

Metabolic Flux Analysis Elucidates the Importance of the Acid-Formation Pathways in Regulating Solvent Production by *Clostridium acetobutylicum*

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Metabolic flux analysis was used to investigate the roles of the acid formation pathways in *Clostridium acetobutylicum*. The acid formation pathways were revealed to serve different roles in wildtype fermentations than previously expected. Specifically, enzymes known to catalyze butyrate formation were found to uptake butyrate without concomitant production of acetone. This role was further corroborated by flux analysis of a recombinant strain overexpressing the butyrate formation enzymes. Analysis of wildtype fermentation data also revealed an important role for the acetate formation enzymes, namely the cycling of carbon between acetate and acetyl-CoA during the stationary phase. Next, metabolic flux analysis was used to compare the patterns of activity in two butyrate kinase deficient strains of *C. acetobutylicum*. The strain developed by gene inactivation, PJC4BK, exhibited a shift in acid formation fluxes toward acetate while the strain developed by antisense RNA strategies, 824(pRD4), did not exhibit such a shift. However, both strains exhibited altered solvent formation patterns. PJC4BK exhibited a strong transient enhancement of solvent formation fluxes. In contrast, 824(pRD4) exhibited relatively lower levels of solvent formation fluxes, although fluxes were sustained over a longer period of time.

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INTRODUCTION

Metabolic flux analysis is a systemic approach developed to assess the roles of individual steps in a network of metabolic pathways. The elucidation of patterns of metabolic activity via metabolic flux analysis is especially important to investigate the complex primary metabolism of the anaerobic, spore-former *Clostridium acetobutylicum*. Historically, the fermentation process using *C. acetobutylicum* accounted for a significant portion of the acetone and butanol market until the emergence of the petrochemical

industry. Active research has since been aimed at developing a better understanding of the complex fermentation with the goal of improving solvent formation characteristics.

The primary metabolic pathway of *C. acetobutylicum* has been reviewed extensively (Jones and Woods, 1986) and summarized in Fig. 1. It involves the glycolysis of glucose to pyruvate, and subsequently, the formation of the key branch point intermediate, acetyl-CoA. Acetate is produced in two steps from acetyl-CoA catalyzed by phosphotransacetylase (PTA) and acetate kinase (AK). Acetyl-CoA is also converted to acetoacetyl-CoA, which is the precursor of acetone, and butyryl-CoA, which is the precursor of butyrate and butanol. Acetone is formed in two steps involving the transfer of the CoA moiety to either acetate or butyrate followed by the subsequent decarboxylation of acetoacetate to acetone and CO₂. Butyrate is formed from butyryl-CoA in two steps catalyzed by phosphotransbutyrylase (PTB) and butyrate kinase (BK). Butanol is formed from butyryl-CoA by two sequential reductions catalyzed by several dehydrogenase enzymes. It should also be noted that the steps catalyzed by AK and BK are primary routes of ATP generation for *C. acetobutylicum*.

The product formation characteristics of *C. acetobutylicum* have been extensively studied in large-scale industrial and small-scale benchtop fermentations. The current paradigm of metabolic activity of the wildtype strain is based upon observed net rates of production (and consumption) and expected roles of various pathways. The pattern of metabolic activity is the formation of acids (acetate and butyrate) during growth followed by acid reutilization and solvent (acetone and butanol) formation during the stationary phase. Typically, it was observed that butyrate was

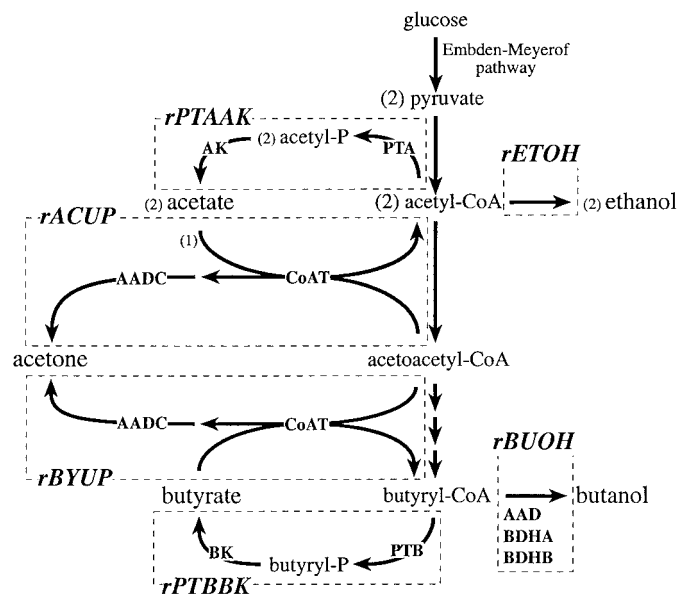


FIG. 1. *C. acetobutylicum* primary metabolic pathways and relevant fluxes. The conversion between major carbon containing species is depicted (no cofactors). Enzymes are indicated in bold and abbreviated as follows: PTA, phosphotransacetylase; AK, acetate kinase; CoAT, CoA Transferase; AADC, acetoacetate decarboxylase; PTB, phosphotransbutyrylase; BK, butyrate kinase; AAD, alcohol/aldehyde dehydrogenase (DH); BDHA & BDHB, butanol DH isozymes A and B. The reactions involved with the fluxes examined here are indicated by dashed boxes.

