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BIOENERGY AND BIOFUELS

Metabolic engineering of *Clostridium tyrobutyricum* for *n*-butanol production: effects of CoA transferase

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Abstract The overexpression of CoA transferase (*ctfAB*), which catalyzes the reaction: acetate/butyrate + acetoacetyl-CoA → acetyl/butyryl-CoA + acetoacetate, was studied for its effects on acid reassimilation and butanol biosynthesis in *Clostridium tyrobutyricum* (Δ*ack*, *adhE*2). The plasmid pMTL007 was used to co-express *adhE2* and *ctfAB* from *Clostridium acetobutylicum* ATCC 824. In addition, the *sol* operon containing *ctfAB*, *adc* (acetoacetate decarboxylase), and *ald* (aldehyde dehydrogenase) was also cloned from *Clostridium beijerinckii* NCIMB 8052 and expressed in *C. tyrobutyricum* (Δ*ack*, *adhE*2). Mutants expressing these genes were evaluated for their ability to produce butanol from glucose in batch fermentations at pH 5.0 and 6.0. Compared to *C. tyrobutyricum* (Δ*ack*, *adhE2*) without expressing *ctfAB*, all mutants with *ctfAB* overexpression produced more butanol, with butanol yield increased to 0.22−0.26 g/g (vs. 0.10−0.13 g/g) and productivity to 0.35 g/l h (vs. 0.13 g/l h) because of the reduced acetate and butyrate production. The expression of *ctfAB* also resulted in acetone production from acetoacetate through a non-enzymatic decarboxylation.

Keywords Aldehyde/alcohol dehydrogenase · Butanol · *Clostridium tyrobutyricum* · CoA transferase · Metabolic engineering

Introduction

With increasing concerns about environmental pollution and the diminishing oil supplies, increased attentions and efforts have focused on the development of next-generation or advanced biofuels (Demirbas 2009; Green 2011; Jiang et al. 2014). Biobutanol, which shares many similar fuel properties with gasoline and has a 30 % higher energy density than ethanol, is one of the most promising advanced biofuels with good prospect as a gasoline substitute (Dürre 2007; Xue et al. 2013). *n*-Butanol can be produced from biorenewable feedstocks in acetone-butanol-ethanol (ABE) fermentation, which was once the second largest industrial fermentation that can be traced back more than 100 years ago (Jones and Woods 1986). However, ABE fermentation processes are limited by low butanol yield, productivity, and titer and generally cannot compete with petroleum-based *n*-butanol that currently dominates in the market (Zhao et al. 2013). Metabolic engineering of solventogenic clostridia, mainly *Clostridium acetobutylicum* and *Clostridium beijerinckii*, have thus been intensively studied and used to manipulate the host strains to better understand their physiology and to develop robust strains for industrial application (Branduardi et al. 2014; Jang et al. 2012; Lee et al. 2008; Lütke-Eversloh 2014; Papoutsakis 2008; Wang et al. 2014).

However, the progress to date has been limited because the biphasic nature of the ABE fermentation and the complex metabolic and regulatory pathways involved are difficult to manipulate and control (Zheng et al. 2009; Lehmann et al. 2012b). To overcome these problems, we have focused on

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the engineering of *Clostridium tyrobutyricum*, an acidogen which naturally can only produce butyrate and acetate (Liu et al. 2005), but not solvents, because of lacking some key enzymes (genes), including CoA transferase (*ctfAB*), acetoacetate decarboxylase (*adc*), and aldehyde dehydrogenase (*ald*), in the pathways leading to ABE production (see Fig. 1). In our recent study, we overexpressed a bifunctional aldehyde/alcohol dehydrogenase gene (*adhE2*) from *C. acetobutylicum* in *C. tyrobutyricum* and turned the mutant into an *n*-butanol producer (Yu et al. 2011). Compared to native solventogenic clostridia, the engineered *C. tyrobutyricum* has a much simpler butanol biosynthesis pathway and potentially can produce more butanol from glucose at a higher yield (>0.3 g/g). However, because large amounts of butyrate and acetate were also produced by this mutant in fermentation, the actual butanol yield was low, only ~0.1 g/g glucose consumed (Yu et al. 2011). Since *ctfAB* is usually overexpressed in solventogenic clostridia during the solventogenic phase to convert acetate and butyrate to acetyl-CoA and butyryl-CoA, respectively, it is desirable to also express *ctfAB*, together with *adhE2*, in *C. tyrobutyricum* to further increase butanol production.

In the present study, *adhE2* and *ctfAB* were co-expressed in *C. tyrobutyricum* strain Ct(Δack) with acetate kinase (*ack*) knockout, which could tolerate and produce butyrate at concentrations higher than 40 g/l (Liu et al. 2008). The *ctfAB* genes from *C. acetobutylicum* were expressed together with *adhE2* in plasmid pMTL007 (Heap et al. 2007), and the effects of overexpressing *ctfAB* on fermentation kinetics were studied in stirred-tank bioreactors at pH 6.0 and 5.0. In addition, co-expressing *ctfAB*, *ald*, and *adc* genes obtained from the *sol* operon of *C. beijerinckii* with *adhE2* in Ct(Δack) was also studied. The results showed that *ctfAB* expression not only significantly increased butanol and reduced acid production but also induced acetone production even in the absence of *adc* gene. This study demonstrated the beneficial effects of *ctfAB* on acid reassimilation and butanol biosynthesis in the non-native solventogenic *C. tyrobutyricum* with potential industrial application for *n*-butanol production. This study also provided new insights on the role of *ctfAB* in controlling acid reassimilation and its effects on solventogenesis.

Materials and methods

Bacterial strains, plasmids, and culture media

Table 1 shows the bacterial strains and recombinant plasmids developed and used in this study. *C. tyrobutyricum* Ct(Δack), a mutant strain of ATCC 25755 with *ack* knockout (Liu et al. 2008), was used as the host for all recombinant plasmids constructed in this work. The *Clostridium* cultures were grown in clostridial growth medium (CGM) with glucose as the carbon source at 37 °C under anaerobic conditions. The CGM contained (g/l): 4 tryptone, 2 yeast extract, 1.0 $K_2HPO_4 \cdot 3H_2O$, 0.5 KH_2PO_4, 2 $(NH_4)_2SO_4$, 0.1 $MgSO_4 \cdot 7H_2O$, and trace minerals (Zhu and Yang 2003). *Escherichia coli* strains used in the cloning were cultivated at 37 °C aerobically in liquid Luria-Bertani (LB) medium with agitation at 250 rpm and on LB agar plates. These media were sterilized by autoclaving at 121 °C for 30 min and after cooling supplemented with appropriate antibiotics: 25 μg/ml chloramphenicol, 45 μg/ml thiamphenicol, or 250 μg/ml cycloserine.

