

# Characterization of Recombinant Strains of the *Clostridium acetobutylicum* Butyrate Kinase Inactivation Mutant: Need for New Phenomenological Models for Solventogenesis and Butanol Inhibition?

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**Abstract:** Two metabolic engineering tools, namely gene inactivation and gene overexpression, were employed to examine the effects of two genetic modifications on the fermentation characteristics of *Clostridium acetobutylicum*. Inactivation of the butyrate kinase gene (*buk*) was examined using strain PJC4BK, while the combined effect of *buk* inactivation and overexpression of the *aad* gene—encoding the alcohol aldehyde dehydrogenase (AAD) used in butanol formation—was examined using strain PJC4BK(pTAAD). The two strains were characterized in controlled pH  $\geq 5.0$  fermentations, and by a recently enhanced method of metabolic flux analysis. Strain PJC4BK was previously genetically characterized, and fermentation experiments at pH  $\geq 5.5$  demonstrated good, but not exceptional, solvent-production capabilities. Here, we show that this strain is a solvent superproducer in pH  $\geq 5.0$  fermentations producing 225 mM (16.7 g/L) of butanol, 76 mM of acetone (4.4 g/L), and 57 mM (2.6 g/L) of ethanol. Strain PJC4BK(pTAAD) produced similar amounts of butanol and acetone but 98 mM (4.5 g/L) of ethanol. Both strains overcame the 180 mM (13 g/L) butanol toxicity limit, without any selection for butanol tolerance. Work with strain PJC4BK(pTAAD) is the first reported use of dual antibiotic selection in *C. acetobutylicum*. One antibiotic was used for selection of strain PJC4BK while the second antibiotic selected for the pTAAD presence. Overexpression of *aad* from pTAAD resulted in increased ethanol production but did not increase butanol titers, thus indicating that AAD did not limit butanol production under these fermentation conditions. Metabolic flux analysis showed a decrease in butyrate formation fluxes by up to 75% and an increase in acetate formation fluxes of up to 100% during early growth. The mean specific butanol and ethanol formation fluxes increased significantly in these recombinant

strains, up to 300% and 400%, respectively. Onset of solvent production occurred during the exponential-growth phase when the culture optical density was very low and when total and undissociated butyric acid levels were  $<1$  mM. Butyrate levels were low throughout all fermentations, never exceeding 20 mM. Thus, threshold butyrate concentrations are not necessary for solvent production in these stains, suggesting the need for a new phenomenological model to explain solvent formation. © 2000 John Wiley & Sons, Inc. *Biotechnol Bioeng* 67: 1–11, 2000.

**Keywords:** *Clostridium acetobutylicum*; flux analysis; metabolic engineering; solvents; butyrate kinase; AAD

## INTRODUCTION

The Gram-positive, spore-forming, obligate-anaerobe *Clostridium acetobutylicum* is typically characterized by two distinct phases of product formation in batch fermentations. During acidogenesis, the first of the two phases, cell growth is exponential, and the primary organic products are acetic and butyric acids. Accumulation of these products, which are toxic to the organism at high concentrations, results in a decrease in culture pH. As cells enter the stationary phase, solvent production predominates. During this second developmental stage, known as solventogenesis, acetone, butanol, and ethanol are generated as the cells take up the previously formed acids. The biochemical pathways for production of acids and solvents in this strain have been long studied (Jones and Woods, 1986). There is currently active research involving the metabolic engineering of *C. acetobutylicum* to develop strains and processes that will facilitate use of this organism in cost-effective acetone-butanol-ethanol (ABE) fermentations. The usefulness of this organism for production of commodity chemicals will depend upon directing the carbon flux during fermentations toward the desired products. Our research presently focuses on de-

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veloping strains with desired patterns of product formation as well as understanding the signals and control mechanisms that ultimately determine the flow of carbon toward specific products.

Of particular interest in metabolic studies are the molecules located at nodes between acid and solvent production pathways. Butyryl-CoA is a branch point intermediate located along the central-metabolic pathway of *C. acetobutylicum* at the node dividing the butyrate-formation branch from the butanol-formation branch. Butyrate is generated from butyryl-CoA during acidogenesis by a two-step, ATP-forming, enzymatic process: phosphotransbutyrylase (PTB) catalyzes the formation of butyryl phosphate, which is then converted to butyrate by the action of butyrate kinase (BK). The two enzymes involved in butyrate production (PTB and BK) are encoded by *ptb* and *buk*, respectively, which comprise a single operon with *ptb* upstream of *buk* (Walter et al., 1993). During solventogenesis, butanol is produced from butyryl-CoA by two successive reductions. The first results in butyraldehyde production by the action of a butyraldehyde dehydrogenase and the second reduction, catalyzed by butanol dehydrogenase, results in butanol formation. Previous experiments (Green and Bennett, 1996; Nair and Papoutsakis, 1994) indicate that the bifunctional AAD (alcohol/aldehyde dehydrogenase), whose gene is first expressed during the beginning of solventogenesis in the wild-type (WT) organism, is the main enzyme responsible for butanol production. The onset of solventogenesis is the time when the first solvent, usually butanol, is detected by measuring concentrations of at least 1–2 mM. Threshold undissociated butyric acid (UBA) concentrations have been implicated in the switch to solvent production. Terracciano and Kashket determined that when the UBA reached 13–18 mM, solvent production began (Terracciano and Kashket, 1986). Husemann and Papoutsakis reported similar results, citing 6–13 mM of UBA as the requirement for initiation of solventogenesis when the external pH was between 3.7 and 5.0 (Husemann and Papoutsakis, 1988).

The ability to selectively inactivate genes in *C. acetobutylicum* using nonreplicative plasmids is a recent one (Green and Bennett, 1996). The butyrate-formation pathway of this organism has been altered by inactivation of the *buk* gene, which encodes the enzyme (BK) responsible for conversion of butyryl phosphate to butyrate. The resulting strain (PJC4BK) was genetically characterized and was shown to produce reduced butyrate amounts, but typical levels of solvents in pH  $\geq$  5.5 fermentations (Green et al., 1996). Here, we show that in pH  $\geq$  5.0 fermentations, the strain is a superproducer with final solvent titers much higher than WT values, and that the solvent ratios were significantly altered. Additionally, a plasmid-(pTAAD) encoded solvent-formation gene, *aad*, was overexpressed in strain PJC4BK in an effort to better understand the effect of increased gene dosage on solvent formation in this strain. Metabolic flux analysis using a recently modified and enhanced method (Desai et al., 1999) demonstrates the profound effect of

these genetic alterations on several primary metabolic fluxes.

## MATERIALS AND METHODS

### Bacterial Strains and Plasmids

Bacterial strains and plasmids are listed in Table I.

### Growth Conditions

*Escherichia coli* was grown aerobically at 37°C in Luria-Bertani (LB) medium, and *C. acetobutylicum* was grown anaerobically at 37°C in 10-mL tube cultures of Clostridium Growth Medium (CGM; Roos et al., 1985; Wiesenborn et al., 1988). Colonies of *E. coli* and *C. acetobutylicum* were obtained on agar-solidified LB or Reinforced Clostridial Medium (RCM; Difco Laboratories, Detroit, MI), respectively. For recombinant strains, liquid media were appropriately supplemented with erythromycin (Em) (100  $\mu$ g/mL), tetracycline (Tc) (10  $\mu$ g/mL), and chloramphenicol (Cm) (35  $\mu$ g/mL); 40  $\mu$ g/mL of erythromycin and 10  $\mu$ g/mL of tetracycline were used in solid media as needed. *E. coli* strains were stored long-term at –85°C in LB medium with 10% glycerol. For long-term storage of *C. acetobutylicum*, strains were maintained as spores on agar-solidified RCM at a pH of 6.8 or frozen at –85°C in CGM with 15% glycerol. In preparation for electroporation, *C. acetobutylicum* was grown under anaerobic conditions at 37°C in liquid 2X YTG (pH 5.2) supplemented with erythromycin as needed.

