Genetic manipulation of acid formation pathways by gene inactivation in Clostridium acetobutylicum ATCC 824

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Integrational plasmid technology has been used to disrupt metabolic pathways leading to acetate and butyrate formation in Clostridium acetobutylicum ATCC 824. Non-replicative plasmid constructs, containing either clostridial phosphotransacetylase (pta) or butyrate kinase (buk) gene fragments, were integrated into homologous regions on the chromosome. Integration was assumed to occur by a Campbell-like mechanism, inactivating either pta or buk. Inactivation of the pta gene reduced phosphotransacetylase and acetate kinase activity and significantly decreased acetate production. Inactivation of the buk gene reduced butyrate kinase activity, significantly decreased butyrate production and increased butanol production.

Keywords: Clostridium acetobutylicum, solvent biosynthesis, acid biosynthesis, metabolic engineering, gene integration

INTRODUCTION

Clostridium acetobutylicum is a Gram-positive, spore-forming, saccharolytic bacterium capable of fermenting a wide variety of sugars to acids (acetate and butyrate) and solvents (acetone, butanol and ethanol). The production of acetone and butanol by C. acetobutylicum was a thriving industrial process but the fermentation can no longer compete with the chemical synthesis of solvents from petroleum. However, renewed interest in biomass combustion has focused research on the genetic manipulation of C. acetobutylicum with the aim of developing a more competitive industrial process.

The biochemical pathways for the conversion of carbohydrates to acids and solvents by C. acetobutylicum have been well reviewed (Jones & Woods, 1986). During acid production, acetyl-CoA and butyryl-CoA function as key intermediates for acetate and butyrate. Although both acids are produced by similar pathways, the enzymes involved are unique to each pathway. Acetyl phosphate and butyryl phosphate are first produced from their corresponding CoA derivatives in reactions catalysed by phosphotransacetylase (PTA) and phosphotransbutyrylase (PTB), respectively. The acyl phosphates are then metabolized to acetate or butyrate. These reactions generate ATP and are catalysed by acetate kinase (AK) and butyrate kinase (BK). During solvent production, acetyl-CoA and butyryl-CoA are first reduced to acetaldehyde and butyraldehyde, and then to ethanol and butanol, respectively. Acetate and butyrate are also reassimilated in a reaction coupled to the irreversible production of acetocacetate from acetoacetyl-CoA by acetoacetyl-CoA:acetate/butyrate transferase. Acetone and carbon dioxide are produced from the decarboxylation of acetocacetate by acetocetate decarboxylase.

The genes encoding BK (buk), PTB (ptb), AK (ack) and PTA (pta) have been identified in C. acetobutylicum ATCC 824 (Walter et al., 1994; Boynton et al., 1996). Both sets of genes exist in tandem on the chromosome and form operons with pta preceding buk and pta preceding ack.

Non-replicative integrational plasmids have been used to advance genetic studies in Gram-positive bacteria but this technique has been limited to bacteria that are easily transformed (Perego, 1993; Kieser & Hopwood, 1991; Chassy & Murphy, 1993). Such plasmids need a DNA fragment from the host and a genetic marker for which selection can be made. After transfer, plasmids may become established by integrating into the homologous region on the host replicon in a Campbell-like fashion (Campbell, 1962). Plasmid integration may result in two direct repeats of the homologous region flanking the
plasmid DNA. Insertion can be mutagenic if the homologous DNA fragment on the integrational plasmid lies entirely within a gene or transcription unit (Piggot et al., 1984). Insertion may also result in stable gene amplification if the homologous DNA fragment on the integrational plasmid lies on the chromosome of E. coli (Young et al., 1984; Leenhouts et al., 1989).

Although most clostridial strains are difficult to transform, reliable and efficient transformation procedures now exist for C. acetobutylicum (reviewed by Young et al., 1989; Mermelstein & Papoutsakis, 1993a), which, together with increased knowledge about gene structure and function (Papoutsakis & Bennett, 1993), make it possible to fully exploit integrational plasmid technology in these strains. Integrational plasmids have recently been established in the chromosome of C. beijerinckii (Wilkinson & Young, 1994) and C. acetobutylicum ATCC 824 (Green & Bennett, 1996). This report describes the use of non-replicative integrational plasmids containing internal _buk_ and _pta_ gene fragments to inactivate _buk_ and _pta_ on the chromosome. By inactivating genes involved in acid formation, it may be possible to redirect carbon flow towards solvent production and increase solvent yields. This paper demonstrates the potential of using integrational plasmids to genetically modify _C. acetobutylicum_ ATCC 824 and also provides valuable information about gene function.