Plasmid construction

Plasmid pMTL007 was the basic vector from which the other constructs were derived. The DNA sequences for *adhE2* (CA_P0035) and *ctfAB* (CA_P0163 and CA_P0164) genes were extracted and PCR-amplified from *C. acetobutylicum* ATCC 824 genomic DNA. The whole *sol* operon containing *ald* (Cbei_3832), *ctfAB* (Cbei_3833 and Cbei_3834), and *adc* (Cbei_3835) genes in pSOL and the truncated sol operon containing only *ald* and *ctfAB* genes in pSV6 were derived from *C. beijerinckii* NCIMB 8052 (ATCC 51743) genomic DNA. The thiolase (*thl*) promoter used to drive the constitutive expression of the genes mentioned above was from *C. tyrobutyricum* ATCC 25755 (Yu et al. 2012). The primers used in PCR amplification of these genes are listed in Table 1. The plasmid pMAD72 for the overexpression of *adhE2* under the control of *thl* promoter has been described in details elsewhere (Yu et al. 2011). The plasmid pMAT was constructed from pMAD72 by inserting *ctfAB* after *adhE2* at the *Sac*II site using the Clontech infusion cloning kit. The plasmid pSOL was constructed by inserting the PCR-amplified *sol* operon from *C. beijerinckii* NCIMB 8052 together with *thl* promoter into pMTL007 between *Xho*I and *Sac*II sites by infusion. The plasmid pSV6 contained hygromycin B resistance gene and the truncated *sol* operon (*ald* and *ctfAB* genes) and *adhE2* under *fac* and *thl* promoters, respectively. The artificial promoter *fac*, which was constructed by combining the operator of the *E. coli lacZ* operon and the promoter of *C. pasteurianum* ferredoxin gene (Fox et al. 1996), was derived from the model *Clostridium* shuttle vector to direct the constitutive expression of heterologous genes in *Clostridium* (Heap et al. 2007). The hygromycin B resistance gene was cut from pGEMT-hygB vector with *Nco*I and ligated into pMAD72, and the PCR-amplified *ald* and *ctfAB* genes from *C. beijerinckii* genome were ligated into the plasmid with *Hin*dIII to generate plasmid pSV6. Figure 2 shows the schematic maps of these plasmids. These plasmids were amplified and stored in *E. coli* DH5α and transformed into *C. tyrobutyricum* Ct(Δack) via the donor cell, *E. coli* CA434, by conjugation described below.

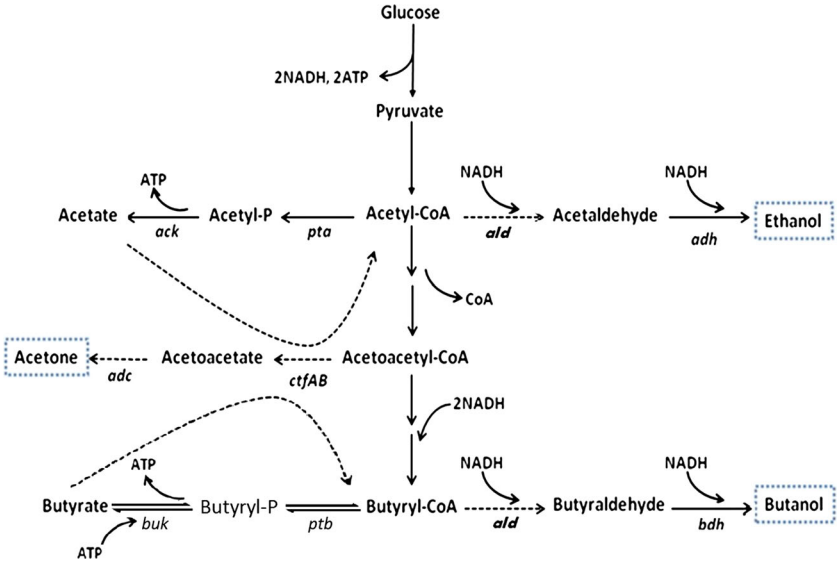


Fig. 1 Metabolic pathway in *C. tyrobutyricum*. The *dotted lines* show the pathways with missing genes in *C. tyrobutyricum* in comparison to other solvent producing *Clostridium* species. The *boxes* show the ABE products produced after introducing *ald* or *adhE2* and *ctfAB* genes. The reversible reactions between butyryl-CoA and butyrate catalyzed by *ptb* and *buk* are hypothetical as these two genes have not been identified or annotated in the published draft genomic sequences of *C. tyrobutyricum* (*ack* acetate kinase, *adc* acetoacetate decarboxylase, *adh* alcohol dehydrogenase, *adhE2* aldehyde/alcohol dehydrogenase, *ald* aldehyde dehydrogenase, *bdh* butanol dehydrogenase, *buk* butyrate kinase, *ctfAB* CoA-transferase, *pta* phosphotransacetylase, *ptb* phosphotransbutyrylase)

Transformation and mutant confirmation

All plasmids were transformed into Ct(Δack) by conjugation as previously described with some modifications (Yu et al. 2011). The plasmids were first transformed into *E. coli* CA434 using the heat shock method. Then, the transformants were cultivated in LB medium containing 25 μg/ml chloramphenicol at 37 °C overnight to reach optical density at 600 nm

Table 1 Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Reference/source
Strains		
E. coli DH5α	Host cells for plasmids amplification	Invitrogen
E. coli CA434	Donor cells in conjugation transformation	Williams et al. 1990
Ct(Δack)	*C. tyrobutyricum* ATCC 25755 with *ack* knockout	Liu et al. 2008
Ct(Δack)-pMAD72	*adhE2* overexpression in Ct(Δack)	Yu et al. 2011
Ct(Δack)-pMAT	*adhE2* and *ctfAB* overexpression in Ct(Δack)	This study
Ct(Δack)-pSOL	*Sol* operon overexpression in Ct(Δack)	This study
Ct(Δack)-pSV6	*ald*, *ctfAB*, and *adhE2* overexpression in Ct(Δack)	This study
Plasmids		
pMTL007	ColE1 ori; Cmr; pCB102 ori	Heap et al. 2007
pMAD72	From pMTL007; P-thl *adhE2*	Yu et al. 2011
pMAT	From pMTL007; P-thl *adhE2 ctfAB*	This study
pSOL	From pMTL007; P-thl *sol* operon (*ald ctfAB adc*)	This study
pSV6	From pMTL007; P-fac *ald ctfAB* Hygr; P-thl *adhE2*	This study
Primers	Sequence (5′-3′)	
ctfAB-for	TTTGCTTCATTATCC AAGGAGGGATTAAAATGAACTCTAAAATAATT	
ctfAB-rev	GTAATTACAAATCCC GTATTTCTTTCTAAACAGCCATGGGT	
SOL-for	CCATGGAGATCTCGA ATTGATAAAAATAATAATAGTGGGTATAATTAAG	
SOL-rev	GTAATTACAAATCCC AATCATATATAACTTCAGCTCTAGGCAATA	
SV6-for	GACACACTAATACCTACAAGCTTAAAGATTTA	
SV6-rev	AGTAAGCTTAGTCATTTGTTACATCAATTAC	