**Table I.** Bacterial strains and plasmids.

Strain/Plasmid	Relevant characteristics <sup>a</sup>	Source or reference
<i>C. acetobutylicum</i>		
ATCC 824 (WT)		ATCC <sup>b</sup>
M5	AAD <sup>-</sup> AADC <sup>-</sup> CoAT <sup>-</sup> <i>spo</i> <sup>-</sup>	Clark et al., 1989
PJC4BK	ATCC 824 <i>buk</i> ::pJC4BK MLS <sup>r</sup>	Green et al., 1996
<i>E. coli</i>		
ER2275	<i>recA</i> <sup>-</sup> <i>lacZ</i> <sup>-</sup> <i>mcrBC</i> <sup>-</sup>	NEB <sup>c</sup>
pAN1	Cm <sup>r</sup> , $\Phi$ 3TI	Mermelstein, 1992
pAM620	Tc <sup>r</sup>	Su et al., 1992
pHXS5	Ap <sup>r</sup> <i>aad</i> <i>ctfA</i> <i>ctfB</i> <i>solR</i>	Nair, 1995
pIMP1	Ap <sup>r</sup> MLS <sup>r</sup>	Mermelstein, 1992
pJC4	MLS <sup>r</sup> Tc <sup>r</sup>	Lee et al., 1992
pJC4BK	MLS <sup>r</sup>	Green et al., 1996
pTAAD	Ap <sup>r</sup> Tc <sup>r</sup> <i>aad</i>	This study
pTLH1	Ap <sup>r</sup> Tc <sup>r</sup>	This study

<sup>a</sup>Abbreviations: AADC, *adc*, acetoacetate decarboxylase; CoAT, *ctfA*, *ctfB*, acetoacetyl-coenzyme A: acetate/butyrate:coenzyme A-transferase subunits; *spo*<sup>-</sup>, asporogenous; MLS<sup>r</sup>, macrolide lincosamide streptogramin B resistant; *recA*<sup>-</sup>, homologous recombination abolished; *lacZ*,  $\beta$ -galactosidase; *mcrBC*, methylcytosine-specific restriction system; Cm<sup>r</sup>, chloramphenicol resistant;  $\Phi$ 3TI,  $\Phi$ 3T methylase; Tc<sup>r</sup>, tetracycline resistant; Ap<sup>r</sup>, ampicillin resistant; AAD, *aad*, alcohol/aldehyde dehydrogenase; *solR*, putative repressor of *sol* operon genes.

<sup>b</sup>American-type culture collection.

<sup>c</sup>New England Biolabs, Beverly, MA.

After electroporation, transformants were grown on 2X YTG plates (pH 5.8) with appropriate antibiotics. 2X YTG contains 16 g of Bacto tryptone, 10 g yeast extract, 4 g sodium chloride, and 5 g glucose/L.

### ***Escherichia coli* DNA Isolation and Manipulation**

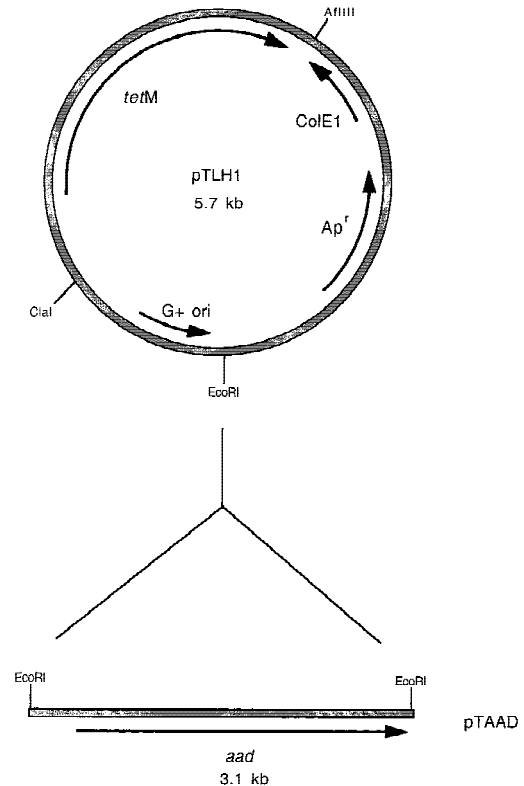
The isolation of plasmids from *E. coli* via the alkaline lysis method and further manipulation of *E. coli* plasmid DNA was performed using standard protocols (Lee and Rasheed, 1990). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) or Pharmacia Biotech, Inc. (Piscataway, NJ) and used according to manufacturer instructions. T4 DNA ligase and alkaline phosphatase were also purchased from Pharmacia. DNA fragments were purified using the Qiaquick kit for DNA extraction from agarose gels (Qiagen, Inc., Chatsworth, MA) and concentrated using Microcon 100 Microconcentrators (Amicon, Inc., Beverly, MA). Previously published methods were used for electrotransformation of *C. acetobutylicum* (Mermelstein and Papoutsakis, 1993) using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA).

### ***Clostridium acetobutylicum* Plasmid DNA Isolation and Manipulation**

A protocol was optimized for isolation of plasmid DNA from *C. acetobutylicum*, a procedure made difficult by the presence of high extracellular-nuclease activity (Awang et al., 1988; Jones and Woods, 1986; Reid et al., 1983; Young and Ehrlich, 1989). Approximately 6 mL of clostridia cells were collected during late-exponential growth by centrifugation at 12,500 rpm (10,500g) in a microcentrifuge. The cells were washed twice in a buffer containing 0.5M KCl, 0.1M EDTA and 0.05M Tris-HCl followed by a single wash in SET (25% sucrose, 0.05M Tris-HCl, 0.05M EDTA) to remove salts before suspending the cells in 450 µL of a 5 mg/mL solution of lysozyme in SET. This lysis solution was incubated at 37°C for 20 min. After the incubation, 350 µL of alkaline SDS was added and the solution mixed by inverting several times. 350 µL of 3M potassium—5M acetate solution was immediately added and mixed by inverting the tube gently. The precipitate was removed by centrifugation and the supernatant containing the plasmid DNA was extracted twice in 1 vol of phenol:chloroform:isoamyl alcohol (25:24:1) and once in 1 vol of chloroform:isoamyl alcohol (24:1). The plasmid DNA was precipitated in isopropanol and washed with 70% ethanol before drying the pellet and suspending it in TE buffer containing 20 µg RNase activity/mL. The washing step is key to successful plasmid DNA isolation and is thought to dislodge non-specific nucleases which may be attached to the surface of the cell by ionic interactions (Hendrix and Welker, 1985).