**METHODS**

**Bacterial strains and plasmids.** Bacterial strains and plasmids are listed in Table 1.

**Growth conditions.** _Escherichia coli_ was grown aerobically at 37 °C in Luria-Bertani (LB) medium. _C. acetobutylicum_ was grown anaerobically at 37 °C in 10 ml tube cultures of Clostridium Growth Medium (CGM; Roos et al., 1985). Colonies were obtained on agar-solidified Reinforced Clostridial Medium (RCM; Difco). LB medium was supplemented, as required, with erythromycin (200 μg ml⁻¹), tetracycline (15 μg ml⁻¹) and chloramphenicol (32 μg ml⁻¹). RCM and CGM media were supplemented with 20 and 40 μg erythromycin ml⁻¹, respectively.

Batch fermentations of _C. acetobutylicum_ were performed in either a Biostat M or a BioFlo II bioreactor (New Brunswick Scientific) with working volumes of 1.8 and 4.1 CGM medium, respectively. The medium was supplemented with additional glucose (80 g l⁻¹), clarithromycin (75 μg ml⁻¹; Mermelstein & Papoutsakis, 1993b) and antifoam C (0.15 % v/v). The growth medium (pH 6.2) was inoculated with 0.1 vols of pre-culture that had reached an OD₆₀₀ of 0.2. After inoculation, the pH was allowed to fall to 5.5, at which point it was controlled through additions of 6 M HCl containing 109 mM sec-butanol (internal standard). Anaerobic conditions were maintained by sparging the vessel with nitrogen. The initial sparge rate was 125 ml min⁻¹, but this was reduced to 25 ml min⁻¹ once the culture OD₆₀₀ reached 0.6. The reactor temperature was maintained at 37 ± 0.1 °C and the agitation rate was controlled at 200 r.p.m.

**DNA isolation and manipulation.** The manipulation, transformation and isolation of plasmid DNA from _E. coli_ ER2275 was performed using standard procedures (Maniatis et al., 1982). Large-scale plasmid isolation was undertaken using a plasmid purification kit (Qiagen). Restriction enzymes and T4 DNA ligase were used in accordance with the supplier's instructions (Promega). Prior to transformation of _C. acetobutylicum_, pJC4BK and pJC4PTA were methylated in _E. coli_ ER2275(pAN1) by the _Bacillus subtilis_ phage φ3T methyltransferase, which protects the plasmid DNA from restriction by the clostridial endonuclease _Cas824I_ (Mermelstein & Papoutsakis, 1993a). The plasmid DNA was desalted and concentrated using Microcon 100 micro concentrators (Amicon). Approximately 15 μg of methylated plasmid DNA was used to transform _C. acetobutylicum_ by electroporation using a previously described method (Mermelstein et al., 1992). Chromosomal DNA was prepared from _C. acetobutylicum_ using a Puregene DNA isolation kit (Gentra Systems). Cells were harvested from a 10 ml CGM culture during exponential growth (OD₆₀₀ ~ 0.8).

**Enzyme assays.** Cell extracts were prepared from 50 ml acidogenic _C. acetobutylicum_ culture. The cells were harvested by centrifugation (20000 g for 20 min at 4 °C) when the OD₆₀₀ reached 1.0. The cell pellet was resuspended in 10 ml 15 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 0.1 mM ZnSO₄. The cells were lysed by two passages through a pre-cooled French pressure cell (SLM model FA-078) at an operating pressure of 10000 p.s.i. and the cell debris was removed by centrifugation (35000 g for 20 min at 4 °C). AK and BK activity was assayed by the procedure of Rose (1955) in the acetyl and butyryl-phosphate-forming direction. PTB activity was assayed in the forward direction by monitoring the formation of CoA using a method reported by Cary et al. (1988). PTA was assayed in the backward direction by following the formation of acetyl-CoA as described by Brown et al. (1977). The protein concentration was determined using the standard Bio-Rad Protein Assay (based on the Bradford dye-binding procedure) in accordance with the manufacturer's instructions.