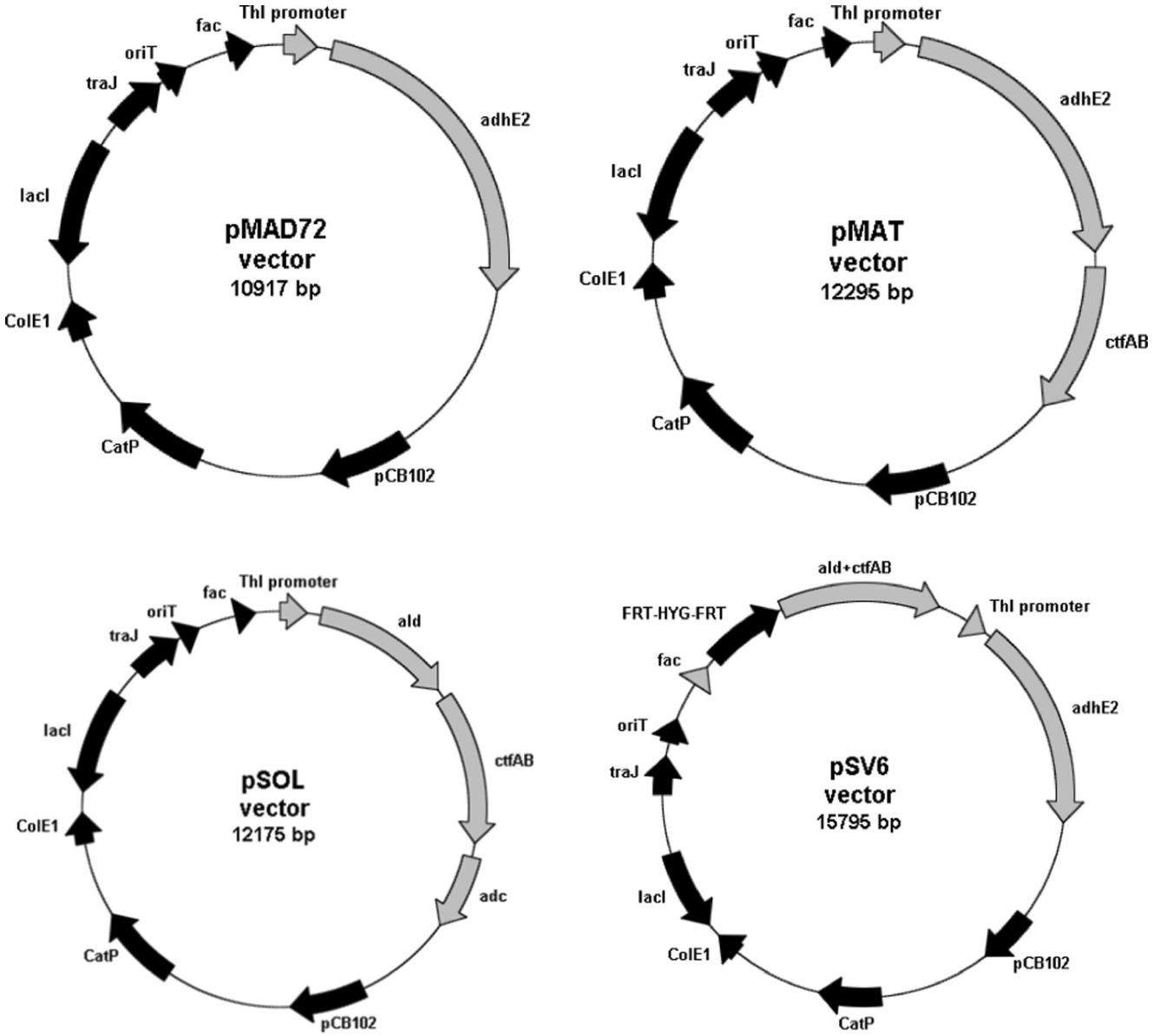


Fig. 2 Plasmid maps of pMAD72, pMAT, pSOL, and pSV6. The *gray arrows* show the promoters and genes (*adc* acetoacetate decarboxylase, *adhE2* aldehyde/alcohol dehydrogenase, *ald* aldehyde dehydrogenase, *ctfAB* CoA-transferase, *CatP* chloramphenicol resistance gene, *ColE1* gram-negative replicon, *fac* artificial *Clostridium* promoter, *oriT* origin of transfer, *pCB102* gram-positive replicon, *thl* thiolase promoter, *traJ* TraJ protein for conjugation)

(OD_{600}) of 1.5−2.0. The collected transformants were washed once using 1 ml sterile phosphate-buffered saline (PBS) and collected by centrifugation at 4000×*g* for 2 min. The transformed donor cells were then mixed with 200 μl of *C. tyrobutyricum* cells precultured at 37 °C overnight, and the mixture was pipetted onto CGM agar plates in an anaerobic chamber and incubated at 37 °C for 8−24 h. Then, cells were recovered and resuspended in 1 ml of PBS and spread onto CGM plates containing 45 μg/ml thiamphenicol and 250 μg/ml cycloserine for 2−3 days to select for positive transformants, which were confirmed by PCR cloning and plasmid extraction. The transformants carrying the plasmids pMAD72, pMAT, pSOL, and pSV6 are designated as mutant strains Ct(Δack)-pMAD72, Ct(Δack)-pMAT, Ct(Δack)-pSOL, and Ct(Δack)-pSV6, respectively, and were obtained and stored at −80 °C.

Enzyme activity assay

The activity of *ctfAB* in cells was assayed under anaerobic conditions following the method previously described (Chen and Blaschek 1999). Each crude cell extract from 50 ml of cells present in an overnight culture was prepared in a buffer containing 50 mM 3-(*N*-morpholino) propanesulfonic acid

(MOPS) (pH 7.0), 500 mM $(NH_4)_2SO_4$, and 20 % (*v*/*v*) glycerol. The cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4 °C. The assay mixture (1 ml) containing 110 mM Tris-HCl (pH 7.5), 5.5 % (*v*/*v*) glycerol, 20 mM $MgCl_2$, 0.1 mM acetoacetyl-CoA, crude cell extract (20 to 100 μg), and 0.32 M potassium acetate (or butyrate) was purged with nitrogen to eliminate O_2. The assay mixture without potassium acetate (or butyrate) was used as blank for negative control. The activity of CoA transferase was measured by monitoring the disappearance of acetoacetyl-CoA at 310 nm in a UV/Vis spectrophotometer (UV-1601, Shimadzu). One unit of enzyme activity is defined as the disappearance of 1 μmol of acetoacetyl-CoA per min. Protein concentration was measured by the Bradford dye-binding assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard. The specific enzyme activity is reported as U/mg protein.

Fermentation kinetics

Batch fermentation kinetics were studied in a stirred-tank bioreactor containing 600 ml of CGM medium with glucose as the carbon source and 45 μg/ml thiamphenicol to prevent culture degeneration or plasmid loss. The bioreactor was sparged with nitrogen for ~30 min to reach anaerobic condition and then inoculated with an overnight culture at a volume ratio of 5 %. Unless otherwise noted, the bioreactor was maintained at 37 °C with the pH controlled at 5.0 or 6.0 by adding 40 % ammonium hydroxide. Samples were collected twice a day at regular intervals for analyses of cell density and concentrations of glucose, acetone, butanol, ethanol, acetate, and butyrate. Each fermentation condition was repeated at least once, and representative data with averages and standard deviations are reported.

Analytical methods

Cell growth was monitored by measuring the OD_{600} with a spectrophotometer (UV-16-1, Shimadzu, Columbia, MD). YSI 2700 Select Biochemistry Analyzer (Yellow Springs, OH) was used to assay the concentration of glucose in samples. Acetone, butanol, ethanol, acetate, and butyrate were analyzed with a gas chromatograph (GC, Shimadzu GC-2014) equipped with a flame ionization detector and a 30-m fused silica column (0.25-μm film thickness and 0.25-mm ID, Stabilwax-DA). The carrier gas was nitrogen at 1.47 ml/min (linear velocity: 35 cm/s). Samples were diluted 20 times with an internal standard buffer solution containing 0.5 g/l isobutanol, 0.1 g/l isobutyric acid, and 1 % phosphoric acid (for acidification) and injected (1 μl each) using an auto injector (AOC-20i Shimadzu). The column temperature was held at 80 °C for 3 min, raised to 150 °C at a rate of 30 °C/min, and held at 150 °C for 3.7 min. Both the injector and detector were set at 250 °C.

Statistical analysis

All batch fermentations were at least duplicated for each condition studied, and the means with standard errors for kinetic parameters such as product yields and productivities are reported. Student's *t* test analysis with JMP software was performed to determine the significant difference ($p<0.05$).

Results

Enzyme activity

To confirm the expression of *ctfAB* in the mutants, the CoA transferase activity was assayed with the parental strain Ct(Δack) as the negative control and *C. acetobutylicum* and *C. beijerinckii* as positive controls, and the results are shown in Table 2. As expected, the strains Ct(Δack) and Ct(Δack)-pMAD72 showed no or negligible CoA transferase activity while Ct(Δack)-pMAT, Ct(Δack)-pSOL, and Ct(Δack)-pSV6 all showed a high specific CoA transferase activity (0.11 to 0.26 U/mg protein) comparable to the positive controls, confirming the expression of *ctfAB* genes in these mutants. With CoA-transferase, these mutants can catalyze the transfer of CoA moiety from acetoacetyl-CoA to either butyrate or acetate, thus allowing the conversion of butyrate and acetate to butanol and ethanol, respectively.