preferentially reutilized and associated with acetone formation. To investigate the roles of various pathways, several investigations on the nature of acid formation and reutilization were carried out. It was suggested that since the levels of acid formation enzymes decrease significantly at the onset of solventogenesis, the uptake of acids does not occur through a reversal of the acid formation pathways. Acetone formation was also linked to uptake of both acetate and butyrate by the CoAT enzyme (Hartmanis *et al.*, 1984). It was concluded that acid uptake and activation occur solely via the acetone formation pathway. In this model of metabolic activity, the acid formation pathways are thought to play important roles primarily during the exponential (growth associated) phase of the fermentation. At the shift to the stationary phase, the relative significance of acid formation pathways is thought to diminish in favor of the solvent formation pathways.

In this study, metabolic flux analysis was used as an investigative tool to quantitate the roles played by the acid formation pathways in the metabolism of *C. acetobutylicum*. Specifically, data from fermentations of the wildtype and a recombinant strain overexpressing PTB and BK were used to estimate the *in vivo* fluxes through the acid and acetone formation pathways. These estimates provide a different view of metabolic activity than previously accepted and

were used to re-evaluate the roles of the acetate and butyrate formation pathways. Next, two recently engineered strains, PJC4BK and 824(pRD4), exhibiting reduced levels of BK were characterized by metabolic flux analysis. The different techniques, gene inactivation and antisense RNA, used for metabolic engineering of these strains were shown to have differing impacts on metabolic activity. In general, downregulation of the BK was shown to impact not only acid formation pathways, but also induction of both acetone and butanol formation pathways. Finally, the implications on identifying potential solventogenesis inducer species are discussed briefly.

METHODS

Bacterial Strains, Culture, and Maintenance

C. acetobutylicum ATCC 824 (referred to as 824) is the wildtype strain available from American Type Culture Collection (Manassas, VA). *C. acetobutylicum* ATCC 824(pN5-1) is a strain overexpressing the butyrate formation enzymes (PTB and BK) (Walter *et al.*, 1994b). *C. acetobutylicum* ATCC 824(pPC1) is a strain carrying a control plasmid to account for host-plasmid interactions (Walter *et al.*, 1994a). *C. acetobutylicum* PJC4BK (referred to as PJC4BK) is the buk knockout mutant developed by Green *et al.* (1996). *C. acetobutylicum* ATCC 824(pRD4) is a BK deficient strain developed using a plasmid-borne antisense RNA (asRNA) strategy (Desai and Papoutsakis, 1999).

C. acetobutylicum strains were cultured at 37°C in clostridial growth medium (CGM) (Wiesenborn *et al.*, 1988) inside an anaerobic chamber (Forma Scientific, Inc., Marietta, OH) and maintained as spores at 4°C in corn mash-glucose medium (CMG) (Roos *et al.*, 1985) or CGM. Colonies were obtained on Reinforced Clostridial Media (Difco Microbiological Media, VWR Scientific Products, Chicago, IL). Spores were activated for vegetative growth by heating to 70–80°C for 10 min in CGM. Media were supplemented with the antibiotics erythromycin (100 µg/ml) or clarithromycin (100 µg/ml) as necessary.

Analytical Methods

Cell growth was monitored by measuring optical density at 600 nm (OD₆₀₀) using a Beckman DU 64 spectrophotometer. Culture supernatants were analyzed for glucose and products (acetate, butyrate, lactate, acetoin, ethanol, acetone, and butanol) as previously described (Desai and Papoutsakis, 1999). Uncertainties for measured quantities were as follows: OD₆₀₀, 5%; glucose, 5%; ethanol, 7%; acetone, 4%; butanol, 8%; acetoin, 10%; butyrate, 10%; and acetate, 15%.

Bioreactor Studies

Benchtop-scale fermentations were carried out in either a Biostat M (B. Braun, Allentown, PA) or a BioFlo II (New Brunswick Scientific, Edison, NJ) bioreactor with working volumes of 1.5 L and 4.0 L CGM medium, respectively, as previously described (Desai and Papoutsakis, 1999). All fermentations were initiated at pH ~6.2 and allowed to drop to the control pH. The control pH in fermentations with 824, 824(pN5-1), and 824(pPC1) was maintained by addition of 2 M hydrochloric acid and 2 M ammonium hydroxide. In contrast, the pH in the fermentations with PJC4BK and 824(pRD4) was controlled only with base addition. The reported time scale (T_N) of these bioreactor experiments has been normalized to account for differences in lengths of lag phases (due to small variations in inoculum preparation, and also due to strain differences) by setting T_N to 0 h when the OD_{600} reached 1.0. On this scale, negative T_N values simply indicate that the OD_{600} was less than 1.0.

