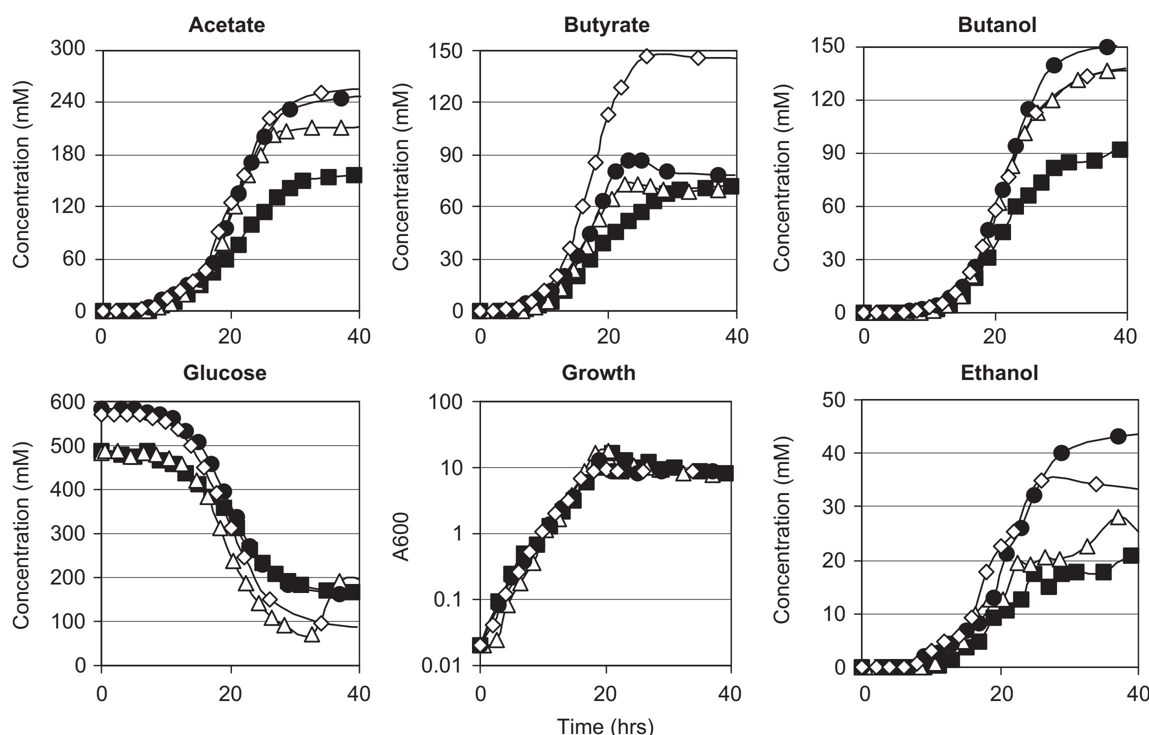


Fig. 2. pH-controlled fermentations of M5(p94AAD3). Product concentrations for the major metabolites and growth characteristics of the M5(p94AAD3) fermentations at various fermentation pH values. pH 5.0 (closed squares), pH 5.5 (open triangles), pH 5.75 (closed circles), and pH 6.0 (open diamonds) are shown. Time has been normalized based on culture absorbance to account for variations in lag time of the cultures (A_{600} of 1.0 occurs at hour 10).

but quickly dropped to near zero by hour 10 (Fig. 3). With increased pH, M5(p94AAD3) exhibited a bimodal glucose utilization pattern, with high utilization early and then again at hour 10. This second period of glucose utilization corresponds with increased fluxes of butanol and acetate formation. The butanol formation flux reached its maximal value at about hour 10 at pH 5.5–6.0. Acetate formation began high, but showed a second large peak in its flux that overlaps with the peak of the butanol flux. The butyrate formation flux was also sustained longer with increasing pH. The ethanol formation flux followed similar trends as the butyrate formation flux displaying a high initial flux, and a more prolonged flux with increasing pH. The rTHL and rHYD fluxes showed very similar patterns to the glucose utilization fluxes, exhibiting high initial values with a second peak apparent at pH 5.5 and above. The NADH from ferredoxin (Fd) formation flux (rFDNH) displayed more complex patterns, mostly bimodal but also some trimodal patterns. These patterns were evident for all pH values and only approximately correspond to the peaks in the butanol or ethanol-formation fluxes, or the rHYD fluxes, thus suggesting unusual complexity in the regulation of electron flow.