Fermentation kinetics

Figure 3 shows the fermentation kinetics for Ct(Δack)-pMAD72, Ct(Δack)-pMAT, Ct(Δack)-pSOL, and Ct(Δack)-pSV6 at pH 6.0. All these mutants were able to produce

Table 2 CoA transferase activities in *C. acetobutylicum* ATCC 824, *C. beijerinckii* NCIMB 8052, and *C. tyrobutyricum* Ct(Δack), Ct(Δack)-pMAD72, Ct(Δack)-pMAT, Ct(Δack)-pSOL, and Ct(Δack)-pSV6

Strain	Specific enzyme activity (U/mg protein)	
	Acetate as substrate	Butyrate as substrate
Ct(Δack)	0.00±0.00	0.00±0.00
Cac ATCC 824	0.31±0.05	NA
Cbei NCIMB 8052	0.17±0.04	NA
Ct(Δack)-pMAD72	0.000±0.001	0.007±0.001
Ct(Δack)-pMAT	0.22±0.02	0.26±0.02
Ct(Δack)-pSOL	0.11±0.04	NA
Ct(Δack)-pSV6	0.18±0.03	NA

Data shown are mean±SD ($n=3$)

NA not available

butanol and ethanol because of the overexpression of *adhE2* or *ald* gene. The former encodes a bifunctional aldehyde/alcohol dehydrogenase, which catalyzes the reaction from butyryl-CoA to butanol and acetyl-CoA to ethanol. It is noted that the genome of *C. tyrobutyricum* contains *adh* (alcohol dehydrogenase) and *bdh* (butanol dehydrogenase) genes (unpublished data). Therefore, overexpressing *ald* (aldehyde dehydrogenase) alone would be sufficient for *C. tyrobutyricum* to produce butanol and ethanol, as evidenced in the case with the mutant Ct(Δack)-pSOL (Fig. 3c).

For the mutant Ct(Δack)-pMAD72 overexpressing only *adhE2*, butanol production reached ~10 g/l, with large amounts of butyrate (~13.7 g/l) and acetate (~6.7 g/l) also produced (Fig. 3a). In contrast, for the mutants also expressing *ctfAB*, more butanol (12.3 to 13.4 g/l) and much less acids (3.1–4.5 g/l butyric acid, 1.6–2.6 g/l acetic acid) were produced (Fig. 3b–d). Clearly, with *ctfAB* genes, which are responsible for transferring the CoA moiety from acetoacetyl-CoA to butyrate and acetate, butyrate and acetate produced by the cells can be reassimilated back into butyryl-CoA and acetyl-CoA and reenter the main metabolic pathway (Wiesenborn et al. 1989a), leading to the production of butanol and ethanol, respectively. Therefore, much less acid accumulation and more butanol production were observed with Ct(Δack)-pMAT, Ct(Δack)-pSOL, and Ct(Δack)-pSV6. Compared to Ct(Δack)-pMAD72, the production of acetate and butyrate decreased 61–76 % and 67–77 %, respectively, while butanol production increased 20.6–31.4 %. While acetate production was significantly reduced, ethanol production did not increase but instead decreased in mutants overexpressing *ctfAB*. This could be due to that *adhE2* overexpression shifted the metabolic flux from C2 (acetate) toward C4

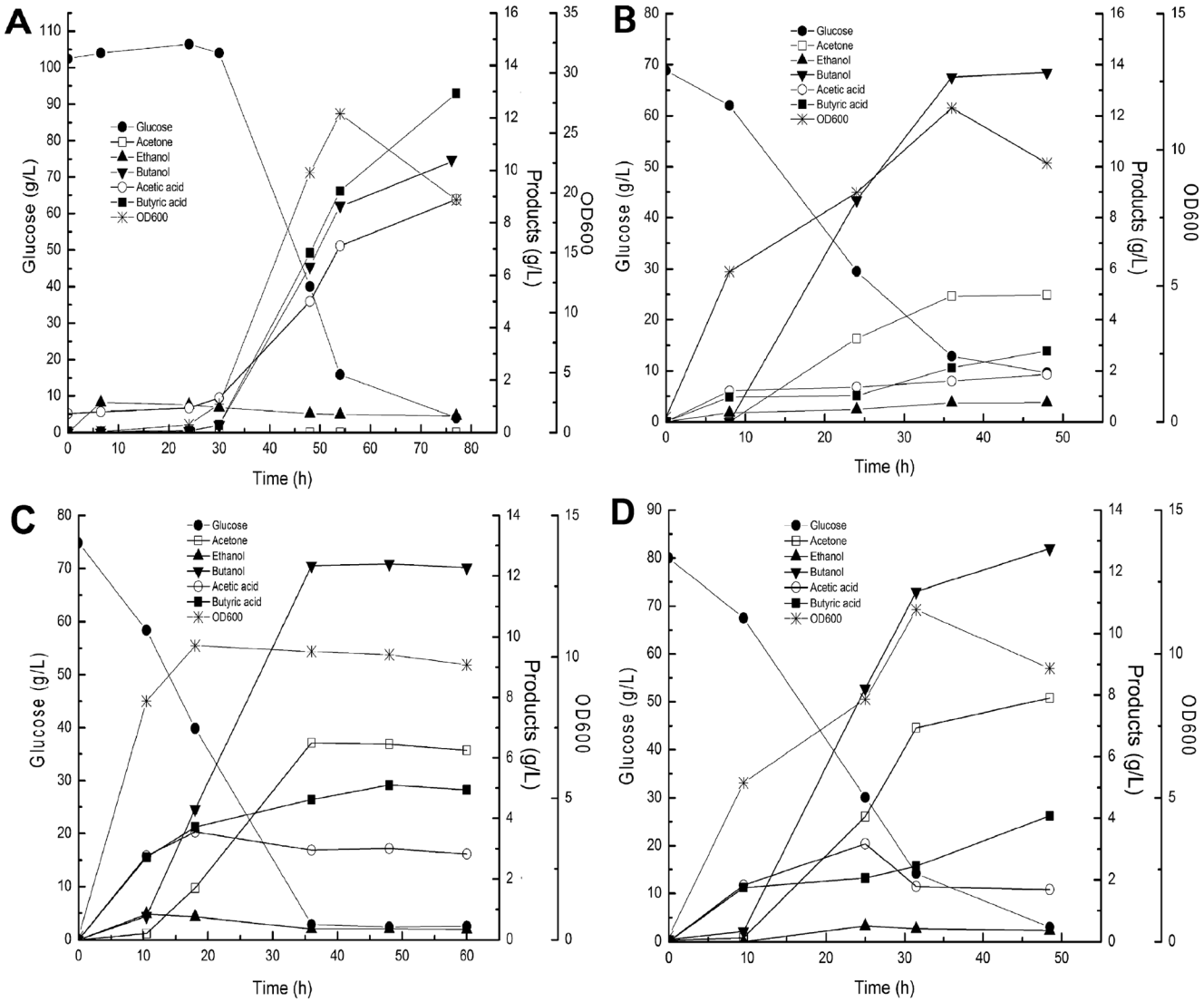


Fig. 3 Fermentation kinetics of *C. tyrobutyricum* at pH 6.0 with various strains. **a** Ct(Δack)-pMAD72, **b** CT(Δack)-pMAT, **c** Ct(Δack)-pSOL, and **d** Ct(Δack)-pSV6

(butyrate) biosynthesis (Yu et al. 2011), which is further discussed later in this paper.

It should be noted that Ct(ack)-pMAD72 consumed ~100 g/l of glucose during the fermentation, while only 60–70 g/l of glucose was consumed by the mutants with CoA transferase expression. The earlier and accelerated butanol production by these mutants led to an early threshold of butanol toxicity (Bowles and Ellefson 1985), which inhibited cell metabolism and resulted in incomplete glucose consumption. Nevertheless, the butanol titer produced by the mutants was higher than that by Ct(ack)-pMAD72 even though less glucose was consumed because of increased butanol yield.