### **Construction of Plasmids**

The tetracycline-resistant cloning vector pTLH1 was created for use in the MLS<sup>r</sup> clostridial strains. pTLH1 (Fig. 1) is a



**Figure 1.** Construction of pTAAD.

5.7 kb circular *E. coli*–*C. acetobutylicum* shuttle vector which contains a Gram-negative origin of replication from pUC9, a Gram-positive origin of replication from pIM13, a multiple cloning site from pUC9, and a *tetM* gene from Tn916 as a selectable marker. To construct pTLH1, the 3.2 kb fragment of pIMP1 (Mermelstein, 1992) from an *AfIII* and *ClaI* double digestion was first isolated. The *tetM* gene was PCR amplified from pAM620 (Su et al., 1992), which contains the complete Tn916 isolated from *Enterococcus faecalis*, using primers designed to incorporate a *ClaI* restriction site upstream from the gene and an *AfIII* restriction site downstream of the gene. The upstream primer (UPTLH2 = 5'-ATAATCAAGTCACGGTATCGATGACAGT -3') was designed by substituting an A for a C at position 11645 to create a *ClaI* restriction site. The downstream primer (DSTLH2 = 5'-ACAGGACACAATATC-CACATGTAGTTTA -3') was designed by substituting an A for a T at position 14143 of the Tn916 Genbank sequence to create an *AfIII* restriction site downstream of the *tetM* gene. Touchdown PCR was used for amplification of the fragment containing *tetM*. Thirty PCR cycles were completed under the following conditions: 1 min at 94°C to denature the DNA, annealing for 1 min at 60–45°C with the temperature decreased 0.5°C per cycle, and 2½ min at 72°C for elongation of the nascent DNA strands. An additional 15 cycles of PCR were then performed at a constant annealing temperature of 45°C. The 2.5-kb-long PCR product was purified, double-digested using *AfIII* and *ClaI*, and ligated to the 3.2-kb pIMP1 fragment resulting in the 5.7-kb pTLH1 master shuttle vector.

To construct the vector pTAAD (Fig. 1), a 3.06-kb *aad* fragment was cloned into pTLH1. The *aad* fragment, which contains the 2.6-kb *aad* open reading frame and its two natural promoters, was PCR amplified out of pHXS5 using primers designed to generate terminal *Eco*RI sites. The upstream primer (UPECOAAD = 5'- TTCTAAATATA CTGAGAATTCCTAAATA -3') substitutes a G for a T at position 1692. The downstream primer (DSECOAAD = 5'- CTAATTATTTTA GAATTC ATTTTAATCC -3') substitutes an A for a G at position 4718. Thirty cycles of PCR were completed under the following conditions: 1 min at 94°C, 1 min at 45°C, and 3 min at 72°C. The resulting fragment was purified, *Eco*RI digested, and ligated with the 5.7-kb *Eco*RI-linearized pTLH1 plasmid. The result was the pTAAD vector containing *aad* oriented in the same direction as the Gram-positive origin of replication.

### Fermentations and Analytical Methods

Fermentations of *C. acetobutylicum* strains at pH  $\geq$  5.0 and product concentration analysis were performed as previously reported (Green et al., 1996). Two antibiotics were used for maintenance of strains PJC4BK(pTLH1) and PJC4BK(pTAAD), erythromycin to select for the host strain PJC4BK and tetracycline for maintenance of the plasmids. Selective pressure was applied up to the time that the reactors were inoculated. However, no antibiotics were used during fermentations because use of tetracycline was found to inhibit solvent production (Harris, 1997 MS Thesis). Comparison studies indicated that the plasmid-carrying strains were stable throughout the course of fermentations without selective pressure. The presence of the plasmid was also confirmed by plasmid DNA isolation from the bacterial cells throughout the fermentations. Approximate measurement errors were: glucose, 3%; acetone, 3%; ethanol, 3%; butanol, 3%; acetoin, 3%; acetate, 5%; and butyrate, 5%.

### Undissociated Acid Calculations

The total extracellular butyrate and acetate concentrations were measured using gas chromatography as discussed in the Analytical Methods section. Because the pH of the broth was known at each sample point, the fraction of the butyrate that was in the undissociated butyric acid form was calculated using the Hendersen-Hasselbalch equation (Husemann and Papoutsakis, 1988). Based on the assumption that undissociated acids can freely traverse the cell membrane, the external and internal undissociated acid concentrations are reported as the same value.

### Metabolic Flux Analysis

Metabolic flux analysis involves the calculation of in vivo fluxes from substrate and product data using a system of linear equations developed from reaction stoichiometry (Papoutsakis, 1984). The analysis was performed using software developed specifically for the analysis of *C. acetobu-*

*tylicum* fermentation data (Desai et al., 1999), and the pathways fluxes considered in the model are depicted in Figure 3. For these analyses, the time scales of the fermentations were re-scaled to account for variable-lag phases. The normalized scale,  $T_N$ , is set such that  $T_N = 0$  h at  $A_{600} = 1$ . On this time scale, the transition from exponential phase to stationary phase typically occurs at  $T_N = 10$  h. Therefore, the stages of the fermentations are classified as "Early" for  $T_N < 10$  h, and "Late" for  $T_N > 10$  h. The variation in calculated metabolic fluxes due to different levels of biomass was also accounted for by using the  $A_{600}$  to calculate specific in vivo fluxes, which are reported in units of  $mM/h^{-1}$  (unit  $A_{600}^{-1}$ ). The calculated metabolic fluxes were examined by two approaches. In the "kinetic" approach, the calculated time-course profiles were examined to reveal the time-dependent patterns of metabolic activity. For this approach, the uncertainties in the calculated fluxes are approximately as follows: rPTBBK, 10%; rPTAAK, 15%; rACUP, 8%, rBYUP, 6%; rETOH, 10%; rBUOH, 10%. In the "integral" approach, the mean specific fluxes during the Early and Late stages of the cultures were calculated. These integral fluxes reflect the overall impact on metabolic pathways driving each stage. For this approach, the variability between fermentations was used to estimate the standard errors of the mean specific fluxes.

## RESULTS

Strain PJC4BK (developed by Green et al., 1996) and newly developed PJC4BK(pTAAD) were extensively characterized using fermentation experiments and metabolic flux analysis. Studies indicated that the fermentations at pH  $\geq$  5.0 were more conducive to solvent production, resulting in higher final solvent titers than fermentations at either pH  $\geq$  5.5 (Green et al., 1996) or pH  $\geq$  4.5 (Harris, 1997 MS Thesis). Analysis of strains PJC4BK and PJC4BK(pTAAD) allowed us to assess the effects of two genetic alterations: the *buk* inactivation alone and in combination with increased *aad* gene dosage using pTAAD.

### Fermentation and Flux Analysis of PJC4BK Versus Wild-Type

The summary of fermentation-product formation by PJC4BK and WT (which serves as a control for the PJC4BK fermentations) is shown in Table II. Representative fermentations are presented in Figure 2 and mean fermentation characteristics are discussed. The doubling time of PJC4BK was 1.4 h, higher than the 1.2-h doubling time of the wild-type organism. The initiation of solvent production (butanol concentration ca. 2 mM) by PJC4BK occurred at very low cell densities ( $A_{600} = 0.4$ ) prior to the accumulation of butyrate [total extracellular butyric acid concentration ca. 1 mM (Table III)]. This is in sharp contrast with conditions at the onset of solventogenesis in WT when the total extracellular butyrate concentration was 45 mM and the undissociated butyric acid (UBA) concentration was calculated to be