Metabolic Flux Analysis

Metabolic flux analysis involves the calculation (or estimation) of *in vivo* fluxes from substrate and product data by using a system of linear equations developed from reaction stoichiometry (Papoutsakis, 1984). While a detailed discussion of the specifics of the metabolic flux analysis of *C. acetobutylicum* fermentations is presented elsewhere (Desai et al., 1999), a brief summary of key ideas is included below. In general, the metabolism of any organism can be represented as a network of chemical reactions that result in the conversion of substrate to biomass and products. The stoichiometric relationships for many of the metabolic reactions have been established and can be used to develop a system of mass balance equations on chemical species that describe metabolic activity. The system of equations can be represented in matrix form as:

$$\mathbf{A} \mathbf{r} = \mathbf{x} \quad (1)$$

where \mathbf{A} is a matrix containing stoichiometric information, \mathbf{r} is a vector containing the *in vivo* fluxes, and \mathbf{x} is the vector containing accumulation rates of the species derived from experimental data. Assumptions, such as the pseudo-steady state approximation on metabolic intermediates, are typically incorporated into the accumulation rate vector. Metabolic flux analysis then becomes a problem of determining a vector of *in vivo* fluxes, $\hat{\mathbf{r}}$, that best-fits (1). The *in vivo* flux estimates are further normalized by dividing by the average OD to calculate specific *in vivo* fluxes.

For the network of metabolic pathways utilized by *C. acetobutylicum*, the acid and acetone formation pathway

fluxes are not calculable because the system of equations is locally underdetermined. Therefore, only the *net* rates of production (or consumption) could be calculated for acetate, butyrate, and acetone. The *specific net rate* (=specific production rate–specific consumption rate) of a given species is calculated below as the rate of change in concentration divided by the average OD between two time points. These *net* rates are linear functions of the *in vivo* fluxes but do not quantitatively reflect the *in vivo* activities of the acid and acetone formation pathways. The *net* rates do indicate the overall status of the fermentation (acidogenic or solventogenic) but do not indicate the level of activity supported by a specific pathway (i.e., acetate formation as catalyzed by PTA and AK). In order to resolve the mathematical singularity and estimate *in vivo* fluxes as opposed to *net* rates, a nonlinear constraint based on *in vitro* studies was developed and incorporated into the model of *C. acetobutylicum*. This reformulated model allowed the calculation of fluxes of acid and acetone formation and was indirectly validated with intracellular measurements (Desai et al., 1999). These calculated *in vivo* fluxes are the best available estimates of the acid and acetone formation fluxes, which cannot be directly measured by techniques such as ^{13}C NMR.

Metabolic flux analysis was performed using software developed specifically for the analysis of *C. acetobutylicum* fermentations (Desai et al., 1999). The metabolic fluxes discussed below are indicated in Fig. 1. The calculated time-course profiles of various metabolic fluxes were examined to reveal the time-dependent patterns of metabolic activity. In the analyses, the growth stage ($T_N < 10$ h) is termed the Early phase and the nongrowth stage ($T_N > 10$ h) is termed the Late phase. It is important to note that the calculation (estimation) of fluxes is dependent on the quality of data. In fact, the uncertainties of the estimated *in vivo* fluxes are similar to those of the respective measured quantity (i.e., r_{PTBBK} , 10%; r_{PTAAK} , 15%; r_{ACUP} , 8%, and r_{BYUP} , 6%). In data sets with relatively high measurement errors, some of the fluctuations in calculated fluxes can be attributed to possible outliers in data. In fact, this feature is characteristic of χ^2 minimization techniques, which assume that the probability of outliers is extremely small.

RESULTS AND DISCUSSION

Acid-Formation Pathways of *C. acetobutylicum*

Data from a batch fermentation of 824 controlled at pH 5.5 (Walter, 1994; Walter et al., 1994b) is depicted in Fig. 2A. As typical for *C. acetobutylicum*, acetate and butyrate were produced primarily during the Early phase, while acetone and butanol were produced primarily during the Late phase. From the product profiles, *specific net rates*