Interestingly, the mutants overexpressing *ctfAB* also produced a significant amount of acetone (6.5–7.4 g/l) even in the absence of *adc*. In solventogenic clostridia, acetone is produced from acetoacetyl-CoA in two steps catalyzed by *ctfAB* and *adc*, respectively (Petersen and Bennett 1990). As expected, Ct(Δack)-pSOL overexpressing *ctfAB* and *adc* was able to produce 7.4 g/l acetone. However, Ct(Δack)-pMAT and Ct(Δack)-pSV6, which did not have the *adc* gene, also showed a comparable acetone production (6.5–7.0 g/l), suggesting a non-enzymatic decarboxylation of acetoacetate. This finding is consistent with a previous study by Han et al. (2011), who knocked out the *adc* gene in *C. beijerinckii* NCIMB 8052 and observed no obvious decrease in acetone production by the knockout mutant. Similarly, the downregulation of *adc* with antisense RNA resulted in 86 % decrease in the decarboxylase activity but only a 17 % reduction in acetone production (Tummala et al. 2003). Clearly, *adc* is not required for acetone production in *C. tyrobutyricum* although its presence appeared to give a slightly higher acetone production compared to the strains without the gene.

The fermentation kinetics for Ct(Δack)-pMAD72, Ct(Δack)-pMAT, Ct(Δack)-pSOL, and Ct(Δack)-pSV6 were also studied at pH 5.0 (see Figure S1 in Supplementary Materials). In general, similar kinetics was observed at both pH 5.0 and 6.0, although butanol and acetone production was lower at pH 5.0. Figures 4 and 5 illustrate the effects of *ctfAB* overexpression and pH on *C. tyrobutyricum* growth and fermentation kinetics, including specific growth rate, product titers, and butanol yield and productivity (also see Table S1 in Supplementary Materials). Compared to Ct(Δack)-pMAD72, the mutants overexpressing *ctfAB* had a much higher butanol yield (0.19–0.22 vs. 0.10 g/g glucose at pH 6.0, 0.18–0.26 vs. 0.14 g/g glucose at pH 5.0) and productivity (0.31–0.35 vs. 0.13 g/l h at pH 6.0, 0.23–0.24 vs. 0.13 g/l h at pH 5.0). These mutants had a comparable specific growth rate but a much lower final cell density compared to Ct(Δack)-pMAD72. The effects of *ctfAB* overexpression and pH on *C. tyrobutyricum* growth and fermentation kinetics are further discussed below.

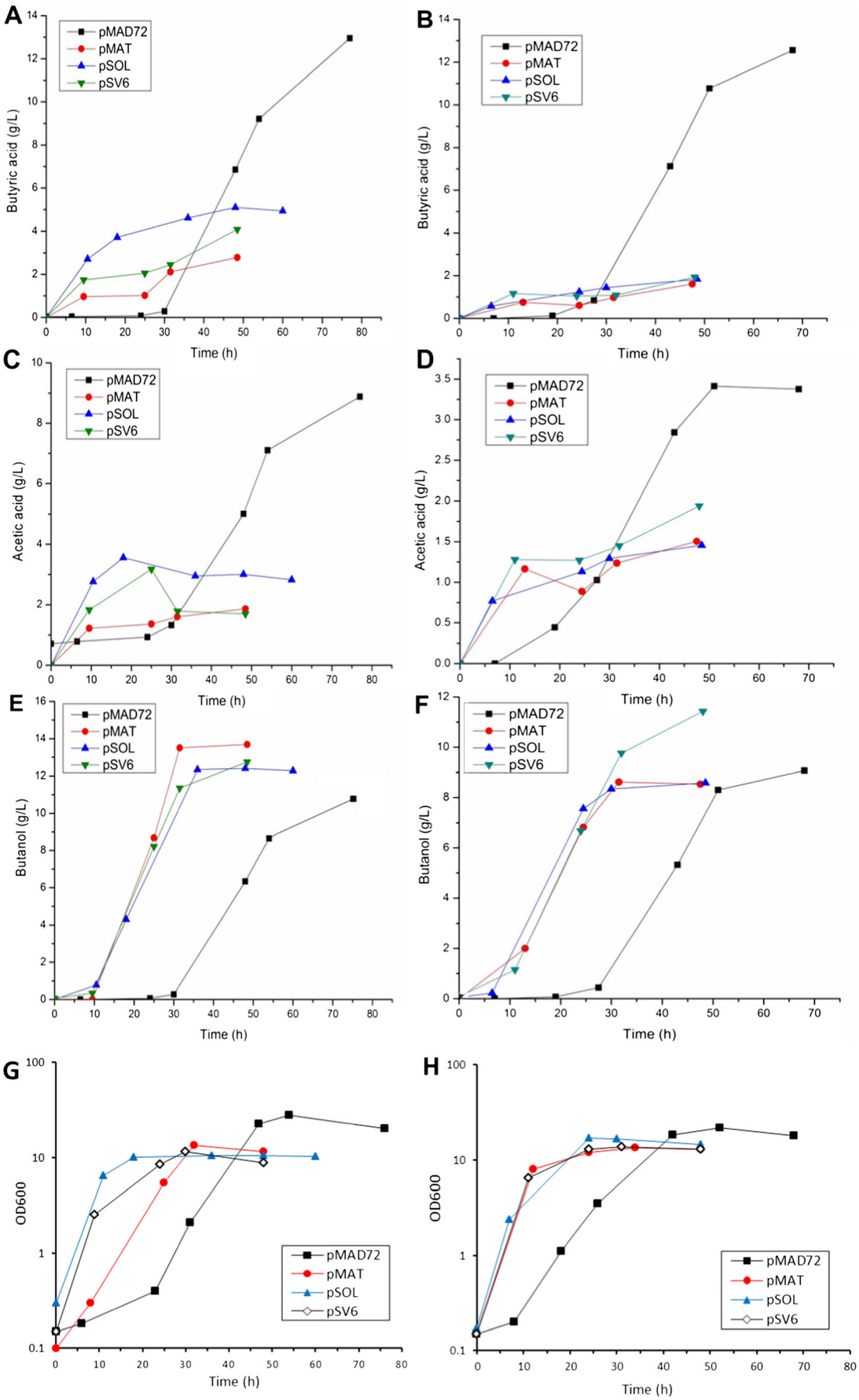
Effects of ctfAB

CoA transferase encoded by *ctfAB* plays an important role in the reassimilation and conversion of acetate and butyrate, produced in the acidogenesis phase, to solvents (acetone, butanol, and ethanol) in the solventogenic phase in solventogenic *Clostridium*. The overexpression of *ctfAB* in *C. tyrobutyricum* Ct(Δack) thus had pronounced effects on cell growth, acid production, and butanol production. Without *ctfAB*, the strain Ct(Δack)-pMAD72 produced much more butyrate (Fig. 4a, b) and acetate (Fig. 4c, d), and less butanol (Fig. 4e, f), compared to the mutants overexpressing CoA-transferase. Ct(Δack)-pMAD72 also grew slower initially with a longer lag phase of ~30 h but reached a higher final cell density (Fig. 4g, h). On the other hand, the overexpression of CoA transferase caused an earlier production of butanol, which was toxic to cells and thus resulted in lower cell density and earlier termination of the fermentation with less glucose consumption and total metabolites (solvents and acids) produced (Fig. 5a, b). Nevertheless, overexpressing *ctfAB* resulted in over 100 % increase in butanol yield (Fig. 5c, d) and productivity (Fig. 5e) but negligible effect on the specific growth rate (Fig. 5f).