**Table II.** Summary of product formation in fermentation experiments.

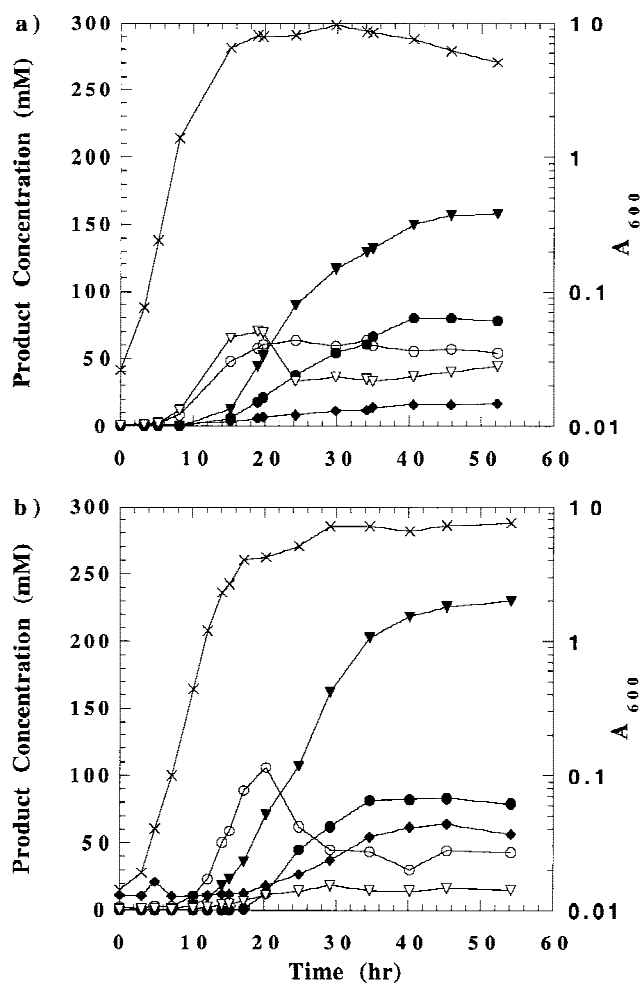
Fermentation characteristics	Strains			
	WT (control)	PJC4BK	PJC4BK(pTLH1) (control)	PJC4BK(pTAAD)
Number of experiments	2	2	2	2
Doubling time (h)	1.2 ± 0.02	1.4 ± 0.1	2.0 ± 0.06	2.2 ± 0.02
Max $A_{600}$	8.8 ± 0.6	8.2 ± 0.4	9.3 ± 0.5	11.4 ± 0.4
Butanol (mM)	158 ± 0	225 ± 4	208 ± 11	226 ± 2
Acetone (mM)	85 ± 3.5	76 ± 5.0	75 ± 4.6	66 ± 1.1
Ethanol (mM)	16 ± 0.7	57 ± 2.5	49 ± 1.1	98 ± 5.7
Acetate (mM)				
Peak	71 ± 5.3	111 ± 3.2	110 ± 4.6	113 ± 8.5
Final	60 ± 3.9	46 ± 2.0	62 ± 3.2	51 ± 2.5
Butyrate (mM)				
Peak	76 ± 3.9	18 ± 0.4	17 ± 1.4	18 ± 1.8
Final	41 ± 2.1	16 ± 0.0	14 ± 1.4	14 ± 1.8
Acetoin (mM)	11 ± 0.4	7 ± 0.4	4.5 ± 0.4	5 ± 0.0
Lactate (mM)	0 ± 0	7 ± 2.1	23 ± 0	4 ± 2.8

Note: Means ± SEM.

17 mM (Table III). As expected, the concentration of butyric acid was low throughout the PJC4BK fermentation, never exceeding 18 mM while WT butyrate concentration peaked at 76 mM. The maximum PJC4BK acetate level (111 mM) exceeded the wild-type value (71 mM) by 56%, yet final acetate levels (46 mM) were 23% lower in the PJC4BK fermentation than in the WT fermentation (60 mM). The maximum PJC4BK butanol concentration of 225 mM was 42% more than produced by the wild-type organism (158 mM), and at 76 mM, the maximum PJC4BK acetone concentration was similar to the WT acetone concentration (85 mM). The final PJC4BK ethanol titer of 57 mM was over 250% greater than WT values. These final PJC4BK solvent values result in a ca. 8:3:2 butanol:acetone:ethanol ratio as compared to 10:5:1 in the WT fermentation. In contrast to results at  $\text{pH} \geq 5.5$  (Green et al., 1996), at  $\text{pH} \geq 5.0$  strain PJC4BK is a solvent superproducer strain with some of the highest reported final butanol titers and altered solvent ratios.

The effect of gene inactivation in PJC4BK was further characterized by metabolic flux analysis via the impact on the following groups of pathways (Fig. 3): acid-formation fluxes (rPTAAK and rPTBBK), alcohol-formation fluxes (rETOH and rBUOH), and the acetone-formation fluxes (rACTN). The acetone-formation flux (rACTN) is the sum of acetone produced by acetate uptake (rACUP) and by butyrate uptake (rBYUP). The time-course profiles of these fluxes in PJC4BK were compared to the corresponding fluxes in the control strain (WT).

The acid-formation kinetic flux analysis in PJC4BK showed significant deviation from the fluxes in WT (Fig. 4). The butyrate-formation fluxes, rPTBBK, were reduced by up to 75% during the Early stage of the PJC4BK fermentations. During the later stages of the fermentation, WT



**Figure 2.** Fermentation profiles of (a) WT and (b) PJC4BK. Optical density  $A_{600}$  (-X-) and product concentrations of Acetate (○-), Acetone (●-), Butyrate (▽-), Butanol (▼-), and Ethanol (◆-).

**Table III.** Conditions at onset of solvent production.

Fermentation characteristics	Strains			
	WT (control)	PJC4BK	PJC4BK(pTLH1) (control)	PJC4BK(pTAAD)
Number of experiments	2	2	2	2
Butanol <sup>a</sup> (mM)	2 ± 0.6	3 ± 0.5	2 ± 0	2 ± 0.1
Butyrate (mM)	45 ± 5.0	1 ± 0.04	1 ± 0	1 ± 0.07
UBA <sup>b</sup> (mM)	17 ± 1.9	0.4 ± 0.02	0.4 ± 0.0	0.4 ± 0.03
Acetate (mM)	24 ± 8.7	9 ± 0.9	5.5 ± 0.4	9 ± 0.7
A <sub>600</sub>	5.1 ± 0.4	0.4 ± 0.05	0.4 ± 0	0.4 ± 0.1

Note: Means ± SEM.

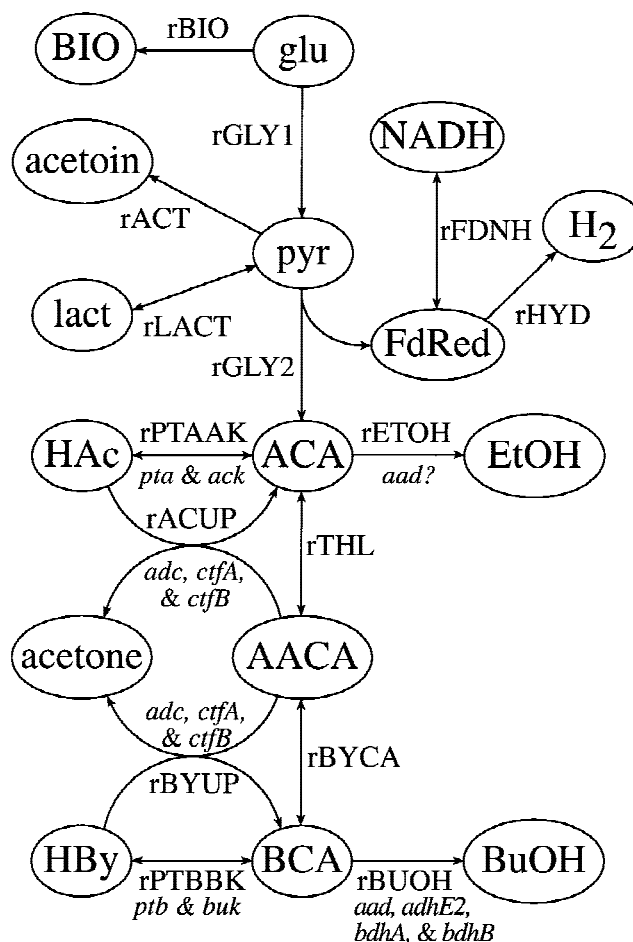
<sup>a</sup>Butanol: Experimental point closest to 2 mM.

