Construction and Characterization of *ack* Deleted Mutant of *Clostridium tyrobutyricum* for Enhanced Butyric Acid and Hydrogen Production

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Clostridium tyrobutyricum produces butyrate, acetate, H_2, and CO_2 as its main fermentation products from glucose and xylose. To improve butyric acid and hydrogen production, integrational mutagenesis was used to create a metabolically engineered mutant with inactivated *ack* gene, encoding acetate kinase (AK) associated with the acetate formation pathway. A non-replicative plasmid containing the acetate kinase gene (*ack*) fragment was constructed and introduced into *C. tyrobutyricum* by electroporation. Integration of the plasmid into the homologous region on the chromosome should inactivate the target *ack* gene and produce *ack*-deleted mutant, PAK-Em. Enzyme activity assays showed that the AK activity in PAK-Em decreased by ~50%; meanwhile, phosphotransacetylase (PTA) and hydrogenase activities each increased by ~40%. The sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) results showed that the expression of protein with ~32 kDa molecular mass was reduced significantly in the mutant. Compared to the wild type, the mutant grew more slowly at pH 6.0 and 37 °C, with a lower specific growth rate of 0.14 h^{-1} (vs 0.21 h^{-1} for the wild type), likely due to the partially impaired PTA-AK pathway. However, the mutant produced 23.5% more butyrate (0.42 vs 0.34 g/g glucose) at a higher final concentration of 41.7 g/L (vs 19.98 g/L) as a result of its higher butyrate tolerance as indicated in the growth kinetics study using various intial concentrations of butyrate in the media. The mutant also produced 50% more hydrogen (0.024 g/g) from glucose than the wild type. Immobilized-cell fermentation of PAK-Em in a fibrous-bed bioreactor (FBB) further increased the final butyric acid concentration (50.1 g/L) and the butyrate yield (0.45 g/g glucose). Furthermore, in the FBB fermentation at pH 5.0 with xylose as the substrate, only butyric acid was produced by the mutant, whereas the wild type produced large amounts of acetate (0.43 g/g xylose) and lactate (0.61 g/g xylose) and little butyrate (0.05 g/g xylose), indicating a dramatic metabolic pathway shift caused by the *ack* deletion in the mutant.

Introduction

Butyric acid production from natural resources by *Clostridium tyrobutyricum* has become an increasingly attractive alternative to petroleum-based chemical synthesis because of its wide applications in food and pharmaceutical industries (*1*−*3*) and concerns of environmental pollution generated by the petrochemical industry. *C. tyrobutyricum* also produces hydrogen, which has a high energy content per unit weight (141.86 kJ/g or 61,000 Btu/lb) and is considered one of the most promising future fuels replacing the depleting fossil energy (*4*). For economical production of butyrate by fermentation, a fibrous-bed bioreactor with immobilized cells of *C. tyrobutyricum* has been developed to increase reactor productivity, butyrate yield, and final product concentration (*5*). However, the production of acetic acid as a byproduct in butyric acid fermentation not only reduces butyrate yield but also increases product recovery and purification costs. While a complete selectivity for butyrate production is possible in glucose-limited fed-batch fermentation (*6*, *7*), the reactor productivity and final product concentration were not high enough for economical production. It is thus desirable to create metabolically engineered mutants that produce mainly butyrate and little or no acetate.

The metabolic pathway for butyrate and acetate production in *C. tyrobutyricum* and *C. acetobutylicum* have been extensively studied (*8*, *9*). In general, glucose is catabolized via EMP (Embden−Meyerhof−Parnas) pathway, and xylose is catabolized via HMP (hexose monophosphate) pathway to pyruvate. Pyruvate is oxidized to acetyl-CoA and carbon dioxide with concomitant reduction of ferredoxin (Fd) to FdH_2, which is then oxidized by hydrogenase to Fd, producing hydrogen and converting NAD^+ to NADH (*10*). Acetyl-CoA is the key metabolic intermediate at the node dividing the acetate-forming branch, catalyzed by phosphotransacetylase (PTA) and acetate kinase (AK), from the butyrate-forming branch, catalyzed by phosphotransbutyrylase (PTB) and butyrate kinase (BK). The metabolic pathway along with these key enzymes are shown in Figure 1.