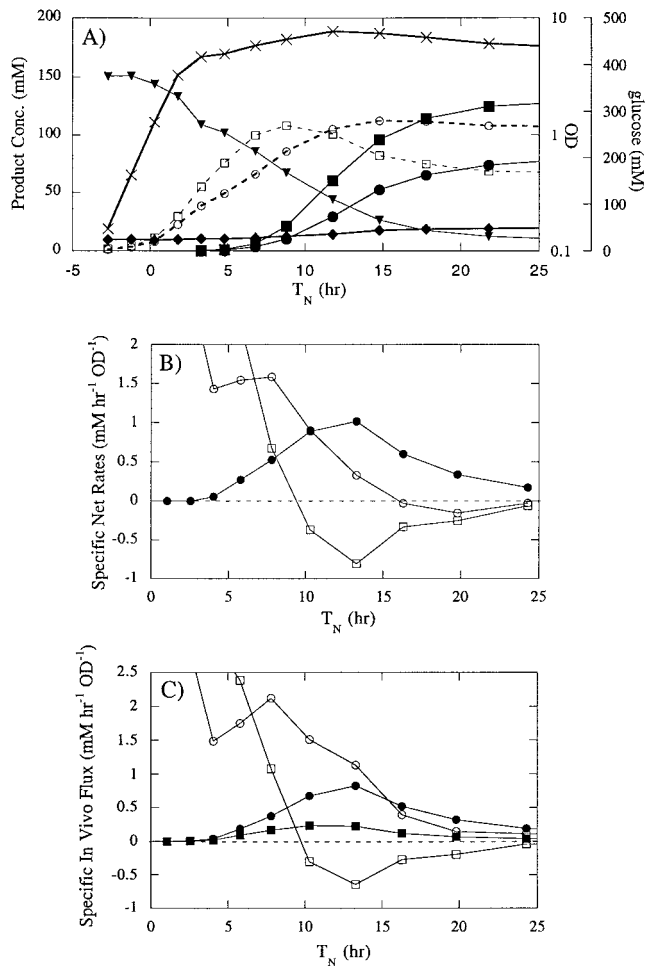


FIG. 2. *C. acetobutylicum* ATCC 824 fermentation data and analysis. (A) Product profiles of glucose (▼), OD (×), acetate (○), butyrate (□), ethanol (◆), acetone (●), and butanol (■). (B) Specific net rates of production (or consumption) derived from raw data for acetate (○), butyrate (□), and acetone (●). (C) Specific *in vivo* fluxes calculated from metabolic flux analysis for rPTAAK (○), rPTBBK (□), rBYUP (■), and rACUP (●). Dashed lines in (B) and (C) indicate zero net rate or *in vivo* flux.

(of production or consumption) of acetate, butyrate, and acetone were calculated (Fig. 2B). The *specific net rates* of acetate and butyrate began at their peak in the Early phase and decreased during the transition to the Late phase. In fact, during the Late phase the *specific net rates* were negative, indicating consumption of both acetate and butyrate. In contrast, the *specific net rate* of acetone increased from zero near the end of the Early phase, and reached a peak near the beginning of the Late phase. An important feature revealed by the *specific net rates* is the production of acetone prior to the observed consumption of either acetate or butyrate during the end of the Early phase. This indicates unobserved uptake of acetate and butyrate and higher acid formation rates than suggested by the

specific net rates. Furthermore, during the Late phase the specific net consumption of butyrate does not completely account for acetone production and thereby also suggests higher acid formation rates than indicated by the *specific net rates*. The *specific net rates* do not accurately describe metabolic activity because acetate or butyrate produced from the respective CoA species is immediately converted back to the CoA species via the acetone formation pathway. This idea is consistent with the hypothesis that acid uptake does not occur by the reversal of acid-formation pathways (Hartmanis *et al.*, 1984). In essence, some cycling from the acyl-CoA to the acid and back to the acyl-CoA species (via acetone formation) does in fact occur during both Early and Late phases but is not quantifiable from the *specific net rates* of acid production. Therefore, the activities of the acid formation pathways are not accurately reflected by the *specific net rates*.

Metabolic flux analysis was then used to reveal the *unobservable* patterns of metabolic activity. The *specific in vivo fluxes* as estimated by metabolic flux analysis are depicted in Fig. 2C. The profile of rPTAAK was consistent with expectations from the *specific net rate* profile of acetate, namely, the flux decreased from its peak in Early phase to low values during the Late phase. However, the acetate formation pathway continues to produce acetate throughout the Late phase in contrast to results suggested by the *specific net rates*. The consumption of acetate observed at the end of the Late phase in Fig. 2B must be a consequence of acetate uptake via the acetone formation pathway, rACUP. In contrast to the acetate formation pathway, the butyrate formation pathway appears to serve different roles during different phases of culture. The positive values of rPTBBK during the Early phase indicate that the butyrate formation pathway produces butyrate. From the onset of the Late phase, however, the butyrate formation pathway serves to uptake butyrate as indicated by the negative values of rPTBBK. In fact, the rate of uptake (of butyrate) via the butyrate formation pathway is greater than uptake via the acetone formation pathway. The profiles of acetone formation fluxes, rACUP and rBYUP, were similar to the profile of the specific net acetone production rate, namely, both fluxes began at zero and reached a peak near the beginning of the Late phase. Interestingly, more acetone was produced via acetate uptake than butyrate uptake in contrast to the previous association of acetone formation and butyrate uptake.