3.4. Comparative analysis of M5(p94AAD3) and M5(pTHLAAD) fermentations shows that thiolase overexpression limits acetate and ethanol formation, but also decreases butanol titers

At pH values below 6.0, butyrate concentration is fairly low in M5(p94AAD3) cultures and only about half the butanol concentration. Acetate concentrations, however, are the highest of all

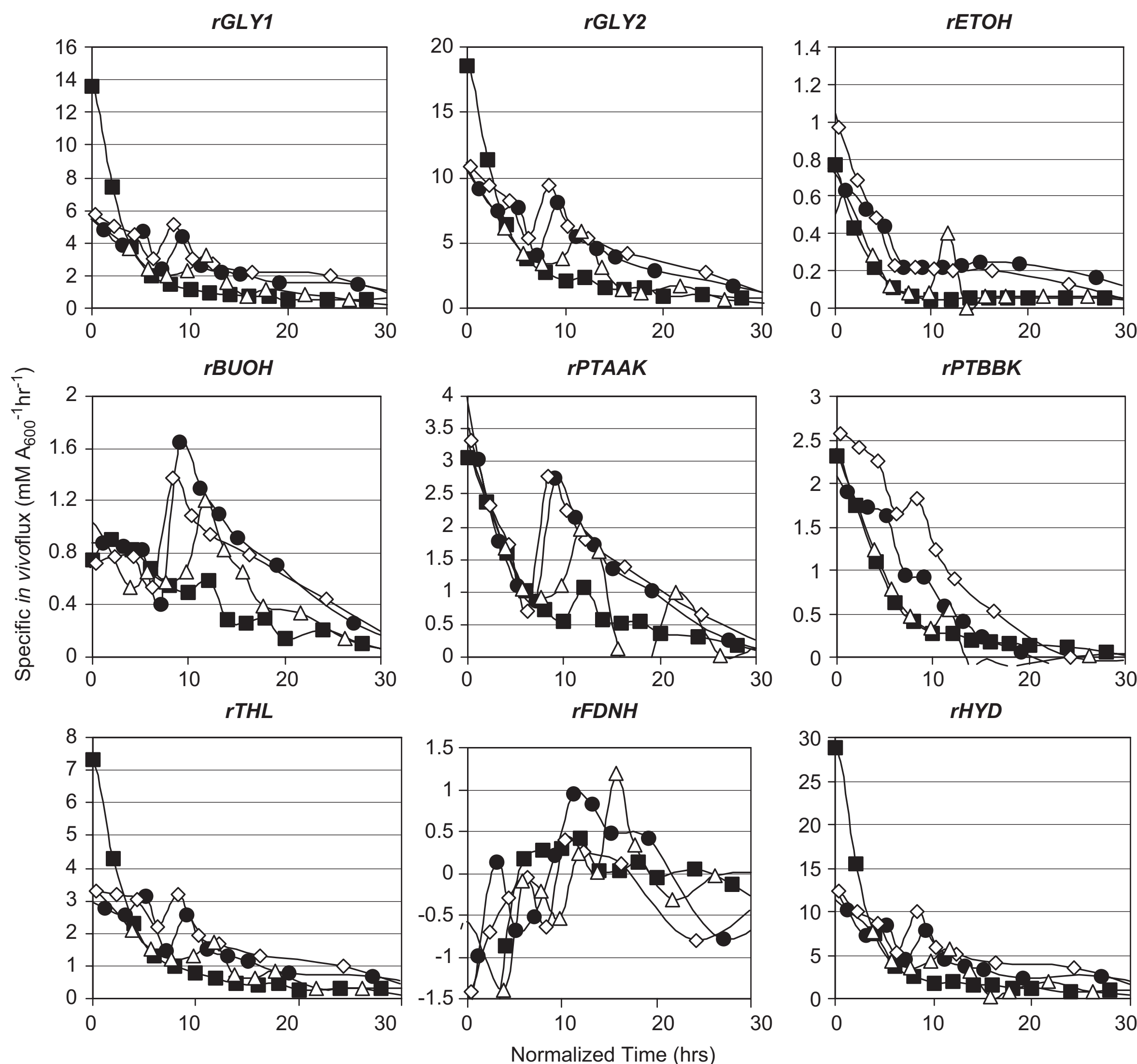


Fig. 3. Metabolic flux analysis of M5(p94AAD3) at various fermentation pH settings. pH 5.0 (closed squares), pH 5.5 (open triangles), pH 5.75 (closed circles), and pH 6.0 (open diamonds) are shown. Time has been normalized based on culture absorbance to account for variations in lag time of the cultures (A_{600} of 1.0 occurs at hour 0).

metabolites in all pH values tested. We thus examined if *thl* overexpression might reduce acetate formation. Increased expression of *thl* might increase the carbon flux from acetyl-CoA to butyryl-CoA (Fig. 1) reducing the formation of acetate and ethanol and increasing butyrate and butanol formation. The *thl* with its natural promoter and the *aad* gene with the *ptb* promoter were combined on a single construct to create the plasmid pTHLAAD.

Fermentations of M5(pTHLAAD) were performed at pH 5.5 and 6.0 based on the performance of the M5(p94AAD3) strains. At pH 5.5, acetate levels were reduced from 222 mM in M5(p94AAD3) to 172 mM in strain M5(pTHLAAD) and ethanol production was reduced from 36 to 19 mM (Table 3). However, the lowered production of acetate and ethanol was not accompanied by increased butanol formation. Butanol production decreased from 140 to 108 mM, and butyrate increased from 73 to 84 mM. At pH 6.0, acetate was reduced from 256 mM in M5(p94AAD3) to 148 mM in M5(pTHLAAD) and ethanol was reduced from 34 to 17 mM. Butanol levels also decreased from 138 to 77 mM while butyrate production increased from 144 to 196 mM.