<sup>b</sup>UBA: undissociated butyric acid.

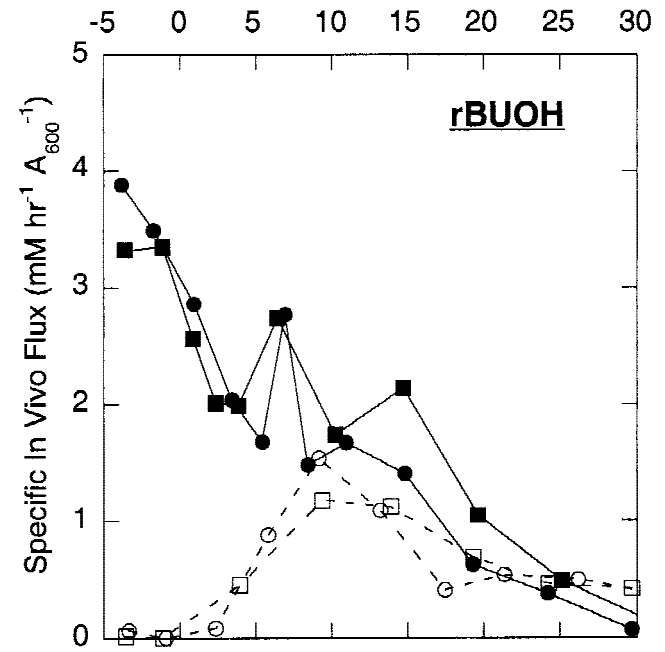
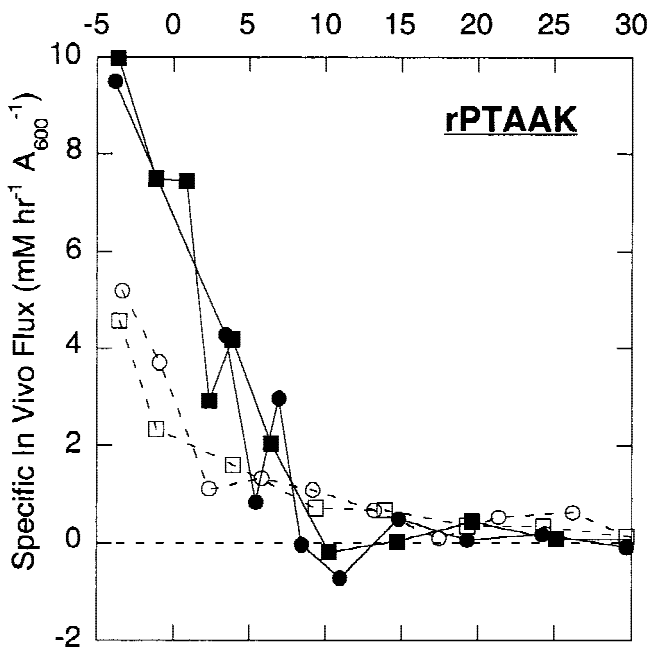
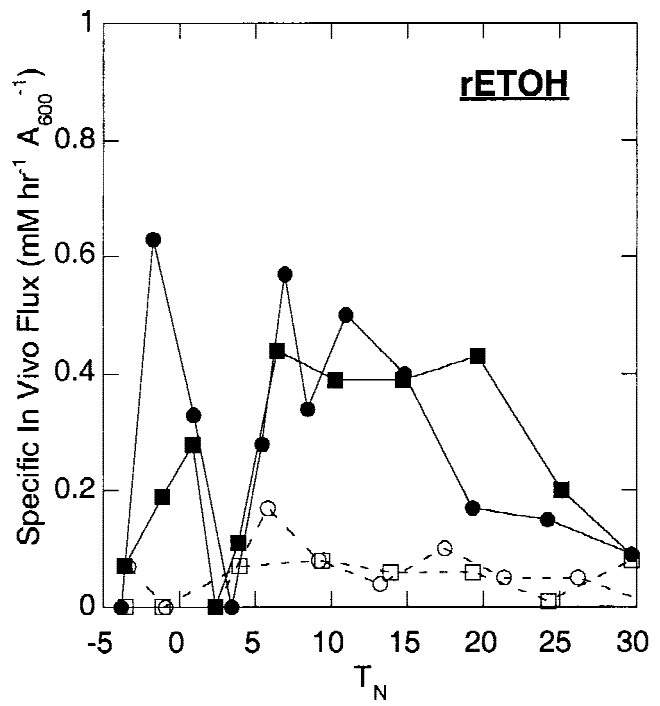
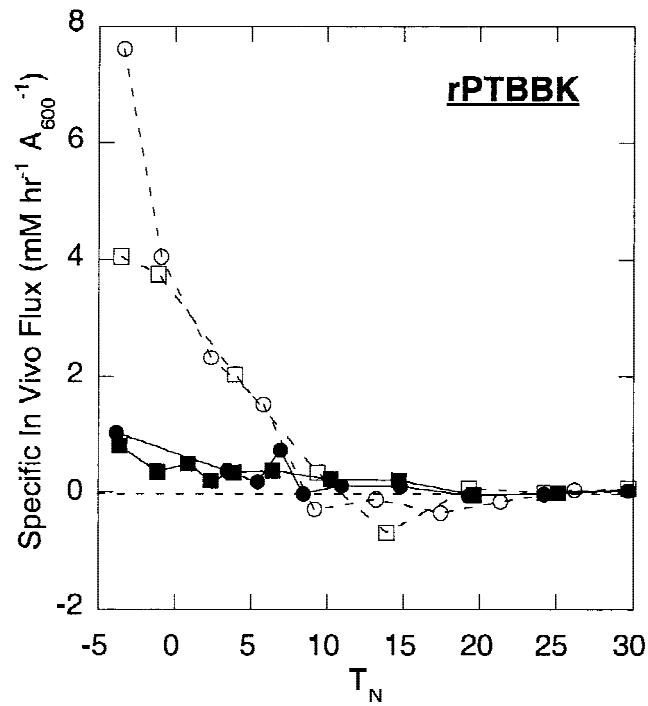
utilized the butyrate formation pathway to uptake butyrate (i.e.,  $r_{PTBBK} < 0$ ), while this pathway was virtually shut down in PJC4BK. In contrast to the decreased butyrate-formation fluxes, the acetate-formation fluxes,  $r_{PTAAK}$ , were up to 100% higher in PJC4BK during the Early stage of the fermentations. The  $r_{PTAAK}$  levels in PJC4BK were similar to, or lower than, levels in WT as the cultures entered the stationary phase.