Clearly, *ctfAB* expression improved butanol production by reassimilating and converting butyrate and acetate to their corresponding alcohols, resulting in 21–31 % higher butanol titer and over 100 % increase in butanol yield (from 0.10 to 0.22 g/g glucose) and productivity (from 0.13 to 0.35 g/l h) at pH 6.0. The mutants with CoA transferase expression also had a much shorter lag phase, although a similar specific growth rate, indicating that CoA transferase expression allowed cells to grow sooner by limiting the accumulation of butyrate, which is an inhibitor to cell growth (Zhu and Yang 2003). However, the accelerated production of butanol by these mutants led to an early threshold of butanol toxicity (Bowles and Ellefson 1985), lower cell density reached in the stationary phase, and earlier termination of the fermentation with less glucose consumption.

Although *ctfAB* had pronounced effects on decreasing butyrate and acetate production and increasing butanol production, it showed negligible effect on ethanol production (Fig. 5a, b). This can be attributed to the fact that *C. tyrobutyricum*, as a native high butyrate-tolerant and producing strain, has a high metabolic flux from acetyl-CoA to butyryl-CoA, which favors butanol production over ethanol production. Therefore, the expression of CoA transferase in *C. tyrobutyricum* increased its butanol production but had little effect on ethanol production.

It is noted that the reduction in butyrate production was much more than the reduction in acetate production in the presence of *ctfAB*. For example, on average, butyrate production decreased 73 and 85 %, while acetate production decreased 67 and 59 % at pH 6.0 and pH 5.0, respectively (see


A
Butyric acid (g/L)
Time (h)
pMAD72
pMAT
pSOL
pSV6
B
Butyric acid (g/L)
Time (h)
C
Acetic acid (g/L)
Time (h)
D
Acetic acid (g/L)
Time (h)
E
Butanol (g/L)
Time (h)
F
Butanol (g/L)
Time (h)
G
OD600
Time (h)
H
OD600
Time (h)

◀ **Fig. 4** Comparison of butyrate, acetate, and butanol production and cell growth (OD) among strains carrying different plasmids pMAD72, pMAT, pSOL, and pSV6 in batch fermentations at pH 6.0 (**a**, **c**, **e**, **g**) and 5.0 (**b**, **d**, **f**, **h**)

Table S1 in Supplementary Materials). Apparently, more butyrate has been converted by CoA transferase than acetate, although the in vitro enzyme activity assay showed a similar rate for CoA transfer to butyrate or acetate (see Table 2). This finding is consistent with a previous study showing that in vivo CoA transferase had a much higher activity toward butyrate than acetate (Wiesenborn et al. 1989a).

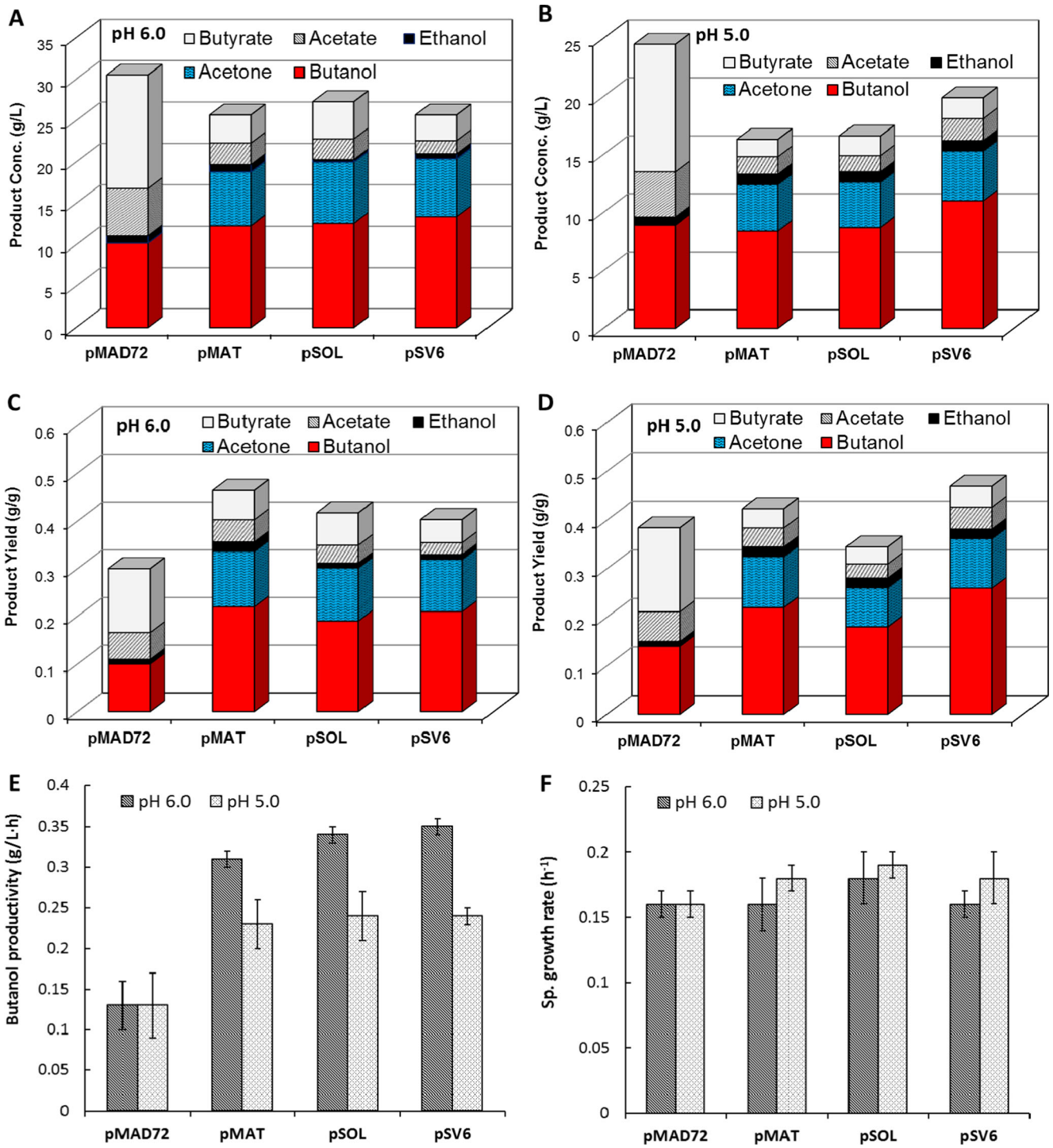


Fig. 5 Comparison of butyrate, acetate, acetone, ethanol, and butanol production and specific growth rate for strains carrying different plasmids pMAD72, pMAT, pSOL, and pSV6 in batch fermentations at pH 6.0 and 5.0