Metabolic flux analysis shows glucose utilization (rGLY1), rGLY2 and rTHL are all significantly higher (by 2–3-fold) early in the fermentation of M5(pTHLAAD) compared to M5(p94AAD3) at both pH values (Fig. 4). Most of the increased glucose utilization and thiolase flux is manifested in butyrate formation indicated by the higher initial fluxes of rPTBBK. The butyrate formation flux in M5(pTHLAAD) was roughly twice the initial flux in M5(p94AAD3). This difference is most evident at pH 6.0 when butyrate formation was sustained the longest. rBUOH was induced much earlier at pH 5.5 in M5(pTHLAAD) than M5(p94AAD3), but this was reversed at pH 6.0. This suggests that even under the *ptb* promoter, AAD expression and/or activity are more pronounced at lower pH, and thus able to compete more effectively for butyryl-CoA for butanol versus butyrate formation. The butanol flux was trimodal in strain M5(pTHLAAD) at pH 6, but less pronounced so at pH 5.5. The acetate fluxes (rPTAAK) were overall lower in strain M5(pTHLAAD), and did not exhibit as pronounced a multimodal pattern as the fluxes in the M5(p94AAD3) strain. As was the case in the M5(p94AAD3) strain, the fluxes for acetate and butanol in M5(pTHLAAD) closely aligned with each other. The ethanol fluxes were consistently lower in strain M5(pTHLAAD) than in strain M5(p94AAD3), thus suggesting that lower pools of acetyl-CoA due to *thl* overexpression (Fig. 1) penalize ethanol formation the most while enhancing butyrate formation. The rFDNH fluxes show distinct but difficult to interpret patterns: by carefully examining

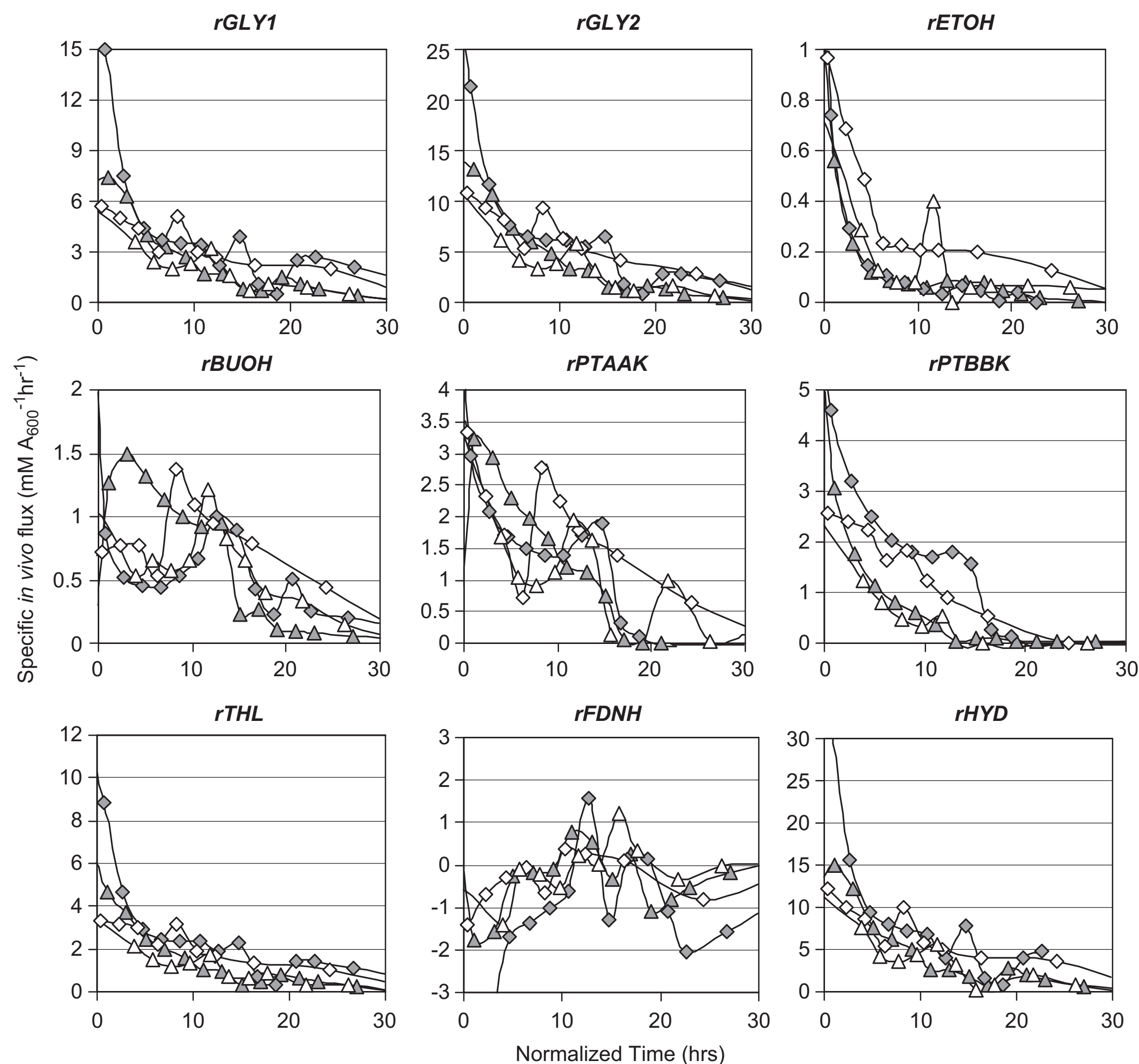


Fig. 4. Metabolic flux analysis of M5(p94AAD3) and M5(pTHLAAD). M5(p94AAD3) fluxes are shown as open symbols at pH 5.5 (triangles) and 6.0 (diamonds). M5(pTHLAAD) fluxes are shown as gray symbols at pH 5.5 (triangles) and 6.0 (diamonds). Time has been normalized based on culture absorbance to account for variations in lag time of the cultures (A_{600} of 1.0 occurs at hour 0).