The comparison of kinetic fluxes involved with alcohol production (Fig. 5) illustrates that the impact of *buk* inactivation on alcohol formation pathways was significant. The patterns of  $r_{BUOH}$  were completely altered in PJC4BK. In contrast to  $r_{BUOH}$  levels in WT that begin at 0 and peak at  $T_N = 10$ –15 h,  $r_{BUOH}$  in PJC4BK actually began at its maximum and decreased as the cultures entered the stationary phase. The peak levels of  $r_{BUOH}$  in PJC4BK were up to 300% higher than the peak levels in WT, but  $r_{BUOH}$  levels in PJC4BK during the stationary phase of the culture decreased to levels similar to those in WT. The ethanol-formation fluxes,  $r_{ETOH}$ , in PJC4BK were 100 to 400% higher than in WT. In contrast to  $r_{BUOH}$  profiles,  $r_{ETOH}$  increased to its peak values near  $T_N = 10$  h and remained high during the stationary phase of the cultures. The acetone formation fluxes were also impacted by the *buk* inactivation (Fig. 6). The formation of acetone via the uptake of butyrate,  $r_{BYUP}$ , was reduced by up to 50% in PJC4BK compared to WT. In addition, acetone formation via the uptake of butyrate was also delayed in PJC4BK compared to WT. In contrast, the peak values of acetone formation via acetate uptake,  $r_{ACUP}$ , were elevated by up to 300% in PJC4BK compared to WT. The profiles of the  $r_{ACUP}$  fluxes in PJC4BK and WT were similar in that they began near 0, increased to their peak values at  $T_N = 10$ –15 h, and then decreased to near 0 during the stationary phase.

An alternative approach (integral flux analysis) for examining metabolic pathway fluxes is to calculate mean specific fluxes during the Early and Late stages of the fermentations. The mean specific fluxes calculated for the pathways depicted in Figure 3 are shown in Table IV. The *buk* inactivation clearly had a global effect on metabolism. During the Early stage,  $r_{PTBBK}$  and  $r_{BYUP}$  were decreased by



**Figure 3.** Model species and pathways. Model species are depicted in circles and abbreviated as follows: glu, glucose; BIO, biomass; pyr, pyruvate; FdRed, reduced ferredoxin; lact, lactate; ACA, acetyl-CoA; HAc, acetate; EtOH, ethanol; AACA, acetoacetyl-CoA; BCA, butyryl-CoA; HBy, butyrate; and BuOH, butanol. Pathway fluxes that convert between species are indicated next to arrows such that irreversible fluxes are represented with unidirectional arrows and reversible fluxes are indicated with double arrows. Gene designations are indicated in italics next to relevant pathways and labeled as follows: *pta*, phosphotransacetylase; *ack*, acetate kinase; *adc*, acetoacetate decarboxylase; *ctfAB*, CoA transferase subunits A & B; *ptb*, phosphotransbutyrylase; *buk*, butyrate kinase; *aad*, alcohol/aldehyde dehydrogenase; *bdhAB*, butanol dehydrogenase isozymes A & B.



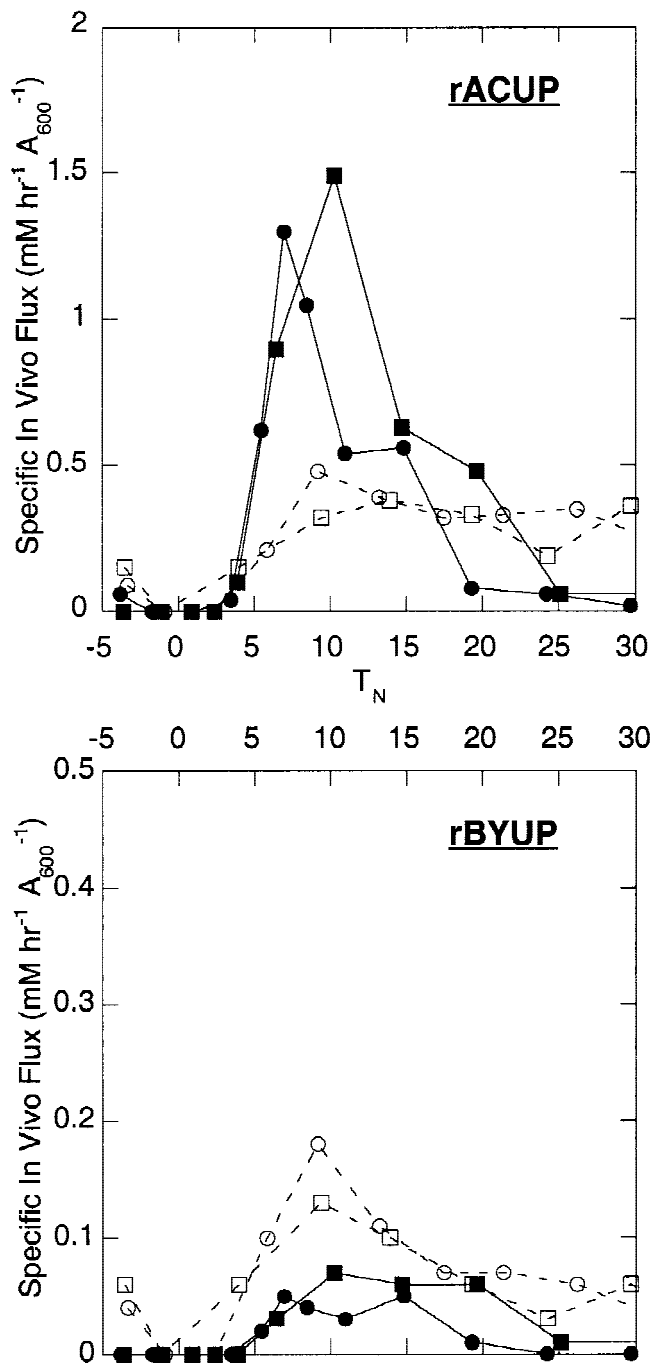
**Figure 4.** Acid-formation fluxes in WT and PJC4BK. Butyrate-formation fluxes (rPTBBK) and acetate formation fluxes (rPTAAK) are indicated for WT (open symbols:  $\square$ - and  $\circ$ -) and PJC4BK (closed symbols:  $\blacksquare$ - and  $\bullet$ -).

**Figure 5.** Alcohol-formation fluxes in WT and PJC4BK. Ethanol-formation fluxes (rETOH) and butanol-formation fluxes (rBUOH) are indicated for WT (open symbols:  $\square$ - and  $\circ$ -) and PJC4BK (closed symbols:  $\blacksquare$ - and  $\bullet$ -).

65% and 85%, respectively. Fluxes through several other pathways were also elevated. The alcohol-formation fluxes, rBUOH and rETOH, were elevated by 385% and 420%, respectively. The fluxes involving acetate, rPTAAK and rACUP, were also elevated by 200% and 75%, respectively. Finally, the glycolysis fluxes, rGLY1 and rGLY2, were elevated by 70% during the Early stage.

After the transition to the Late stage, several pathways

maintained the trends observed during the Early stage while others exhibited changes. The fluxes involving alcohol formation, rBUOH and rETOH, remained elevated by 60% and 450%, respectively. The acid-formation-pathway fluxes, rPTBBK and rPTAAK, changed trends. Specifically, the flux rPTBBK was similar in magnitude in both PJC4BK and WT but in opposite directions. In contrast, rPTAAK was reduced by 80% in PJC4BK compared to WT.



**Figure 6.** Acetone-formation fluxes in WT and PJC4BK. Acetone formation via acetate-uptake fluxes (rACUP) and acetone formation via butyrate-uptake fluxes (rBYUP) are indicated for WT (open symbols: □ and ○) and PJC4BK (closed symbols: ■ and ●).