**Southern hybridization.** Chromosomal DNA was digested to completion with _SspI_, separated on an agarose gel, transferred to a Hybond-N+ nylon membrane (Amersham) by capillary blotting (Southern, 1975) and then probed with radiolabelled gene fragments isolated from pJC4PTA or pJC4BK (Table 1). The gene fragments were labelled with [α-³²P]dATP using a random primer DNA labelling system (Gibco BRL) and unincorporated nucleotides were removed by exclusion chromatography on Sephadex G-50. The pre-hybridization, hybridization and washing steps were performed at 42 °C in accordance with the membrane manufacturer's instructions and the radioactive membranes were visualized after exposure to X-ray film.

**Analytical methods.** Cell growth was monitored at 600 nm using a Beckman DU 64 spectrophotometer with cuvettes of 1 cm path length. Samples were diluted in distilled water, if necessary, so that the OD₆₀₀ did not exceed 0.4. Enzyme assays were performed in a Cary 118 spectrophotometer. Fermentation samples were analysed for acetate, butyrate, ethanol, acetone and butanol by gas chromatography using a Vario Vista 6000 Gas Chromatograph. Samples of culture supernatant and a standard solution (17 mM ethanol, 14 mM aceton, 11 mM butan-1-ol, 17 mM acetate and 11 mM butyrate) were acidified with 0.1 vols 3 M HCl containing 109 mM HCl (internal standard). Acidified samples (0.8 μl) were injected onto a 5% SP-2330 stationary phase of Carbowax 20M and a support phase of Carbograph mesh (80/120 mesh); 5% loading. Separation of sample components was achieved using a temperature program. The initial column temperature was 35 °C. The temperature was increased by 10 °C min⁻¹ until it reached 110 °C and then held for 3 min. The temperature was then increased by 15 °C min⁻¹ until it reached 155 °C and then increased by 20 °C min⁻¹ until it reached 200 °C. It was held at this temperature for 7.5 min.
**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics*</th>
<th>Source/reference</th>
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</thead>
<tbody>
<tr>
<td><strong>C. acetobutylicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 824</td>
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<td>ATCC, Rockville, MD, USA</td>
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<tr>
<td>PJ C4PTA</td>
<td>PTA&lt;sup&gt;+&lt;/sup&gt; MLS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Insertion of pJC4PTA into ATCC 824; this study</td>
</tr>
<tr>
<td>PJ C4BK</td>
<td>BK&lt;sup&gt;-&lt;/sup&gt; MLS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Insertion of pJC4BK into ATCC 824; this study</td>
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<td><strong>E. coli</strong></td>
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<td>ER2275</td>
<td>RecA&lt;sup&gt;-&lt;/sup&gt; McrBC&lt;sup&gt;-&lt;/sup&gt;</td>
<td>New England Biolabs</td>
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**Plasmids**

<table>
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<tbody>
<tr>
<td>pAN1</td>
<td>Cm&lt;sup&gt;-&lt;/sup&gt; 3T I</td>
<td>Mermelstein &amp; Papoutsakis (1993a)</td>
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<tr>
<td>pJC4</td>
<td>MLS&lt;sup&gt;-&lt;/sup&gt; Te&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Lee et al. (1992)</td>
</tr>
<tr>
<td>pJC7</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; PTB&lt;sup&gt;-&lt;/sup&gt; BK&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Cary et al. (1988)</td>
</tr>
<tr>
<td>pPUC-PTAK</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; PTA&lt;sup&gt;+&lt;/sup&gt; BK&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Boynton et al. (1996)</td>
</tr>
<tr>
<td>pJC4PTA</td>
<td>MLS&lt;sup&gt;-&lt;/sup&gt; AK&lt;sup&gt;-&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJC4BK</td>
<td>MLS&lt;sup&gt;-&lt;/sup&gt;</td>
<td>This study</td>
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</table>

* McrBC<sup>-</sup>, lacking methylcytosine-specific restriction system; RecA<sup>-</sup>, homologous recombination abolished; Ap<sup>+</sup>, ampicillin resistance; Cm<sup>-</sup>, chloramphenicol resistance; MLS<sup>-</sup>, macrolide, lincosamide, streptogramin B resistance; Te<sup>-</sup>, tetracycline resistance; 3T I, 3T methylase.