the areas under the curves, the most significant observation is that this flux remains mostly negative, especially for M5(pTHLAAD) (compare against the fluxes in Fig. 3). This means that NADH is used to produce reduced Fd and eventually H_2. *thl* overexpression limits the formation of 2-C compounds (acetate and ethanol) while maintaining similar levels of 4-C compounds (butyrate and butanol), dramatically increasing the selectivity of 4-C compounds.

3.5. *Can disruption of the ack and buk genes reduce acetate and butyrate formation?*

3.5.1. *A method to KO genes in the asporogenous M5 strain*

As efforts thus far were unable to reduce the formation of acids by *aad* and/or *thl* overexpression, gene KOs were used to inactivate the acetate and butyrate formation pathways separately. A non-replicative plasmid method was used, utilizing a cloning vector lacking an origin of replication for *C. acetobutylicum.* As previous efforts utilizing antibiotic resistance markers had limited success in generating KO mutants, it was thought that increasing the expression of the antibiotic resistance genes might result in greater efficiency of KO strain generation. Although antibiotic markers for erythromycin and thiamphenicol resistance have been used successfully with replicating cloning vectors, these plasmid-borne markers have multiple gene copies in each cell, while chromosomal integrants would have only one gene copy. To increase expression and translation of the thiamphenicol resistance gene, an optimized Shine-Dalgarno sequence (AGGAGG) replaced the endogenous sequence and the gene was put under the control of the high expression *ptb* promoter. The thiamphenicol resistance marker was used for compatibility with the other plasmids used in this study. The transformants were initially screened using colony PCR. After the confirmation of the genomic integration, one clone (M5 AKKO) for the *ack* disruption and one (M5 BKKO) for the *buk* disruption were chosen for further study. Genomic DNA was isolated from each strain and the chromosomal locus spanning the genomic integration plus upstream and downstream sequences were PCR amplified and sequenced. The sequencing data confirmed the expected single crossover event that has previously been observed with similar methods (Green et al., 1996). The schematic of the genomic crossover and the sequencing data are shown as Supplementary Fig. S1.

3.5.2. *Characterization of the ack and buk M5 KO strains*

The growth and metabolic profiles of M5 AKKO and M5 BKKO strains were compared against the parental M5 strain in 200 mL

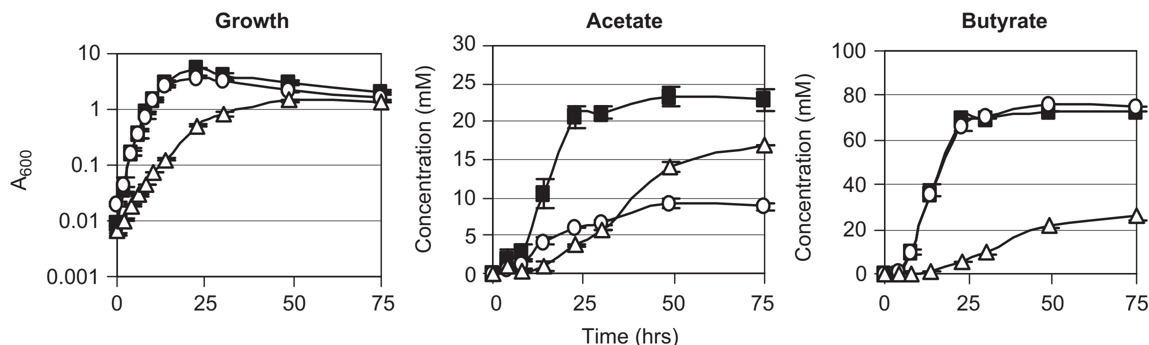


Fig. 5. Growth profiles and product analysis of M5 AKKO, M5 BKKO, and M5. Growth profiles and acetate and butyrate production in static flask cultures without pH control. M5 (closed squares), M5 AKKO (open circles), and M5 BKKO (open triangles). Results shown are averages of two experiments. Error bars show the standard deviation between experiments. Error bars not visible are smaller than the symbols used.

flask cultures without pH control (Fig. 5). The growth rates of the M5 and the M5 AKKO strains were fairly similar with doubling times of 1.20 and 1.48 h, respectively. M5 BKKO displayed a dramatic growth inhibition, with a doubling time of 2.93 h, which makes it nearly 2.5 times slower than the M5 and twice slower than the M5 AKKO strain. Product formation was altered in both recombinant strains compared to the parental strain, largely as would be expected. Acetate production was reduced by >60% from 23 mM in M5 to just 9 mM in M5 AKKO. Butyrate production in the M5 and M5 AKKO strains was virtually the same at 73 and 76 mM, respectively. Butyrate was produced at very low levels in the M5 BKKO strain, reaching a maximum of 26 mM after 75 h of growth. This is a reduction of 64% compared with M5. Acetate production was also reduced in M5 BKKO, reaching a final value of 17 mM. This level is more than the M5 AKKO could produce suggesting that the acetate pathway is less inhibited than the butyrate pathway in M5 BKKO.