In order to further corroborate the roles of the butyrate formation pathway, fermentation data using a strain over-expressing the enzymes involved in butyrate formation (strain 824(pN5-1) (Walter *et al.*, 1994b)) was analyzed. The rPTBBK fluxes in 824(pN5-1) were compared to fluxes in the wildtype strain, 824, and a strain carrying a control plasmid, 824(pPC1) (Fig. 3). Surprisingly, no

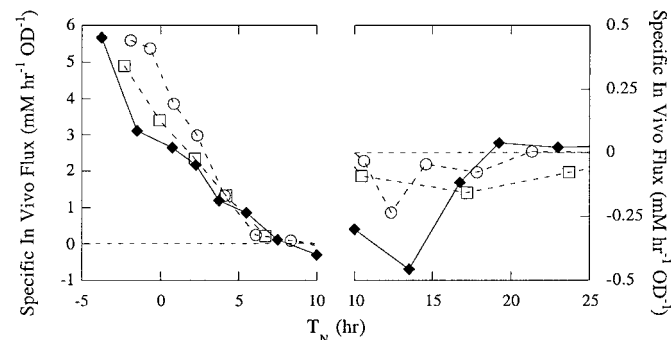


FIG. 3. Butyrate formation pathway fluxes (rPTBBK) in fermentations with 824 (○), 824(pPC1) (□), and 824(pN5-1) (◆). Early ($T_N \leq 10$ h) and Late ($T_N \geq 10$ h) phases are separated and depicted with different scales. Horizontal dashed line indicates zero flux.

significant increase in butyrate formation fluxes was observed during the Early phase. However, butyrate uptake via the butyrate formation pathway in 824(pN5-1) was nearly 2-fold greater than in either of the other control strains during the early Late phase. In essence, overexpression of the enzymes involved in butyrate formation resulted in an increased uptake of butyrate near the beginning of the Late phase. These results confirm the role of the butyrate formation enzymes in catalyzing butyrate uptake without acetone formation.

Metabolism of BK Deficient Strains

In order to further investigate the role of acid formation pathways in the metabolism of *C. acetobutylicum*, two BK deficient strains were examined. PJC4BK is a BK deficient strain of 824 developed by inactivation of *buk* (Green *et al.*, 1996). PJC4BK was reported to exhibit reduced levels of BK but elevated levels of the other enzymes involved in acid formation (PTB, PTA, and AK). 824(pRD4) is a BK deficient strain developed by an antisense strategy targeted against the *buk* gene (Desai and Papoutsakis, 1999). 824(pRD4) was reported to exhibit reduced levels of all enzymes involved in acid formation (PTB, BK, PTA, and AK). Representative product profiles from bioreactor experiments are depicted in Fig. 4. Data from several fermentations with each strain (summarized in Table 1) were analyzed and the fluxes involved with acid and solvent production were examined to evaluate the importance of acid formation pathways. It should be noted that several factors are responsible for the apparent variability in fermentation results, namely, reactor size and feeding strategy. In light of these factors, the calculated specific fluxes for different fermentations of the same strain were remarkably similar.

The acid formation fluxes in PJC4BK show significant deviation from the fluxes in 824(pRD4) (Fig. 5). During

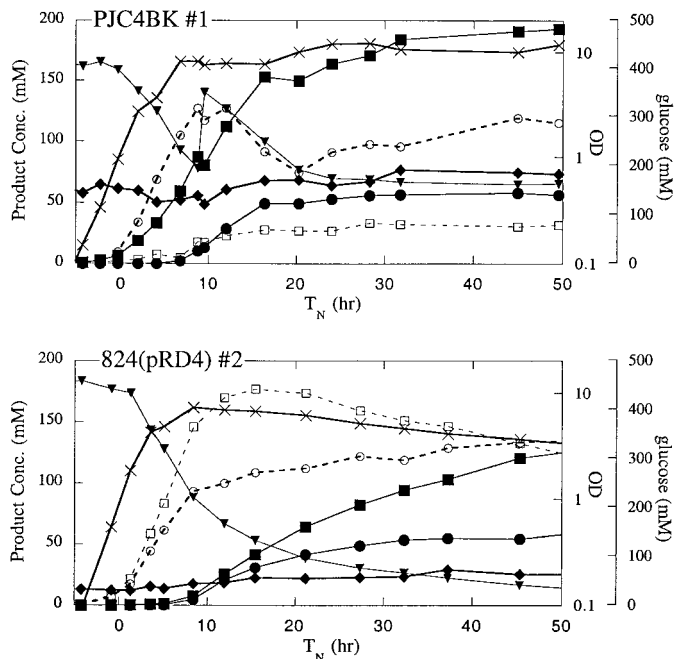


FIG. 4. Product profiles from batch fermentations with PJC4BK and 824(pRD4): glucose (▼), OD (×), acetate (○), butyrate (□), ethanol (◆), acetone (●), and butanol (■).