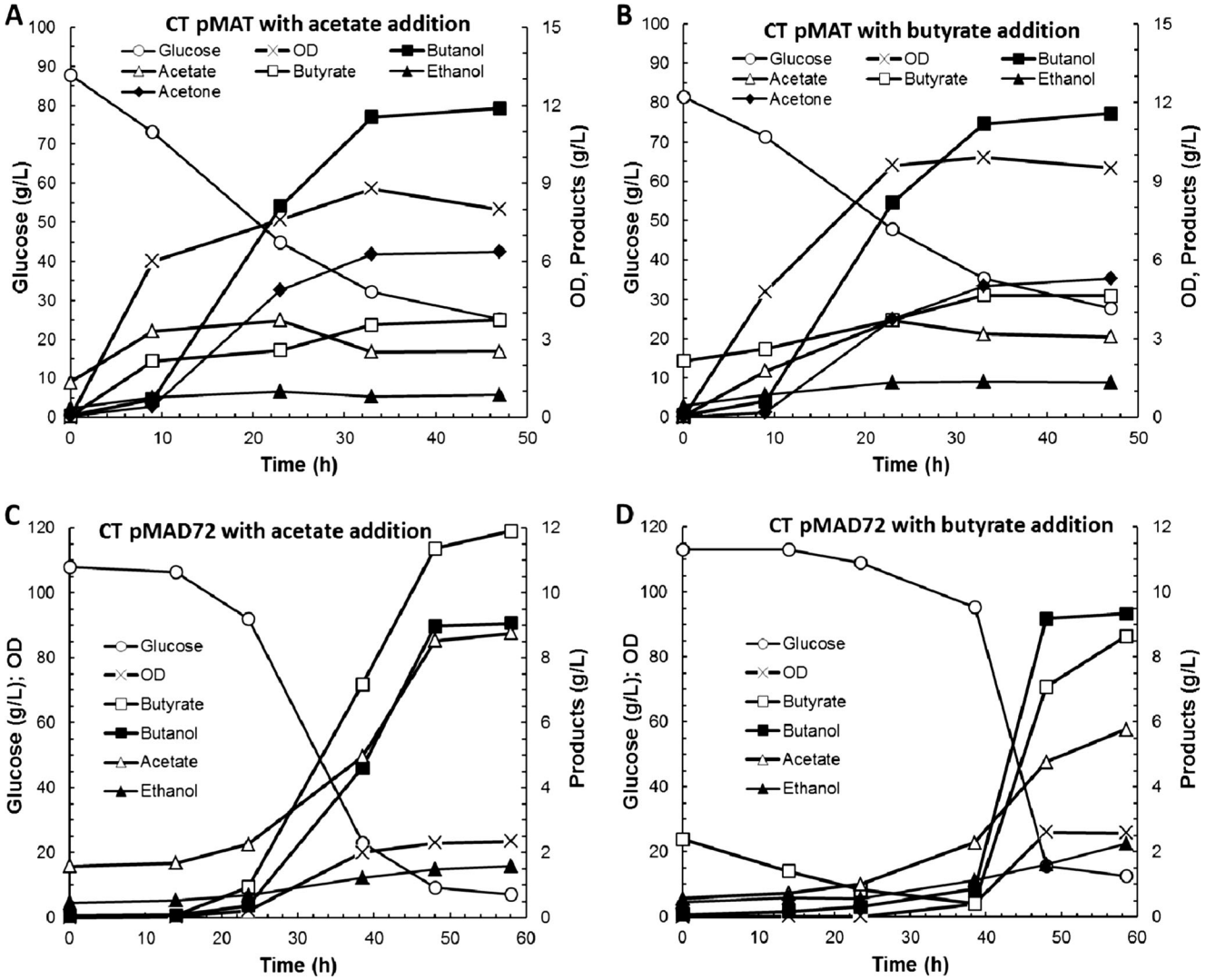


Fig. 6 Fermentation kinetics of *C. tyrobutyricum* Ct(Δack)-pMAT and Ct(Δack)-pMAD72 at pH 6.0 with acetate or butyrate addition in the medium

Effects of pH

In general, more butanol was produced at a higher rate at pH 6.0 than at pH 5.0 because the optimal pH for aldehyde/alcohol dehydrogenase (*adhE2*) is around 6.5 (Fontaine et al. 2002). In addition, at pH 5.0, most acids would be present in the undissociated form, which is toxic to cells (Maddox et al. 2000). On the other hand, the CoA transferase did not seem to be much affected by the pH between 5.0 and 6.0, as their effects on decreasing acid production and increasing butanol yield and productivity were similar at both pHs. The lack of effect on increasing butanol titer at pH 5.0 by the mutants Ct(Δack)-pMAT and Ct(Δack)-pSOL as compared to Ct(Δack)-pMAD72 could be attributed to the low aldehyde/alcohol dehydrogenase activities at the acidic pH. This problem was alleviated by co-expressing *ald* and *adhE2* in Ct(Δack)-pSV6, which produced significantly more butanol (11 vs. <9 g/l) compared to the other mutants.

For all mutants expressing *ctfAB*, more acetone was also produced at pH 6.0 than at pH 5.0 (see Table S1 in Supplementary Materials), because of higher cell activity at pH 6.0. Moreover, the butanol/acetone ratio was lower at pH 6.0 (1.7–1.9 g/g) than at pH 5.0 (2.1–2.6 g/g), suggesting that pH 6.0 was more favorable for acetone production, probably because CoA transferase activity was higher at pH 6.0 than at 5.0 (Wiesenborn et al. 1989a).

Effects of different ctfAB and ald genes

No significant difference in the fermentation kinetics were found for Ct(Δack)-pMAT and Ct(Δack)-pSOL. The former expressed *adhE2* and *ctfAB* from *C. acetobutylicum* ATCC 824, while the latter expressed *ald*, *ctfAB*, and *adc* from *C. beijerinckii*. Different from *C. acetobutylicum*, *C. beijerinckii* does not bear any mega-plasmid and *ald*, *ctfAB*, and *adc* are located on its chromosome. Also, the *ald*

gene in *C. beijerinckii*, unlike *adhE* gene from *C. acetobutylicum*, only has aldehyde dehydrogenase activity. Nevertheless, butanol and acid production levels in both mutant strains were similar, suggesting that the native *bdh* and *adh* genes in *C. tyrobutyricum* genome are functional. In addition, both strains produced acetone at a similar level, indicating that the *adc* gene encoding an acetoacetate decarboxylase is not required for acetone production from acetoacetate, as also found for *C. beijerinckii* by Han et al. (2011). Overexpressing both *adhE2* and *ald* in Ct(Δack)-pSV6 gave the best butanol production among the mutants studied, probably because of the increased aldehyde dehydrogenase activity. The effect was more pronounced at pH 5.0, at which the activities of aldehyde/alcohol dehydrogenase might be limited because the optimal pH for the enzyme activity is around neutral (Fontaine et al. 2002).

Acid reassimilation

To further illustrate the effects of CoA-transferase on acid reassimilation, batch fermentations of Ct(Δack)-pMAD72 and Ct(Δack)-pMAT were studied at pH 6.0 in media initially also containing ~20 mM acetate or butyrate. As expected, for the strain Ct(Δack)-pMAT expressing *ctfAB*, both acetate and butyrate were kept at a relatively low level (less than 3–5 g/l) compared to the control strain Ct(Δack)-pMAD72 without *ctfAB*, which produced large amounts of acetate and butyrate (Fig. 6). In fact, a notable decrease in the acetate level after peaking at ~24 h was observed for Ct(Δack)-pMAT (Fig. 6a, b) but not for Ct(Δack)-pMAD72 (Fig. 6c, d). These results clearly demonstrated that *ctfAB* played an important role in acid reassimilation. It is noted that without *ctfAB*, there was a long lag phase of ~24 h, especially when ~20 mM butyrate was added in the medium. Interestingly, the butyrate concentration decreased from 2.4 to 0.4 g/l during the lag phase (Fig. 6d). The apparent butyrate uptake by Ct(Δack)-pMAD72 suggested the existence of a reverse reaction from butyrate to butyryl-CoA, possibly catalyzed by phosphotransbutyrylase (Ptb) and butyrate kinase (Buk), which has also been proposed for *C. acetobutylicum* (Jiang et al. 2009; Lehmann et al. 2012a; Jang et al. 2012; Millat et al. 2014). Nevertheless, this butyrate uptake pathway seemed to work only in the lag phase, not during the exponential growth phase, and required energy (ATP). Once the butyrate level was reduced to a non-inhibiting level, normal cell growth started and butyrate (and acetate) was produced, which generated more ATP to support fast cell growth. No acetate uptake by Ct(Δack)-pMAD72 was observed (Fig. 6c), again confirming that acetate reassimilation required the CoA transferase (*ctfAB*).