The main objective of this study was to improve butyric acid production by constructing a metabolically engineered *C. tyrobutyricum* mutant with inactivated acetate formation pathway. Integrational mutagenesis can selectively inactivate undesired genes from the host chromosome with non-replicative integrational plasmids and has been successfully applied to create metabolically engineered mutants of clostridial strains

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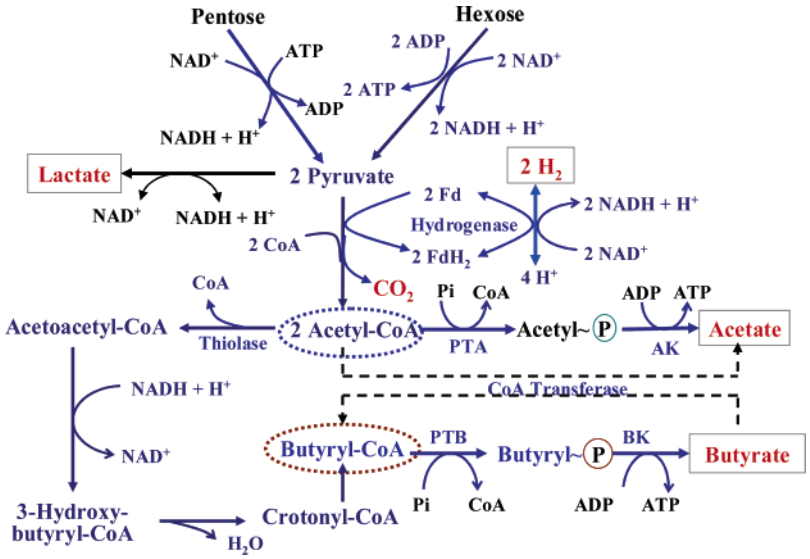

Figure 1. The metabolic pathway in *Clostridium tyrobutyricum*. In general, glucose and xylose are catabolized to pyruvate by the EMP pathway and the HMP pathway, respectively. Pyruvate is oxidized to acetyl-CoA and carbon dioxide with concomitant production of hydrogen. Acetyl-CoA is either oxidized to acetate through the PTA-AK pathway or butyrate through the PTB-BK pathway. Abbreviations: PTA, phosphotransacetylase; AK, acetate kinase; PTB, phosphotransbutyrylase; BK, butyrate kinase.

(*11*, *12*). Our recent study has shown that integrational mutagenesis inactivated the chromosomal *pta* gene, which encodes PTA, and resulted in significantly reduced PTA and AK activities and acetate production (*11*). In this work, we targeted the inactivation of only *ack* gene and studied its effects on butyric acid fermentation. The *ack* gene fragment was cloned, characterized, and used to construct an integrational plasmid for the transformation of *C. tyrobutyricum*, resulting in an *ack*-disrupted mutant, PAK-Em. The activities of key acid-forming enzymes in the fermentation pathway and SDS−PAGE analysis of protein expression in the mutant were examined and compared to those of the wild type. The effects of *ack* mutation on cell growth and butyric acid, acetic acid, and hydrogen production from glucose and xylose in both free-cell and immobilized-cell fementations were studied and the results are reported here. The potential of using PAK-Em for butyrate and hydrogen production are also discussed in this paper.

Materials and Methods

Cultures and Media. *C. tyrobutyricum* ATCC 25755 was cultured anaerobically at 37 °C in a previously described synthetic clostridial growth medium (CGM) (*13*). Colonies were maintained on Reinforced Clostridial Medium (RCM; Difco, Kansa City, MO) plates in an anaerobic chamber. The stock culture was maintained in serum bottles under anaerobic condition at 4 °C. The *C. tyrobutyricum* mutant was selected by supplying the media with 40 μg/mL of erythromycin (Em; Fisher Scientific, Hampton, NH). *E. coli* was grown on Luria-Bertani (LB, Fisher Scientific) medium supplemented with 100 μg/mL of ampicillin (Amp, Fisher Scientific) aerobically at 37 °C.

Cloning of *ack* Deleted Mutant. The amplification of the partial *ack* sequence from *C. tyrobutyricum* genomic DNA and the construction of the non-replicative plasmid used in the transformation to obtain *ack*-disrupted mutant all followed similar procedures as described in our previous work (*11*). Chromosomal DNA from *C. tyrobutyricum* was prepared using QIAGEN genomic DNA kit (Qiagen, Valencia, CA). The isolation of plasmid DNA from *E. coli* was undertaken using QIAprep Miniprep plasmid purification kit (Qiagen). DNA fragments were purified from gel using QIAquick gel extraction kit. Restriction enzymes, T4 ligase, and shrimp alkaline phosphatase were used in accordance with the supplier's instruction (Amersham, Piscataway, NJ).