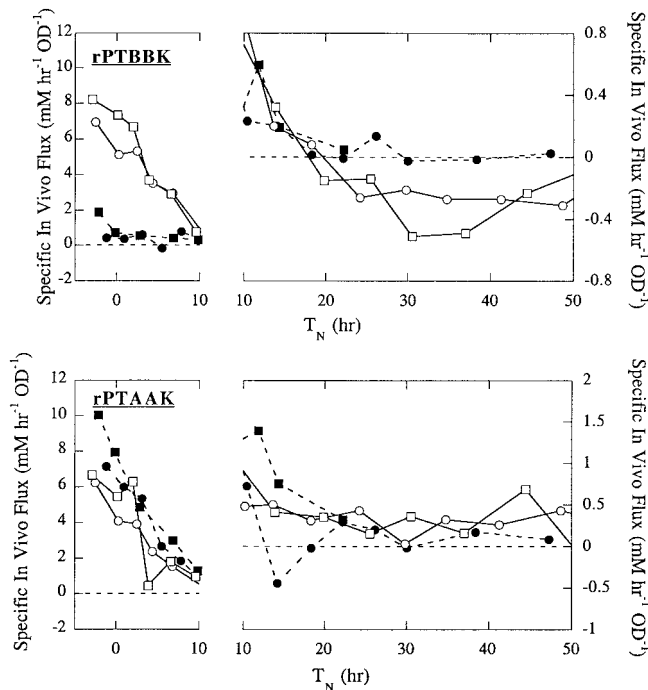


FIG. 5. Acid formation pathway fluxes (rPTBBK and rPTAAK) in fermentations with PJC4BK (#1 (●) and #2 (■)) and 824(pRD4) (#1 (○) and #2 (□)). Early ($T_N \leq 10$ h) and Late ($T_N \geq 10$ h) phases are separated and are depicted with different scales. Horizontal dashed lines indicate zero flux.

TABLE 1
Product Formation Summary in Controlled pH Fermentations

Fermentation characteristics	PJC4BK #1 ^a	PJC4BK #2	824(pRD4) #1	824(pRD4) #2	824
Growth rate, μ (h ⁻¹)	0.47	0.54	0.51	0.54	0.68
Max OD	12.1	8.2	7.25	6.9	7.7
Ethanol (mM)	9	15	13	15	20
Acetone (mM)	56	39	60	89	78
First detected at T_N	6.8	6.9	1.3	-1.0	4.8
Butanol (mM)	193	144	133	174	130
First detected at T_N	-4.2	-7.4	-0.8	-1.0	3.3
Acetate (mM): peak	127	148	135	128	112
final	115	140	131	120	106
Butyrate (mM): peak	33	38	177	185	108
final	32	38	120	101	65

^a Exogenous glucose added to maximize solvent productivity at $T_N \sim 9$ h.

the Early phase, rPTBBK was up to 8–10-fold higher in 824(pRD4) than in PJC4BK. Similarly, during the Late phase rPTBBK was significant in 824(pRD4) while rPTBBK was virtually zero in PJC4BK. In essence, the butyrate formation pathway supported greater fluxes in 824(pRD4) than in PJC4BK. In contrast, rPTAAK was 25–50% higher in PJC4BK than in 824(pRD4) during the Early phase. During the Late phase, rPTAAK was lower in PJC4BK than in 824(pRD4), although the differences may not be significant. These differences in acid formation pathway fluxes can be directly attributed to the different enzyme activity patterns exhibited by each strain (Desai and Papoutsakis, 1999; Green *et al.*, 1996).

The alcohol formation fluxes in PJC4BK and 824(pRD4) are compared in Fig. 6. During the Early phase, PJC4BK exhibited 50–100-fold higher butanol formation fluxes than 824(pRD4). After the shift to the Late phase, rBUOH in PJC4BK decreased to low values while rBUOH in 824(pRD4) remained constant at values ~ 2 -fold higher than in PJC4BK. In contrast to the differences in butanol formation fluxes, rETOH in PJC4BK and 824(pRD4) were very similar. PJC4BK clearly exhibits strong early induction of butanol formation that quickly decreased while 824(pRD4) exhibits low early induction of butanol formation followed by sustained production.

Finally, the acetone formation fluxes also revealed differences between PJC4BK and 824(pRD4) (Fig. 7). In both strains, acetone formation was essentially zero during the Early phase and was initiated near the transition to the Late phase. In both strains, acetone formation via butyrate uptake (rBYUP) reached peak levels at $T_N = 10$ h and slowly decreased throughout the Late phase. However, rBYUP was 4–5-fold higher in 824(pRD4) than in PJC4BK, most likely due to elevated butyrate levels in

824(pRD4) fermentations. In contrast, higher peak levels of acetone formation via acetate uptake (rACUP) were achieved in PJC4BK than in 824(pRD4). In PJC4BK, peak levels of rACUP were reached at $T_N = 10$ h and quickly decreased to low levels. In 824(pRD4), peak levels of rACUP were reached at $T_N = 10$ h and slowly decreased throughout the Late phase. The acetone formation patterns are quite similar to the patterns of butanol formation.