† pJC4PTA was constructed by subcloning a 0.65 kb BamHI-Hind111 pfa fragment from pPUC-PTAK into pJC4.

‡ pJC4BK was constructed by subcloning a 0.8 kb PvuII-Hind111 buk fragment from pJC7 into pJC4.

The injector and flame ionization detector temperatures were 225 and 300 °C, respectively. The carrier gas (N<sub>2</sub>) flow rate was 30 ml min<sup>-1</sup>. The glucose concentrations were determined using a YSI 2700 Select Biochemistry Glucose Analyzer (Yellow Springs Instrument Co.) in accordance with the manufacturer’s instructions. The instrument was calibrated with a YSI dextrose and l-lactate dual standard mixture.

**Measurement of the recombination frequency.** Strains harbouring integrational plasmids were grown overnight in CGM with erythromycin. The cultures were diluted approximately 1000-fold in 50 ml pre-warmed CGM lacking erythromycin and incubated until the OD<sub>600</sub> reached 1.0. The cultures were serially diluted in CGM and plated on non-selective RCM medium. Fifty of the resulting colonies were transferred to a RCM master plate, incubated overnight and then transferred onto two RCM plates, one with and one without erythromycin. The proportion of antibiotic-resistant cells was determined by dividing the number of colonies found on RCM medium with erythromycin by the number of colonies found on RCM medium without erythromycin. Sub-culturing steps and colony counts were repeated until each bacterial population had undergone about 30 generations of growth. The segregation frequency was calculated by dividing the proportion of antibiotic-sensitive colonies by the number of generations of growth (Noirot et al., 1987).

**RESULTS**

**Electrotransformation**

* C. acetobutylicum* ATCC 824 was transformed by electroporation with the methylation plasmid (pAN1) and plasmid mixtures containing pAN1 and either pJC4, pJC4PTA or pJC4BK. The DNA in these mixtures was methylated *in vivo* by the pAN1-encoded methylase which protects vector DNA from Cac824I restriction (Mermelstein & Papoutsakis, 1993a). The methylation plasmid (pAN1) did not transform *C. acetobutylicum*. This plasmid lacks a suitable origin of replication and an erythromycin marker and cannot, therefore, propagate in *C. acetobutylicum* or be maintained in selective media. Plasmid pJC4 and its derivatives pJC4PTA and pJC4BK lack suitable replicons that would enable them to replicate in *C. acetobutylicum*. They do, however, contain erythromycin markers and selective pressure can be used to detect those individual cells in which plasmid DNA has spontaneously integrated into the chromosome. Plasmid pJC4 did not transform *C. acetobutylicum* ATCC 824, whereas pJC4PTA and pJC4BK transformed at a low frequency [0.8 and 0.9 colonies (pg DNA)<sup>-1</sup>, respectively]. This suggests that a small proportion of the incoming pJC4PTA and pJC4BK molecules integrated into the chromosome by homologous recombination.

**Southern hybridization**

Chromosomal DNA from the integrants and parental strain was digested to completion with *ScaI* and then characterized by Southern hybridization (Fig. 1). *ScaI* was chosen because this enzyme has a single site in the backbone of the vector pJC4 but none in the clostralid pta or bak insert. If a single copy of either pJC4PTA or pJC4BK integrated into the chromosome, digestion with *ScaI* should generate two fragments, whose combined size...
Fig. 1. Hybridization analysis of chromosomal DNA from *C. acetobutylicum* strains. (a) Hybridization of a 0.8 kb *pta* fragment to *ScaI*-digested chromosomal DNA from *C. acetobutylicum* PJC4PTA integrant and the parental strain ATCC 824. Lanes 1, 2 and 3 are plasmid PJC4BK, ATCC 824 and PJC4BK, respectively. (b) Hybridization of a 0.65 kb *pta* fragment to *ScaI*-digested chromosomal DNA from *C. acetobutylicum* PJC4PTA integrant and the parental strain ATCC 824. Lanes 1, 2 and 3 are PJC4PTA, ATCC 824 and the plasmid pJC4PTA, respectively. Size markers (kb) of *Bst*EI-digested λ DNA were used and the positions of the bands are shown on the right of each part.

equals the combined size of the integrational vector and the *ScaI* fragment on the parental chromosome that contains the homologous gene.