Discussion

The *sol* operon containing *adhE* or *ald* and *ctfA* and *ctfB* (encoding two protein subunits for the CoA-transferase) is responsible for the production of ABE in solventogenic clostridia (Cornillot et al. 1997; Nair et al. 1999; Nair and Papoutsakis 1994). The ability to reassimilate acetate and butyrate is critical to the biphasic ABE fermentation. Failure to do so by solventogenic clostridia can cause acid crash, a phenomenon often observed in industrial ABE fermentation (Wang et al. 2011; Maddox et al. 2000). As evidenced in this study and many other studies, the CoA transferase encoded by *ctfAB* is responsible for transferring CoA from acetoacetyl-CoA to butyrate and acetate, forming acetoacetate, butyryl-CoA, and acetyl-CoA, which are then converted to ABE in the reactions catalyzed by the enzymes encoded by *adc* and *adhE*, respectively (Lee et al. 2008). Also, acetone production is usually coupled with the reassimilation of acids, as mutants with disrupted acetone-producing pathway also showed a significantly increased acid production (Sillers et al. 2008; Lee et al. 2008, 2009; Jang et al. 2012). It is thus generally believed that the reassimilation of acids in *C. acetobutylicum* is controlled by the expression of *ctfAB* during the metabolic shift from acidogenesis to solventogenesis (Lehmann et al. 2012b).

However, studies with *ctfAB*-disrupted mutants also suggested the existence of a CoA-transferase-independent butyrate uptake pathway involving Ptb and Buk, which normally catalyze the reactions from butyryl-CoA to butyryl phosphate and then to butyrate, respectively (Jiang et al. 2009; Lehmann et al. 2012a; Jang et al. 2012). Butyrate uptake through the reverse Ptb-Buk pathway was demonstrated with a mutant of *C. acetobutylicum* overexpressing *ptb* and *buk* (Walter et al. 1994), as well as by using a mathematical model simulating the metabolic pathways in ABE-producing network (Millat et al. 2014). In addition, purified Ptb from *C. acetobutylicum* ATCC 824 also showed an increased catalytic activity for the reverse reaction of butyryl phosphate to butyryl-CoA as the pH decreased below 6.0 (Wiesenborn et al. 1989b). For the first time, our study also showed the possible existence of the reverse Ptb-Buk pathway for butyrate uptake by a native butyrate-producing *C. tyrobutyricum*, although *ptb* and *buk* genes have not been found or annotated in the recently published draft genome of *C. tyrobutyricum* ATCC 25755 (Bassi et al. 2013; Jiang et al. 2013), probably because of the incomplete annotation (only ~50 %). The existence of Ptb and Buk in *C. tyrobutyricum* was partially proved by testing their enzyme activities in a previous study (Zhang et al. 2012); however, further verification would be necessary.

All previous studies on *ctfAB* and acid reassimilation were conducted with type strains of solventogenic clostridia, such as *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052, which have complex biphasic physiology involving

highly regulated metabolic and transcriptional networks (Alsaker et al. 2010; Dürre et al. 2002; Girbal et al. 1995; Janssen et al. 2012; Rydzak et al. 2011; Thormann et al. 2002; Schwarz et al. 2012; Wang et al. 2013). Several genes located on two operons (*sol* and *adc*) are involved in the biphasic ABE fermentation (Fischer et al. 1993; Gerischer and Dürre 1990; Petersen and Bennett 1990), and they are tightly regulated by several transcription factors, including *spo0A* and *solR* (Alsaker et al. 2004; Thormann et al. 2002; Nair et al. 1999; Ravagnani et al. 2000; Steiner et al. 2011; Tomas et al. 2004). However, the regulatory mechanism is highly complicated, involving many additional genes and transcription factors controlling not only acidogenesis and solventogenesis but also sporulation and clostridia life cycle and remains unclear (Nicolaou et al. 2010; Xu et al. 2015). In contrast, *C. tyrobutyricum*, as a native *ctfAB* and *adc* deficient strain without the complex biphasic physiology, provides a novel (simpler) system to study acid reassimilation by *ctfAB* and its effects on cell growth and solvent production. This cannot be easily done with *C. acetobutylicum* as its *ctfAB* disruption would also influence the expression of *adhE* located within the same cistronic operon, compromising alcohol production by the mutant (Tummala et al. 2003; Sillers et al. 2009).

The metabolically engineered *C. tyrobutyricum* can also be used as a novel host for *n*-butanol production with several advantages over conventional solventogenic clostridia (Ma et al. 2015). Its high tolerance to butyrate, as well as butanol, and strong carbon flux toward C4 products would favor the production of *n*-butanol, instead of ethanol, when *adhE2* is overexpressed (Yu et al. 2011). Butanol is the desirable product as it has superior biofuel properties compared to ethanol. While overexpressing *adhE* in *C. acetobutylicum* ATCC 824 increased both ethanol and butanol production, increasing the flux from acetyl-CoA to acetoacetyl-CoA by also overexpressing *thl* (encoding thiolase) decreased C2 metabolites (acetate and ethanol) and increased acetone and butyrate production (Sillers et al. 2009). Clearly, an enhanced intracellular butyryl-CoA pool could improve butanol production and selectivity. On the other hand, a butyrate-negative mutant strain of *C. acetobutylicum* ATCC 824 showed elevated ethanol titer with depressed butanol production (Lehmann et al. 2012b). Constitutively expressing *adhE2* in *C. tyrobutyricum* also enables the mutant strain to continuously produce *n*-butanol throughout the fermentation without subjecting to life cycle regulation and acid crash as often encountered by *C. acetobutylicum*. Further expression of CoA transferase in *C. tyrobutyricum* not only increased its butanol production, with more than 100 % increase in butanol yield and productivity, but also facilitated the production of acetone. A previous study also showed that overexpressing *adc* and *ctfAB* in *C. acetobutylicum* led to earlier induction of acetone formation, with enhanced acetone (95 %), butanol (37 %), and ethanol (90 %) production (Mermelstein et al. 1993).

Although overexpressing *ctfAB* increased butanol yield and productivity by more than 100 %, the final butanol titer in the fermentation only increased 20 to 30 %. This is because butanol production is also limited by butanol toxicity and the availability of NADH (see Fig. 1). Butanol toxicity can be alleviated by removing butanol in situ during fermentation (Xue et al. 2012, 2014), increasing butanol tolerance via adaptation (Yang and Zhao 2013) and metabolic engineering (Lütke-Eversloh and Bahl 2011; Tomas et al. 2003), whereas NADH availability can be increased by inhibiting hydrogen production (Datta and Zeikus 1985), redox engineering (Ventura et al. 2013; Wang et al. 2012), and using artificial electron carriers such as methyl viologen (Du et al. 2015) and more reduced substrates such as mannitol (Yu et al. 2012). With further metabolic and process engineering, it is possible to produce butanol at a higher titer of ~20 g/l using *C. tyrobutyricum* Ct(Δack) overexpressing *ctfAB* and *adhE2*.

In conclusion, overexpressing *ctfAB* facilitated the reassimilation of butyrate and significantly increased butanol production from glucose by *C. tyrobutyricum* Ct(Δack) overexpressing *adhE2*, resulting in over 100 % increase in butanol yield and productivity. Co-expressing *ctfAB* with *adhE2* also led to the production of acetone to a high level of ~50 % of that for butanol, turning the native acidogenic *C. tyrobutyricum* into an ABE producer with high yields. Further improvement in butanol production can be achieved by engineering the cells for higher butanol tolerance and increasing the NADH level available for butanol biosynthesis during the fermentation. This study demonstrated the essential role of CoA-transferase in acetate and butyrate reassimilation and also suggested possible existence of an exclusive Pta-Buk reverse pathway for butyrate uptake by *C. tyrobutyricum*.

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Compliance with ethical standards This research does not involve human participants or animals.

Conflict of interest The authors declare no conflict of interests.

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