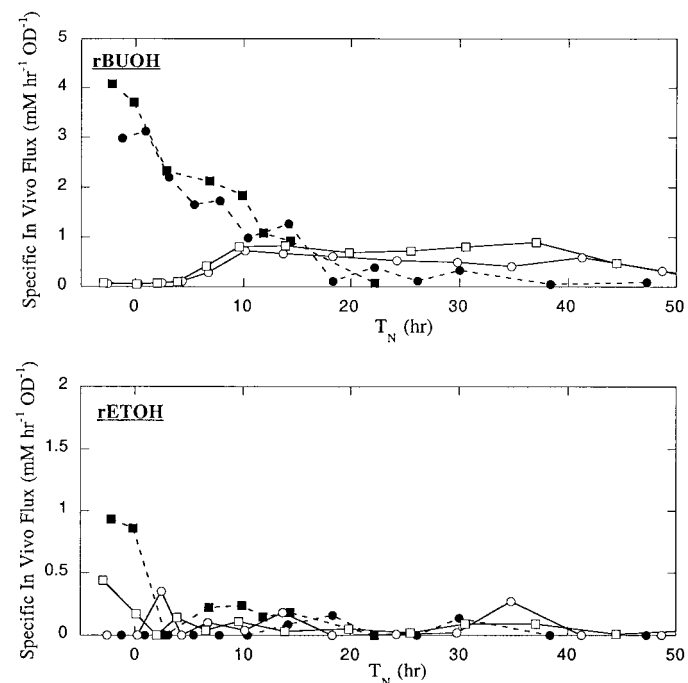


FIG. 6. Alcohol formation pathway fluxes (rBUOH and rETOH) in fermentations with PJC4BK (#1 (●) and #2 (■)) and 824(pRD4) (#1 (○) and #2 (□)).

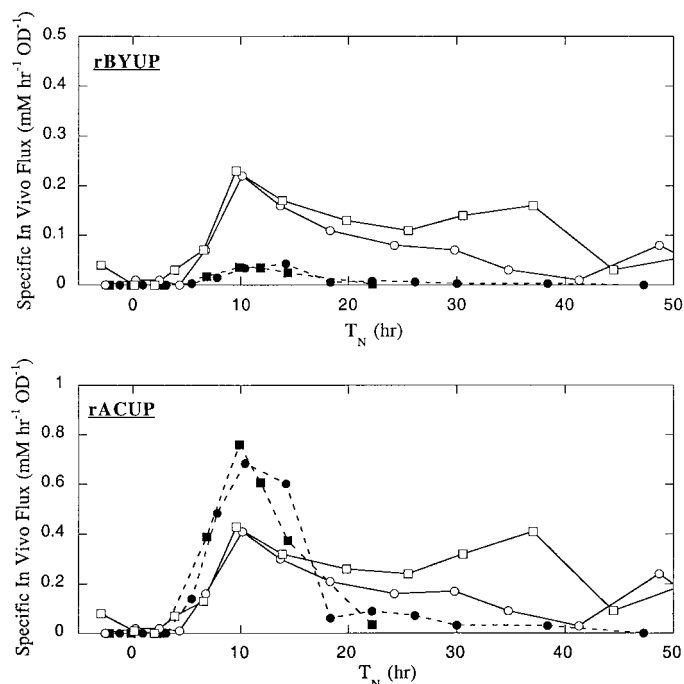


FIG. 7. Acetone formation pathway fluxes (rBYUP and rACUP) in fermentations with PJC4BK (#1 (●) and #2 (■)) and 824(pRD4) (#1 (○) and #2 (□)).

PJC4BK exhibits strong induction followed by a quick decrease while 824(pRD4) exhibits modest induction followed by sustained production.

The metabolic patterns exhibited by PJC4BK and 824(pRD4) revealed major differences in acid and solvent formation fluxes. The impact on acid formation may be explained by the direct impact on levels of the acid formation enzymes. However, the impact on solvent formation fluxes may only be due to indirect effects, such as levels of solventogenesis inducer species.

By examining the patterns of solvent formation we can speculate on the nature of the induction of solventogenesis. Historically, the induction of solventogenesis has been linked to intracellular levels of undissociated butyric acid (UBA) (Hüsemann and Papoutsakis, 1988; Terracciano and Kashket, 1986). However, butanol formation from the very beginning of the fermentations with both PJC4BK and 824(pRD4) suggest that alternate factors, butyryl-phosphate and/or butyryl-CoA, may be the inducer species. Butyryl-phosphate seems to be the most-likely candidate by the following rationale. Both PJC4BK and 824(pRD4) exhibited reduced levels of BK, the enzyme catalyzing the conversion of butyryl-phosphate to butyrate. Also, the reduction in PTB levels by antisense RNA in another strain did not result in early or sustained butanol production (Desai and Papoutsakis, 1999). The differences in induction patterns