The *buk* probe hybridized to two *ScaI* fragments (approx. 3 and 6.2 kb) from the PJC4BK mutant strain, one 5.2 kb *ScaI* fragment from the parental strain and the 4 kb integrational plasmid PJC4BK (Fig. 1a). Since the combined size of the *ScaI* fragments from the mutant strain equaled the combined size of the integrational plasmid and the *ScaI* *buk* fragment on the chromosome, it appears that a single copy of PJC4BK integrated into the *buk* gene on the chromosome. The difference in hybridization intensity between wild-type DNA (lane 2) and PJC4BK DNA (lane 3) was probably due to differences in DNA concentration. The difference in hybridization intensity between the two bands from PJC4BK (lane 3) was probably due to the efficiency of DNA transfer from the agarose gel to the nylon membrane. Smaller DNA fragments tend to blot more efficiently than larger ones (unpublished observation).

The *pta* probe also hybridized to two *ScaI* fragments (approximately 3.6 and 8.4 kb) from the PJC4PTA mutant strain and a 6.5 kb *ScaI* fragment from the parental strain (Fig. 1b). Since PJC4PTA is 5.5 kb and the *pta* gene resides within a 6.5 kb *ScaI* fragment on the chromosome, it appears a single copy of PJC4PTA also integrated into the *pta* gene on the chromosome. The difference in hybridization intensity between the two bands from PJC4PTA (lane 1) was probably due to the efficiency of the DNA transfer. Chromosomal DNA from the parental strain was also probed with pJC4 (vector without insert). The vector probe did not hybridize to DNA from the parental strain (results not shown). This result indicates that the hybridization patterns observed with gene fragment probes originated from clostridial chromosomal DNA.

### Enzyme assays

Cells were harvested from 50 ml cultures of wild-type and mutant strains during the exponential growth phase. Cell-free extracts were prepared and assayed for AK, BK, PTA and PTB (Table 2). Strain PJC4BK displayed approximately twofold higher PTA activity, threefold higher AK activity, fourfold higher PTB activity and fivefold lower BK activity than the wild-type. Strain PJC4PTA displayed approximately threefold higher PTB activity, twofold higher BK activity, sevenfold lower PTA activity and threefold lower AK activity than the wild-type.

### Fermentation studies

The optical density and product profiles from controlled pH batch fermentations of PJC4BK and PJC4PTA are shown in Fig. 2. Following inoculation, PJC4BK grew exponentially for 10 h (Fig. 2a). Cell growth then slowed and the ODγ reached a maximum value of 8.6 after 20 h. During exponential growth, relatively large quantities of acetate were produced and the acetate concentration reached a maximum value of 149 mM after 20 h. Little butyrate was produced and the butyrate concentration reached a maximum value of 37 mM after 36 h. During stationary phase, approximately 5% of the acetate was reassimilated and the culture pH increased from 5.5 to 5.7 (results not shown). Butanol was produced during exponential growth and reached a maximum value of 146 mM after 23 h. Acetone was produced after 14 h and reached a maximum value of 39 mM after 36 h.

Following inoculation, PJC4PTA grew exponentially for 12 h (Fig. 2b). Cell growth then slowed and the ODγ reached a maximum value of 8.3 after 16 h. During

### Table 2. Enzyme activities of *C. acetobutylicum* strains

The assays were performed as described in Methods. Each assay was performed in duplicate and the numbers shown are mean values from three different assays ± SEM. One unit (U) is defined as the amount of enzyme needed to convert 1 µmol substrate to product min⁻¹.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific activity [U (mg protein)⁻¹] ± SEM</th>
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<tbody>
<tr>
<td></td>
<td>PTA</td>
</tr>
<tr>
<td>ATCC 824</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>PJC4BK</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>PJC4PTA</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>
Genetic manipulation of acid formation pathways

0.0
1

Time (h)

10 20 30 40

Fig. 2. Optical density and product profiles in pH 5.5 batch fermentation of C. acetobutylicum strains PJC4BK (a) and PJC4PTA (b). , OD₆₆₀; ○, acetone; △, ethanol; ■, butanol; ●, butyrate; ▲, acetate.

Stability of derived strains

The stability of strains harbouring integrational plasmids was measured after approximately 30 generations of growth without selective pressure. The segregation frequencies (determined as described in Methods) of erythromycin-sensitive PJC4BK and PJC4PTA recombinants were 1.8 × 10⁻³ and 3.0 × 10⁻³, respectively.