Both the M5 AKKO and M5 BKKO could still produce low levels of the acids targeted by the genomic disruptions. This has been observed in other clostridia mutants involving the inactivation of the acid-formation pathways where parallel and analogous pathways (e.g., acetate and butyrate) exist (Green et al., 1996; Liu et al., 2006; Zhu et al., 2005). The formation of the product in the inactivated pathway is attributed to the cross reactivity of the enzymes in the alternate pathway.

3.6. *Overexpression of aad in the M5 AKKO strain: antibiotics and pH impact product formation, but butanol formation remains severely inhibited*

Following the successful inactivation and characterization of the *ack* and *buk* genes in the M5 strain, we aimed to transform the two KO strains with the *aad* overexpression plasmid p94AAD3. The goal was to examine the combined effect of AAD overexpression with the downregulation of either the acetate or butyrate formation pathways. Despite repeated attempts to transform the M5 BKKO strain with p94AAD3, no transformants could be isolated. This is likely due to the very low growth rate achieved by these cells; it is also possible that the *buk* inactivation may impact the competence of the cell. Correct transformants containing p94AAD3 were however isolated from the M5 AKKO strain.

Initial results from tube cultures without pH control that used both erythromycin and thiamphenicol for strain maintenance showed that glucose uptake was inhibited and suggested that the cells could not grow well when using multiple antibiotics. In an earlier study (Harris et al., 2001), it had been determined that the use of tetracycline drastically inhibits solvent formation in *C. acetobutylicum*, but the effect of simultaneous usage of thiamphenicol and erythromycin has not been examined. In order to assess the impact of antibiotic usage on butanol production, cultures were first inoculated in the presence of both thiamphenicol and erythromycin and then diluted 100-fold into media containing either both thiamphenicol and erythromycin, thiamphenicol alone, erythromycin alone, or no antibiotic. The thiamphenicol resistance gene is integrated into the genome disrupting the *ack* gene, while the erythromycin is used for the plasmid maintenance for *aad* overexpression.

Cultures produced the highest level of butanol when grown with erythromycin and nearly as much was produced when both antibiotics were used (Fig. 6). When no antibiotic or thiamphenicol alone was used, the cells produced low butanol levels and increased levels of butyrate. These results indicate that erythromycin is needed to maintain the plasmid and ensure high butanol production, while the dual antibiotic usage has a small inhibitory effect on butanol formation, due to the decreased glucose utilization. In subsequent pH-controlled fermentation experiments, clarithromycin only was used for plasmid maintenance (clarithromycin is a pH stable derivative of erythromycin). Genomic integrations of this type have been previously studied and have been shown to be stable (Green et al., 1996).

Product formation by M5 AKKO(p94AAD3) in pH 5.5 and 6.0 fermentations is summarized in Table 3. Corresponding data for strain M5(p94AAD3) are shown in order to comparatively assess the impact of *aad* overexpression with *ack* inactivation. At pH 5.5, acetate production was reduced from 222 mM in M5 (p94AAD3) to 180 mM in M5 AKKO(p94AAD3), but unexpectedly butanol production was also significantly reduced from 140 to 92 mM. Butyrate production was only slightly increased from 73 mM in M5 (p94AAD3) to 75 mM in M5 AKKO(p94AAD3). Changes in product formation were more dramatic at pH 6.0. Final acetate levels were reduced from 256 to 179 mM. Butanol production was reduced from 138 mM to just 51 mM. The most extreme product shift was the final butyrate concentrations. In M5(p94AAD3), butyrate is produced at 144 mM while in M5 AKKO(p94AAD3) butyrate was doubled to 287 mM, the highest levels ever observed in this organism. The shift in acid products can be seen clearly by comparing the ratio of acetate versus butyrate produced. At pH 5.5, M5(p94AAD3) had an acetate to butyrate ratio of 3.05, whereas strain M5 AKKO(p94AAD3) exhibited a reduced ratio of 2.40. At pH 6.0, M5(p94AAD3) displayed an acetate to butyrate ratio of 1.78, but this was reduced by two-thirds to 0.62 in M5 AKKO(p94AAD3).

Metabolic flux analysis was also performed to determine differences between M5 AKKO(p94AAD3) and M5(p94AAD3).