PCR Amplification of ack Gene. Synthetic oligonucleotides were designed as degenerate primers using the codon usage preference for *C. tyrobutyricum* (http://www.kazusa.or.jp/codon) following previous study (*14*). The sequences of the PCR primers for *ack* gene amplification were 5′- GAT AC(A/T) GC(A/T) TT(C/T) CA(C/T) CA(A/G) AC-3′ (forward) and 5′- (G/C)(A/T)(A/G) TT(C/T) TC(A/T) CC(A/T) AT(A/T) CC(A/T) CC 3′ (reverse). The partial *ack* fragment was amplified using wild-type *C. tyrobutyricum* chromosomal DNA as template. The PCR amplification was performed in a DNA engine (MJ Research, Reno, NV) with a previously developed PCR buffer containing 2.5 mM Mg^{2+} (*11*). Thermal cycling was initiated with a denaturation step (94 °C for 3 min) followed by a 40 cycle program with template denaturation (94 °C for 50 s), primers annealing (42 °C for 50 s), and extension (72 °C for 1 min). At the end of the program, the deoxyadenosine (A) was added to the 3′ end of the PCR products at 72 °C for 10 min. The low primer annealing temperature resulted in multiple PCR products. The PCR product with expected size of ~560 base nucleotides was cut and purified from gel and cloned to T-A cloning vector pCR 2.1 (3.9 kb) using TA cloning kit (Invitrogen, Carlsbad, CA), and the produced plasmid pCR-PAK (4.5 kb) was used to determine the DNA sequence of the *ack* gene fragment.

Construction of Integrational Plasmid. Figure 2 shows the general design in constructing the integrational plasmid pAK-Em. A 1.6-kb *Hind*III fragment containing the Em^r cassette obtained from pDG 647 was used as the antibiotic gene for mutant selection (*15*). The size of pCR-PAK (4.5 kb) was reduced by removing a 1.5-kb fragment with *Sph*I digestion, and the remaining vector backbone was religated to form plasmid pCR-PAK1 (3.0 kb). The *Hind*III digested plasmid pCR-PAK1 was then ligated with the *Hind*III ended Em^r cassette, producing the non-replicative integrational plasmid pAK-Em (4.6 kb) to be used to inactivate the *ack* gene in *C. tyrobutyricum*. The pAK-Em was amplified in *E. coli* and harvested according to the manufacturer's instruction (Invitrogen).