DISCUSSION

Southern hybridization results revealed that the non-replicative plasmids pJC4PTA and pJC4BK integrated into homologous regions on the chromosome. Integration, assumed to have taken place by homologous recombination involving a Campbell-like mechanism, resulted in a single copy of the homologous gene fragment provided by the non-replicative plasmid. Enzyme assay results suggested that pta was inactivated in mutant PJC4PTA and butk was inactivated in mutant PJC4BK. Mutant PJC4PTA also had little AK activity, so it also appears that expression of ak was also essentially abolished. This observation is not surprising since ak lies downstream from pta in the same operon (Boyon et al., 1996). Integration into butk did not affect the expression of pta because butk lies downstream of pta in the same operon (Walter et al., 1994).

Gene integration did not completely eliminate acid formation or enzyme activity. Inactivation of butk reduced butyrate formation during exponential growth but the mutant PJC4BK still produced butyrate, primarily during the stationary (solventogenic) growth phase (Fig. 2a). PJC4BK produced significantly lower levels of BK but elevated levels of PTA, AK and PTB (Table 2). Similarly, pta inactivation reduced acetate formation during exponential growth, but the mutant PJC4PTA still produced some acetate during the stationary growth phase (Fig. 2b). PJC4PTA produced significantly lower amounts of PTA and AK but elevated amounts of BK and PTB (Table 2). This suggests that the enzymes involved in acid formation have broad substrate specificities. These findings are supported by enzyme studies performed on purified PTB and BK from C. acetobutylicum. PTB and BK were found to exhibit a broad substrate specificity with C₂- to C₄-chained acyl-CoA and carboxylic acid compounds. PTB exhibited about 2% relative activity with acetyl-CoA compared with butyryl-CoA (Wiesenborn et al., 1989) and BK exhibited about 6% relative activity with acetate compared with butyrate (Hartmanis, 1987). No such studies have been performed on PTA or AK from C. acetobutylicum.

Gene integration had a significant effect on cell doubling time (Table 3). The mutant strains grew slower than wild-type but reached higher biomass concentrations. It appears that during exponential growth, the mutant cultures compensated for the lack of ATP production via one acid pathway by increasing production of the other acid with no significant loss in overall cell growth.

Gene integration also had a significant effect on product concentration and distribution (Table 3). In wild-type culture, the butyrate/acetate ratio dropped from 1 at the end of the exponential phase to 0.6 at the end of the fermentation because 40% of the butyrate and only 6% of the acetate was reassimilated. In contrast, the maximum butyrate/acetate ratio produced by PJC4BK was 0.2. The butyrate concentration was significantly lower than the wild-type and the acetate concentration was significantly
higher. Butyrate was not reassimilated and the acid ratio did not change during the stationary phase. The maximum butyrate/acetate ratio produced by PJC4PTA at the end of the exponential phase was 1.8. The butyrate concentration was significantly higher than the wild-type and the acetate concentration was significantly lower. The acid ratio dropped to 0.9 by the end of the fermentation because 60% of the butyrate was reassimilated. The mutant PJC4PTA and the wild-type produced comparable amounts of solvents and the final and maximum butanol/acetone ratios were similar. However, the solvent ratios produced by mutant PJC4BK were approximately twice as high. This mutant produced 10% more butanol and 50% less acetone than the wild-type.

The concentration of butyryl-CoA fluctuates markedly during the switch from acid to solvent production (Grupe & Gottschalk, 1992; Boynton et al., 1994) and elevated concentrations of either butyryl phosphate or butyryl-CoA may actually trigger solvent production (Gottwald & Gottschalk, 1985). Mutant PJC4BK produced significant concentrations of butanol during exponential growth. After 12 h, the strain had produced 42 mM butanol, 0 mM acetone, 94 mM acetate and 12 mM butyrate (Fig. 2a). During stationary phase, the butanol and butyrate concentrations reached maximum values of 146 and 37 mM, respectively (Table 3). During exponential growth, most of the butyryl-CoA was reduced to butyraldehyde and then butanol by butyraldehyde dehydrogenase and butanol dehydrogenase, respectively. Disruption of BK probably increased the intracellular concentration of butyryl phosphate and butyryl-CoA. Since both enzymes involved in butanol formation are normally only induced during early stationary phase (Durre et al., 1987), these results also suggest that high concentrations of butyryl phosphate or butyryl-CoA may trigger the expression of butanol formation genes (Nair et al., 1994; Walter et al., 1992). However, since mutant PJC4BK also exhibited a fourfold increase in PTB activity (Table 2) it is possible that high butyryl-CoA levels enhance the transcription or translation of ptb. High butyryl phosphate levels may, therefore, play an important role in the regulation of the solventogenic genes.