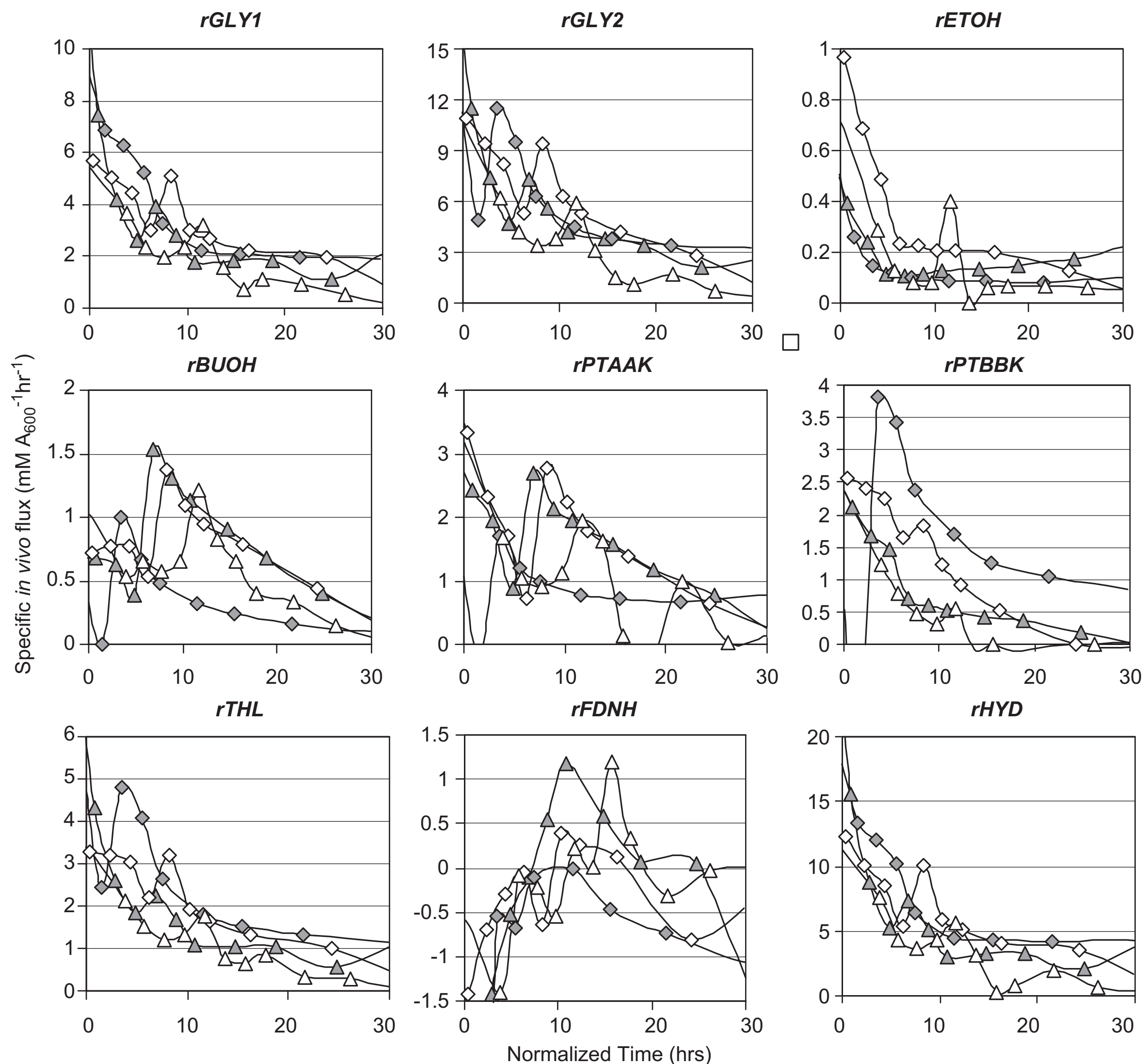


Fig. 6. Metabolic flux analysis of M5(p94AAD3) and M5 AKKO(p94AAD3). M5(p94AAD3) fluxes are shown as open symbols at pH 5.5 (triangles) and 6.0 (diamonds). M5 AKKO(p94AAD3) fluxes are shown as gray symbols at pH 5.5 (triangles) and 6.0 (diamonds). Time has been normalized based on culture absorbance to account for variations in lag time of the cultures (A_{600} of 1.0 occurs at hour 0).

Glucose utilization was increased early in the M5 AKKO (p94AAD3) compared to M5(p94AAD3). Peak butanol formation occurred earlier in M5 AKKO(p94AAD3) than in M5(p94AAD3), reflecting the impact of *ack* deactivation presumably leading. Corresponding to the low butanol flux at pH 6.0 in M5 AKKO(p94AAD3), the butyrate formation flux was increased dramatically and was sustained at high levels much longer than in M5(p94AAD3). The thiolase flux showed a small induction in M5 AKKO(p94AAD3) at pH 6.0, primarily due to the large increase in butyrate formation flux compared to M5(p94AAD3). Once again, the acetate formation flux aligned with the butanol formation flux. Ethanol formation was significantly reduced in strain M5 AKKO(p94AAD3) despite the reduction in acetate formation, which might have anticipated a compensatory ethanol formation. There are also important differences in electron flow: for example, at pH 6, M5 AKKO(p94AAD3) displayed much higher levels of the rHYD flux and all negative values for the rFDNH flux (formation of reduced Fd from NADH), which means all electrons were shuffled to H_2 production rather than butanol/ethanol formation even late in culture, and this is an extreme pattern never previously observed, and was accompanied by extremely high levels of butyrate formation. It appears that M5 AKKO (p94AAD3) cannot effectively utilize NADH for alcohol formation, it thus uses butyrate formation to minimize NADH generation, and still produces substantial levels of H_2 to balance the electron flow. It appears as if other plasmid-coded proteins are necessary for making possible the beneficial use of reduction energy to produce butanol and ethanol.