### Fermentation and Flux Analysis of PJC4BK(pTAAD) Versus PJC4BK(pTLH1)

The plasmid pTAAD was introduced into strain PJC4BK to determine the impact of increased *aad* gene dosage. The expression of *aad* from pTAAD was confirmed by complementation of strain M5 (which lacks the *aad* gene and therefore produces no butanol (Cornillot et al., 1997; Nair and Papoutsakis, 1994)). Butanol formation was restored in

M5(pTAAD) as a result of functional AAD production (Harris, 1997 MS Thesis). Product formation in strain PJC4BK(pTAAD) and the control strain PJC4BK(pTLH1) are summarized in Table II, and mean values are discussed. Representative fermentations are presented in Figure 7. PJC4BK(pTAAD) cultures grew to a maximum  $A_{600}$  of 11.4 with a doubling time of 2.2 h while the PJC4BK(pTLH1) maximum  $A_{600}$  was 9.3 with a 2 h doubling time. Butyrate concentrations were low throughout both the PJC4BK(pTAAD) and PJC4BK(pTLH1) fermentation, never exceeding 18 mM, and butanol formation began at very low cell densities ( $A_{600} = 0.4$ ) when the butyrate concentration was only 1 mM. Final acetone and butanol concentrations in PJC4BK(pTAAD) were 66 mM and 226 mM, respectively, compared to 75 mM and 208 mM, respectively in PJC4BK(pTLH1). Maximum PJC4BK(pTAAD) ethanol concentrations (98 mM) were 100% higher than in strain PJC4BK(pTLH1) (49 mM) and 500% higher than in WT (16 mM). PJC4BK(pTLH1) produced 23 mM of lactate as compared to only 4 mM by PJC4BK(pTAAD) and 0 mM by WT.

Metabolic flux analysis was also carried out to determine the impact of *aad* overexpression in PJC4BK(pTAAD). Kinetic and integral analysis of PJC4BK(pTAAD) and PJC4BK(pTLH1) revealed only one significant effect due to *aad* overexpression. Specifically, the ethanol formation flux, rETH, was elevated by 70% during the Late stage in PJC4BK(pTAAD) when compared to PJC4BK(pTLH1) (Table IV). We conclude that overexpression of *aad* under the present fermentation conditions enhances the ethanol fluxes and titers but not those of butanol. This suggests that the levels of AAD under these conditions did not limit butanol formation.

### DISCUSSION

Inactivation of *buk* had profound global effects on the primary metabolism of *C. acetobutylicum*. The butyrate-formation fluxes in PJC4BK strains were decreased by 65–75% resulting in significantly lower butyrate levels. This decrease in butyrate production corresponds well with the 80% lower BK activity reported by Green et al. (1996) for PJC4BK. Because the butyrate-formation pathway is also responsible for generating energy (ATP) for cells, a reduced butyrate-formation flux may impose a metabolic burden on the cells. A feasible cellular response to this metabolic burden is elevation of the flux through the alternate ATP-generation pathway, namely acetate formation. Acetate-formation fluxes were elevated during the exponential phase of the fermentations resulting in 40–60% higher maximum-acetate levels. The increase in rPTAAK again corresponds well with the 100% increase in activity of the acetate-formation enzymes reported by Green et al. (1996). Acetone formation, which is associated with acid uptake, was also altered in strain PJC4BK. The decreased levels of butyrate and increased levels of acetate easily explain the decrease in rBYUP and the increase in rACUP. The sensitivity of ac-

**Table IV.** Integral analysis of metabolic fluxes.

Strain Stage	WT		PJC4BK		PJC4BK(pTLH1)		PJC4BK(pTAAD)	
	Early	Late	Early	Late	Early	Late	Early	Late
Mean specific fluxes ( $\text{mM h}^{-1} A_{600}^{-1}$ )								
rGLY1	4.02 ± 0.53	0.73 ± 0.02	6.74 ± 0.19	1.13 ± 0.25	4.11 ± 0.17	1.05 ± 0.07	4.36 ± 0.19	1.08 ± 0.14
rGLY2	7.77 ± 0.99	1.41 ± 0.06	13.09 ± 0.28	2.20 ± 0.50	8.01 ± 0.15	1.97 ± 0.09	8.46 ± 0.28	2.12 ± 0.27
rPTAK	1.85 ± 0.33	0.26 ± 0.05	5.62 ± 0.29	0.06 ± 0.04	4.03 ± 0.31	0.09 ± 0.02	4.48 ± 0.38	0.09 ± 0.02
rETOH	0.06 ± 0.00	0.04 ± 0.00	0.26 ± 0.05	0.23 ± 0.03	0.18 ± 0.00	0.17 ± 0.01	0.21 ± 0.05	0.28 ± 0.04
rTHL	3.00 ± 0.33	0.67 ± 0.02	3.76 ± 0.29	1.11 ± 0.28	1.88 ± 0.09	0.99 ± 0.04	1.89 ± 0.08	1.00 ± 0.14
rBYCA	2.77 ± 0.33	0.38 ± 0.01	3.46 ± 0.23	0.78 ± 0.15	1.85 ± 0.10	0.68 ± 0.04	1.87 ± 0.10	0.72 ± 0.10
rACUP	0.16 ± 0.00	0.24 ± 0.03	0.28 ± 0.06	0.30 ± 0.13	0.03 ± 0.01	0.30 ± 0.01	0.02 ± 0.01	0.26 ± 0.04
rBYUP	0.07 ± 0.00	0.05 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
rACTN	0.23 ± 0.00	0.29 ± 0.03	0.029 ± 0.06	0.33 ± 0.14	0.03 ± 0.01	0.31 ± 0.01	0.03 ± 0.01	0.27 ± 0.04
rPTBK	2.28 ± 0.33	-0.04 ± 0.00	0.79 ± 0.31	0.05 ± 0.02	0.28 ± 0.18	0.03 ± 0.01	0.28 ± 0.21	0.04 ± 0.01
rBUOH	0.55 ± 0.01	0.47 ± 0.00	2.69 ± 0.08	0.75 ± 0.14	1.57 ± 0.08	0.66 ± 0.04	1.59 ± 0.11	0.69 ± 0.08
rHYD	8.36 ± 1.26	1.09 ± 0.14	12.92 ± 0.23	0.88 ± 0.32	8.27 ± 0.54	0.94 ± 0.04	9.23 ± 0.51	0.80 ± 0.14
rFDNH	-0.59 ± 0.27	0.32 ± 0.09	0.18 ± 0.05	1.33 ± 0.18	-0.26 ± 0.39	1.03 ± 0.05	-0.77 ± 0.23	1.32 ± 0.13

Note: Mean ± standard error of the mean (SEM).

etone formation to acid levels is due to the high  $K_m$  values exhibited by the enzyme responsible for acetone formation (Wiesenborn et al., 1989). The uptake of acetic acid for use in acetone production was greater in the PJC4BK strains, as evidenced by an increase in the differences in maximum and final values and by increased rACUP values. However, the final acetone titers did not differ significantly because the increase in acetate uptake was offset by the decrease in butyrate uptake.