between PJC4BK and 824(pRD4) may also be explained with butyryl-phosphate as the inducer species. PJC4BK exhibited elevated levels of PTB while 824(pRD4) exhibited reduced levels of PTB. The differences in the ratio of PTB/BK in PJC4BK and 824(pRD4) may have resulted in different levels of butyryl-phosphate. Different levels of butyryl-phosphate in PJC4BK and 824(pRD4) may then be responsible for the strong early butanol formation in PJC4BK and the strong sustained butanol formation in 824(pRD4). The possibility of butyryl-phosphate as the inducer of solventogenesis has also been suggested recently by Chen and Blaschek (1999). In fact, these results on fluxes through the butyryl-CoA branch point seem to fit well with the rigid-node classification described by Stephanopoulos and Vallino (1991).

CONCLUSIONS

Flux analysis has revealed the different roles played by acid formation enzymes in the complex primary metabolism of solventogenic clostridia. The butyrate formation pathway was revealed to play an important role both in the production of butyrate and in butyrate uptake without concomitant acetone formation. The acetate formation pathway was shown to play an important role (ATP generation) during both growth and nongrowth stages of culture. Flux analysis was also used to quantitate and compare the impact of different strategies used for the downregulation of the BK. Strains PJC4BK and 824(pRD4) both exhibited strong induction of butanol formation and acetone formation, although at different stages of the fermentations. In fact, the induction patterns revealed by flux analysis suggest the possibility of butyryl-phosphate as an inducer of solventogenesis. Therefore, metabolic flux analysis has proven to be an important tool to investigate and quantitate the role of metabolic pathways and the impact of genetic manipulations.

REFERENCES

- Chen, C. K., and Blaschek, H. P. (1999). Effect of acetate on molecular and physiological aspects of *Clostridium beijerinckii* NCIMB 8052 solvent production and strain degeneration. *Appl. Environ. Microbiol.* **65**, 499–505.
- Desai, R. P., Nielsen, L. N., and Papoutsakis, E. T. (1999). Metabolic flux analysis of *C. acetobutylicum* fermentations using nonlinear constraints. *J. Biotech.* **71**, 191–205.
- Desai, R. P., and Papoutsakis, E. T. (1999). Antisense RNA strategies for the metabolic engineering of *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.* **65**, 936–945.
- Green, E. M., Boynton, Z. L., Harris, L. M., Rudolph, F. B., Papoutsakis, E. T., and Bennett, G. N. (1996). Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. *Microbiology* **142**, 2079–2086.

- Hartmanis, M. G. N., Klason, T., and Gatenbeck, S. (1984). Uptake and activation of acetate and butyrate in *Clostridium acetobutylicum*. *Appl. Microbiol. Biotechnol.* **20**, 66–71.
- Hüsemann, M. H. W., and Papoutsakis, E. T. (1988). Solventogenesis in *Clostridium acetobutylicum* fermentations related to carboxylic-acid and proton concentrations. *Biotechnol. Bioeng.* **32**, 843–852.
- Jones, D. T., and Woods, D. R. (1986). Acetone-butanol fermentation revisited. *Microbiol. Rev.* **50**, 484–524. [Review]
- Papoutsakis, E. T. (1984). Equations and calculations for fermentations of butyric acid bacteria. *Biotechnol. Bioeng.* **26**, 174–187.
- Roos, J. W., McLaughlin, J. K., and Papoutsakis, E. T. (1985). The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentations of *Clostridium acetobutylicum*. *Biotechnol. Bioeng.* **27**, 681–694.
- Stephanopoulos, G., and Vallino, J. J. (1991). Network rigidity and metabolic engineering in metabolite overproduction. *Science* **252**, 1675–1681. [Review]
- Terracciano, J. S., and Kashket, E. R. (1986). Intracellular conditions required for the initiation of solvent production by *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.* **52**, 86–91.
- Walter, K. A. (1994). “Molecular Characterization of *Clostridium acetobutylicum* Genes Involved in Butanol and Butyrate Formation”. Ph.D. thesis, Northwestern University, Evanston.
- Walter, K. A., Mermelstein, L. D., and Papoutsakis, E. T. (1994a). Host-plasmid interactions in recombinant strains of *Clostridium acetobutylicum* ATCC 824. *FEMS Microbiol. Lett.* **123**, 335–342.
- Walter, K. A., Mermelstein, L. D., and Papoutsakis, E. T. (1994b). Studies of recombinant *Clostridium acetobutylicum* with increased dosages of butyrate formation genes. *Ann. N.Y. Acad. Sci.* **721**, 69–72.
- Wiesenborn, D. P., Papoutsakis, E. T., and Rudolph, F. B. (1988). Thiolase from *Clostridium acetobutylicum* ATCC 824 and its role in the synthesis of acids and solvents. *Appl. Environ. Microbiol.* **54**, 2717–2722.