4. Discussion

In this study, we were able to achieve WT levels of butanol production without acetone production in a non-sporulating culture by expressing *aad* from the *ptb* promoter. It has been suggested that this level of butanol production in the pSOL1 deficient background was unlikely due to the inability of the strains to re-assimilate acid products (Fontaine et al., 2002). Re-assimilation of acid products would replenish butyryl-CoA pools within the cell for butanol production. We have shown here that by manipulating the genetic regulation of *aad* by replacing the natural promoter with the *ptb* promoter that WT levels can be achieved without acid re-assimilation. It has also been thought that the pSOL1 deficient strains may be more sensitive to butanol

than the WT sporulating strain. A previous study has shown that the *spo0A* overexpressing *C. acetobutylicum* strain enters sporulation earlier than the WT strain and that this early sporulation may explain its enhanced butanol tolerance (Alsaker et al., 2004). Sporulating cultures were thought to be more tolerant to solvents than non-sporulating cultures perhaps due to unknown genes on the pSOL1 plasmid (Fontaine et al., 2002). We have shown here that M5 derivative strains were not only able to withstand WT levels of butanol, but were also subjected to elevated levels of butyrate suggesting that tolerance of these strain is comparable to their sporulating counterparts.

As these strains are unable to re-assimilate acids, high levels of acids were produced. In one strategy, high acetate production was countered using *thl* overexpression combined with the *aad* overexpression. The strain overexpressing both *thl* and *aad* did produce less acetate than the strain overexpressing *aad* alone and produced a higher ratio of four-carbon products (butanol and butyrate) to two-carbon products (ethanol and acetate), but less butanol was produced and higher concentrations of butyrate were formed. In a second strategy, the acetate kinase and butyrate kinase genes were inactivated using homologous recombination via a non-replicating plasmid. Both of these strains successfully reduced the product formation of the targeted pathway, but the inactivation of the butyrate kinase resulted in a dramatic inhibition of growth that has blocked further genetic manipulations. Earlier studies were unable to achieve a *buk* disruption in the pSOL1 deficient strain DG1, and it was thus suggested that a mutant producing only acetate would not be viable (Gonzalez-Pajuelo et al., 2005). Here, we have created a *buk* disruption mutant, but the strain produced low butyrate levels and had a very low growth rate.

The acetate kinase mutant M5 AKKO was successfully complemented with *aad* overexpression and acetate levels were reduced in this strain, although this was accompanied by a reduction in butanol formation as well. This strain had a significant shift in the ratio of acetate to butyrate produced, particularly at higher pH. Overall, *thl* overexpression and inactivation of acetate kinase resulted in the production of higher levels of butyrate and lower levels of acetate. This shift in acid production has also been accompanied by lower butanol titers.

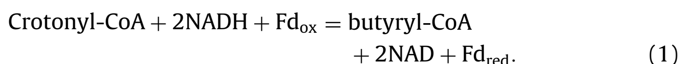
The slow growth rate of the butyrate kinase mutant suggests that M5 primarily balances NADH formation/use by producing butyrate (Fig. 1). The alternate route for NADH oxidation is the reduction of Fd ultimately leading to hydrogen production (Fig. 1). This alternate route of NADH oxidation has been suggested as thermodynamically unfavorable (Gonzalez-Pajuelo et al., 2005). Here, while the rFDNH and rHYD appear overall operational (Figs. 3, 4, and 6), the patterns of the rFDNH flux are very different (mostly negative) than in strains based on the WT pSOL1-containing genetic background (Sillers et al., 2008). This suggests that this pathway for NADH utilization to form H_2 cannot be increased to compensate for the lack of butyrate formation.

The introduction of the *aad* gene into the M5 strain adds still another route for NADH oxidation. During cellular metabolism, the conversion of pyruvate to acetyl-CoA results in the reduction of Fd, which must then be oxidized. If during metabolism Fd is oxidized to generate H_2, acetate formation would result in an excess amount of NADH, while butanol production would result in an NADH deficit. In the WT strain, H_2 production decreases relatively early in the fermentation and thus subsequently NADH can be produced from the oxidation of reduced Fd (Fig. 1). This reaction can provide the NADH necessary for solventogenesis. Here, it was observed that butanol production is correlated with acetate production in several recombinant strains in the M5 genetic background. It seems the FDNH flux is tightly regulated and the NADH necessary for butanol production must come from additional acetate production. This once again highlights the difficulty the M5 strain has in manipulating NADH generation/consumption to achieve the necessary electron balance. Unlike the parent, WT 824 strain, the reactions necessary to achieve the electron balance in the cells appear to be stiff, resisting the changes necessary to achieve an electron balance as necessitated by genetically imposed changes on the metabolic pathway, such as the elimination of butyrate formation.