The delayed and reduced formation of butyrate was probably catalysed by the corresponding enzymes for acetate formation, although the possibility of normal expression for BK and of the existence of BK and PTB isozymes cannot be ruled out. The production of butanol during stationary phase by the mutant PJC4PTA supports the hypothesis that early butanol formation by PJC4BK resulted from the accumulation of butyryl phosphate or butyryl-CoA. It was also noted that the timing of acetone formation was not affected either. The delayed and reduced formation of acetate was probably catalysed by the corresponding enzymes for butyrate formation, although other possibilities cannot be discounted. It would be interesting to understand the cellular basis of this delayed formation of acetate and butyrate by the two mutants.

Approximately 8% of the carbon source (glucose) was converted to lactate by PJC4BK and PJC4PTA (results not shown). The lactic acid pathway is not normally operational and serves as a less efficient mechanism for the oxidation of NADH when the generation of molecular hydrogen is blocked (Simon, 1947). Disruption of the acid pathways probably resulted in a build up of acetyl-CoA. Since insignificant amounts of acetyl-CoA were reduced to ethanol, it is probable that high pyruvate concentrations built up. Pyruvate may then have been reduced to lactate.

Mutants PJC4PTA and PJC4BK were relatively stable after approximately 30 generations. The segregation frequencies for PJC4PTA (3.0 × 10⁻³) and PJC4BK (1.8 × 10⁻³) were comparable to values reported for C. acetobutylicum (3.5 × 10⁻³) (Green & Bennett, 1996) and C. beijerinckii (0.37 × 10⁻³–1.3 × 10⁻³) (Wilkinson & Young, 1994). The mutants were less stable than other Gram-positive integrants. The segregation frequencies were approximately one order of magnitude lower than values reported for Lactobacillus lactis (Leenhouts et al., 1989) and B. subtilis (Young & Ehrlich, 1989).

Integrational plasmid technology provides valuable information about gene function. Gene integration can also be used to disrupt genes encoding enzymes involved in acid production, thus redirecting a greater proportion of the carbon flow toward solvent production and im-

### Table 3. Fermentation characteristics of C. acetobutylicum strains in batch cultures controlled at pH 5.5

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maximum glucose consumption (OD₆₀₀)</th>
<th>Doubling time (h)</th>
<th>Product concentration (mM)*</th>
<th>Product ratios†</th>
<th>Residual glucose (mM)</th>
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<tr>
<td>ATCC 824 anyway</td>
<td>7.7</td>
<td>1.0</td>
<td>11/11</td>
<td>79/74</td>
<td>131/131</td>
</tr>
<tr>
<td>PJC4BK</td>
<td>8.6</td>
<td>1.4</td>
<td>16/16</td>
<td>39/34</td>
<td>146/142</td>
</tr>
<tr>
<td>PJC4PTA</td>
<td>8.3</td>
<td>1.5</td>
<td>13/13</td>
<td>72/61</td>
<td>133/147</td>
</tr>
</tbody>
</table>

*Maximum product values during the fermentation/final product values at end of the fermentation.
† Ratios calculated from the maximum product values/ratios calculated from the final product values.
‡ Data from Walter (1993).
proving both the product yield and selectivity. Product levels may be further enhanced by the addition of glucose since both mutant cultures were glucose-limited (Table 3). Butanol production in PJC4BK may also be improved by increasing the dosage of the butanol pathway genes. Genes may be duplicated on the chromosome using integrational plasmids or introduced into the cell on replicative plasmids. Acetate or butyrate production cannot be completely eliminated (due to competing enzymes), but the results demonstrate that gene inactivation can be used to reduce acid production and increase solvent production. We believe non-replicative integrational plasmid technology can be used to engineer several different strains with altered product ratios.

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