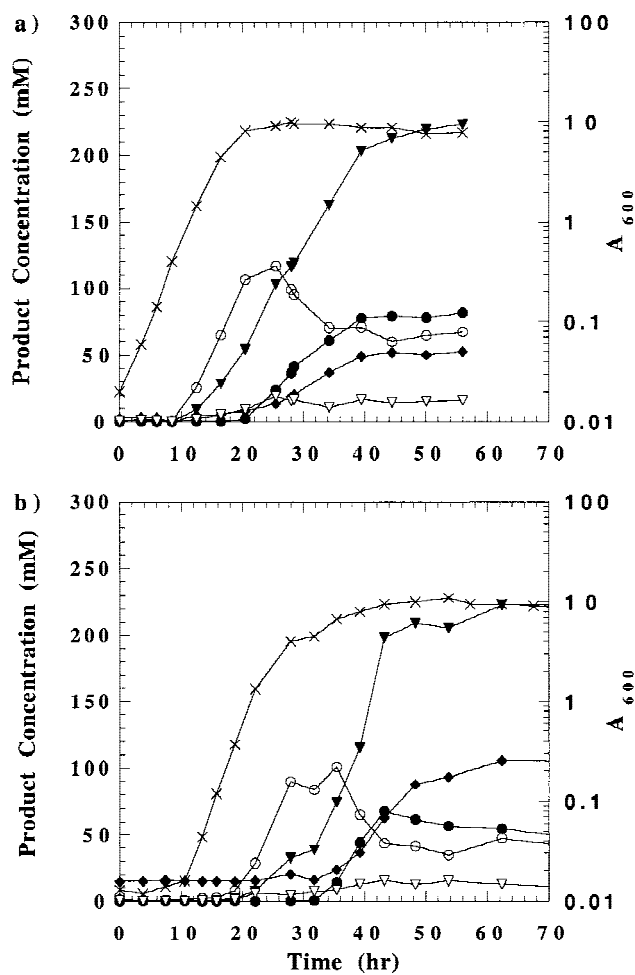
In addition to altered acid-formation patterns, the recombinant strains [PJC4BK, PJC4BK(pTLH1) and PJC4BK(pTAAD)] exhibited alcohol-formation characteristics which were distinct from those of WT. Butanol-formation fluxes were higher by up to 300% compared to the WT, and ethanol-formation fluxes were up to 400% higher.

The effect of increased dosage of AAD on solvent formation in PJC4BK was studied using the plasmid pTAAD. PJC4BK(pTLH1) served as the control strain in this study to screen out potential plasmid effects. Neither of the plasmids caused further increases in butanol production in strain PJC4BK. The presence of the pTAAD resulted in increased ethanol production, but did not affect butanol production. This suggests that AAD is not limiting butanol production in PJC4BK under these fermentation conditions. PJC4BK(pTAAD) showed a 100% increase in ethanol concentrations as compared to PJC4BK(pTLH1), resulting in significantly altered solvent ratios. In addition to its primary role as a source of butyraldehyde and butanol dehydrogenase activity for butanol production, AAD has also been previously shown to possess acetaldehyde and ethanol dehydrogenase activity for production of ethanol from acetyl-CoA (Nair et al., 1994). Therefore, the increase in ethanol production in PJC4BK(pTAAD) is consistent with the known properties of AAD. In this strain, the butanol:acetone:ethanol ratio is approximately 7:2:3 compared to 10:5:1 in WT. For the first time in a high-solvent-producing

derivative of *C. acetobutylicum*, ethanol concentrations exceeded acetone concentrations. Though several studies have shown that metabolic engineering in this organism can result in marked increases in butanol or acetone production, this study also reports a significant enhancement of ethanol production. While *C. acetobutylicum* is not of biotechnological interest as an ethanol producer, four other Clostridial species are: *C. thermocellum*, *C. thermosaccharolyticum*, *C. thermohydrosulfuricum*, and *C. sacchorolyticum* (Rogers and Gottschalk, 1993). The maximum ethanol concentration obtained during the PJC4BK(pTAAD) fermentation (98 mM = 4.5 g/L) is nearly equivalent to the typical ethanol concentration reached during *C. thermocellum* fermentations (5 g/L) (Rogers and Gottschalk, 1993).

Both butanol and ethanol were generated in excess of what was produced during WT fermentations. Peak levels of butanol concentrations (225 mM, 16.7 g/L) in PJC4BK strains consistently exceeded the 180 mM previously believed to be the upper limit of butanol tolerance in the WT strain (Jones and Woods, 1986). This is even more impressive when one considers that these high-butanol titers were obtained with dramatically increased ethanol titers [98 mM (4.5 g/L) in PJC4BK(pTAAD) compared to 16 mM (0.7 g/L) of ethanol produced by WT]. It is assumed that both butanol and ethanol inhibit growth by their effect on the cell's membrane (Linden and Kuhn, 1989). These dramatically increased alcohol titers (a total of 323 mM or 21.2 g/L), which were obtained without any effort to select for cells with increased tolerance, suggest that the membrane-related hypothesis of alcohol inhibition cannot account for the observed results and thus must be reconsidered. The present data and suggestion are further supported by other recent data (Nair et al., 1999) whereby a specific genetic mutation (namely inactivation of the *solR* gene encoding for a putative repressor of the *sol* locus genes) resulted in very high titers (240 mM) of butanol.

In all recombinant-strain fermentations, butanol produc-



**Figure 7.** Fermentation profiles of (a) PJC4BK(pTLH1) and (b) PJC4BK(pTAAD). Optical density  $A_{600}$  (-X-) and product concentrations of Acetate (-○-), Acetone (-●-), Butyrate (-▽-), Butanol (-▼-), and Ethanol (-◆-).

tion began during the exponential phase of cell growth when the cell density was less than  $A_{600}$  of 0.5. This unique characteristic of these strains is in sharp contrast to WT fermentations where the onset of solvent production occurs toward the end of exponential growth. The results presented here do not conform to the classical model correlating high-undissociated butyric acid (UBA) concentrations with the switch from acidogenesis to solventogenesis (Husemann and Papoutsakis, 1988; Terracciano and Kashket, 1986;). This study indicates that butyrate (or its transport) is apparently not involved in inducing solventogenesis. In all of the PJC4BK strains studied, the total external butyrate concentration was 1 mM, and the UBA was estimated at 0.4 mM when butanol (ca. 2 mM) was first detected (Table III). These total external butyrate and UBA levels were well below the 45 mM and 17 mM present, respectively, in the WT fermentation when solvent production began, and below the 6–18 mM UBA level suggested by established phenomenological models (Husemann and Papoutsakis, 1988; Terracciano and Kashket, 1986).

The possibility exists that the accumulation of a meta-

bolic precursor of butyrate (either butyryl CoA or butyryl-P) and not butyrate itself may be responsible for triggering solvent production, as has already been suggested (Gottwald and Gottschalk, 1985). In WT, elevated intracellular-butyrate concentrations during the late exponential/early stationary phase may result in increased concentrations of butyryl phosphate and butyryl-CoA by feedback enzyme inhibition, thus triggering solvent formation. Inactivation of the *buk* in PJC4BK strains does not interfere with expression of *ptb*, which encodes the enzyme (PTB) responsible for formation of butyryl-P from butyryl-CoA. Therefore, butyryl phosphate is likely to accumulate in strains with inactivated *buk*. In fact, PTB activity was found to increase by a factor of 3 in this recombinant strain (Green et al., 1996). Studies indicate that acyl phosphates such as acetyl-P may act as agents that can phosphorylate regulatory proteins of the two-component signal transduction system resulting in modification of gene expression. Much of this work was done in *E. coli* where intracellular acetyl-P has been shown to affect flagellar and chemotactic protein (McCleary et al., 1993), and also stimulates glutamine-synthetase synthesis (Feng et al., 1992). In fact, it has been proposed that in *E. coli*, acetyl-P may function as a global signal. Though much work in *C. acetobutylicum* remains to be done before this theory can be proven, the results presented in this work indicate that it is possible that butyryl-P serves an analogous role in regulation of solvent production.

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