This suggests that the pSOL1 megaplasmid carries some gene or genes of importance to making the electron flow flexible and easy as it occurs in the WT 824 strain, which contains the pSOL1 megaplasmid. There are several oxidoreductase genes residing on pSOL1, and these may be involved in this reaction. Alternatively, a large number of genes encoding transcription factors and DNA-binding proteins also reside on pSOL1, and these may be involved in the regulation of the electron flow. Another noteworthy observation that supports the important role of other pSOL1 genes in providing product formation and electron-balance flexibility is that when the pPTBAAD plasmid (which is identical to the p94AAD3 plasmid) is used to overexpressed *aad* in the WT 824 strain, large quantities of ethanol are formed in addition to butanol resulting in abnormally high ethanol/butanol ratios (Sillers et al., 2008). This does not happen however in the M5 strain upon *aad* overexpression from the same plasmid. Further efforts to increase butanol production should focus on the identification of the gene or genes involved in this complex regulation of electron flow in the M5 genetic background.

A short discussion is also in order regarding the stoichiometric model (Desai et al., 1999; Papoutsakis, 1984) used for the estimation of fluxes. First, both in its original form (Papoutsakis, 1984) and the modified form (Desai et al., 1999) (which introduces a non-linear constraint based on observed ratios of acetate and butyrate re-uptake to solve an otherwise underdetermined singularity), acetate and butyrate are re-uptaken based on the reaction catalyzed by CoAT and leading to acetone formation. In the absence of acetone formation and of the CoAT enzyme (as in strain M5), the CoAT-driven acetate and or butyrate uptake fluxes become zero automatically according to the model, and thus no model changes are necessary for the M5 strain. The reversal of the acetate and butyrate formation pathways is still possible and valid, but as seen in Figs. 3, 4, and 6 are indeed positive (forward direction; within the 10% error of the estimation), except perhaps for a short period for the acetate flux in Figs. 3 and 6. It is however doubtful if this represents a real flux reversal as it barely exceeds to range of error, and comes relatively late in the fermentation.

Second, regarding the issue of NADH balance in view of the recent publication on a somewhat related organism (*Clostridium kluyveri*), whereby the reduction of crotonyl-CoA to butyryl-CoA, which is catalyzed by BCD (Fig. 1, for more details, see Fig. 1 of Desai et al. (1999), has been shown to require two equivalents of NADH, one of which is used for crotonyl-CoA reduction, and the other of which is used for reduction of Fd (Herrmann et al., 2008; Li et al., 2008):



$$\text{Crotonyl-CoA} + 2\text{NADH} + \text{Fd}_{\text{ox}} = \text{butyryl-CoA} + 2\text{NAD} + \text{Fd}_{\text{red}}. \quad (1)$$

This is proposed as one of the two possible models for electron transport in clostridia, although no other clostridia have been tested yet. The other model is the reaction as was written in our stoichiometric model (Desai et al., 1999; Papoutsakis, 1984), namely,

$$\text{crotonyl-CoA} + \text{NADH} = \text{butyryl-CoA} + \text{NAD}. \quad (2)$$

In either case, the electron stoichiometry for the reaction from crotonyl-CoA to butyryl-CoA remains the same as in our model

(Desai et al., 1999; Papoutsakis, 1984). Also, in either case, the electron exchange is mediated by the Etf(s) (electron-transfer flavoprotein(s)), which in the case of *C. acetobutylicum* at least, are coded together with the BCD, CRO, BHBD gene on a single operon (Boynton et al., 1996).

Note that if one adds Eq. (2) above with the equation shown in the upper right part of Fig. 1, namely,

$$\mathrm{NADH} + \mathrm{Fd}_{\mathrm{ox}} = \mathrm{NAD} + \mathrm{Fd}_{\mathrm{red}}, \tag{3}$$

one obtains Eq. (1). Thus, the stoichiometric model (Desai et al., 1999; Papoutsakis, 1984) covers both proposed mechanisms, the only difference being the obligatory coupling of Fd reduction upon butyryl-CoA formation. This however, does not affect the stoichiometric model which has been extensively validated by cross-checking of the predicted fluxes against steady state, integrated batch data as well as transient data, such as in McLaughlin et al. (1985), Papoutsakis (1984), Meyer et al. (1985, 1986a, b), Meyer and Papoutsakis (1989a–c), and Roos et al. (1985). By our estimation, this is the most extensively tested complex stoichiometric model.

Even if the model of Eq. (1) holds for *C. acetobutylicum*, it is almost certain that there are additional proteins catalyzing the reaction of Eq. (3). This is based on the observation that strong reversal of the reaction is observed in a large set of experiments whereby hydrogen production is low to zero while solvent production in very high (McLaughlin et al., 1985; Meyer et al., 1985, 1986b; Meyer and Papoutsakis, 1989a; Roos et al., 1985). In any case, the electron balance must be satisfied and thus reduced electron carriers, like NADH and $\mathrm{Fd}_{\mathrm{red}}$, must be re-oxidized mostly by alcohol and H_2 formation. It is possible that some of the proteins for the exchange of electrons between NAD, Fd and the generation of H_2 are mediated by proteins coded on the pSOL1 megaplasmid and this, as suggested above, might explain the resistance of the M5 strain to ME compared to the parent WT strain.

Acknowledgments

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2008.07.005.

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