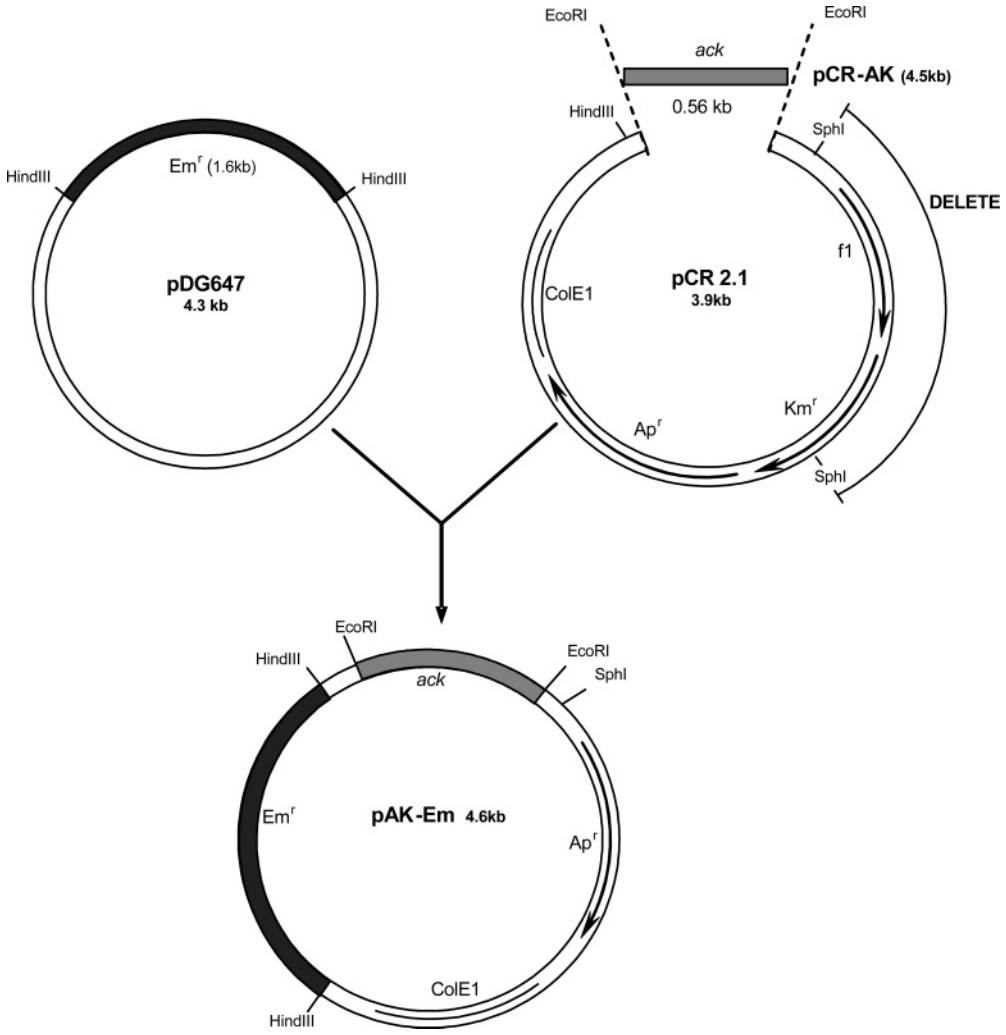

Figure 2. Construction of integrational plasmid pAK-Em with one 0.56-kb *ack* fragment cloned from *C. tyrobutyricum*. Abbreviations: *ack*, partial *ack* gene; f1, f1 filamentous phage origin of replication with helper phage; Ap^r, ampicillin resistance gene; Em^r, erythromycin resistance gene; ColE1, compatibility group origin of replication in *E. coli*.

Transformation and Mutant Selection. The competent cells of *C. tyrobutyricum* were prepared by growing cells in the CGM medium containing 40 mM DL-threonine, which helped to weaken the cell wall of the Gram-positive microorganism. The optimized electroporation SMP buffer (270 mM sucrose, 1 mM $MgCl_2$, 7 mM sodium phosphate, pH 7.4) was used to wash the harvested cells. The protoplast was prepared to examine the presence of the restriction system in *C. tyrobutyricum* on pAK-Em before transformation (*16*). The transformation of the integrational plasmid into *C. tyrobutyricum* was carried out in an anaerobic chamber using a Bio-Rad Gene pulser (Model II) under the optimized pulsing conditions (2.5 kV, 600 Ω, and 25 μF). In electroporation, about 0.5 mL of cell suspension was chilled on ice for 5 min in a 0.4-cm electroporation cuvette (Bio-Rad, Hercules, CA), and plasmid DNA (10−15 μg of plasmid pAK-Em) was added into the cold competent cell suspension. The transformed cells were transferred to 5 mL of prewarmed CGM and incubated at 37 °C for 3 h prior to plating on RCM plates containing 40 μg/mL Em. Plates were incubated to develop the mutant colonies in 37 °C anaerobic incubator for 3−5 days. As a negative control, a non-replicative plasmid (15 μg) with an Em^r cassette but without the *ack* fragment was also used to transform the *C. tyrobutyricum* cells. The transformant or *ack* inactivated mutant, PAK-Em, was selected from the agar plate and maintained in the CGM medium containing 40 μg/mL Em. The mutant was characterized for its protein expression, AK, PTA, and hydrogenase activities, butyrate tolerance, and fermentation kinetics with glucose and xylose as the substrate at two different pHs (6.0 and 5.0).

Enzyme Assays and SDS−PAGE. The bacterial cells were cultivated in serum bottles with 100 mL of CGM (without erythromycin) at 37 °C and harvested by centrifugation after having grown to the exponential phase (OD_{600} = ∼1.5). The cell pellets were washed, suspended in 10 mL of 25 mM Tris-HCl buffer (pH 7.4), and broken by sonication. The protein extract was then collected for protein electrophoresis and PTA and AK activity assays under ambient conditions. For hydrogenase activity assay, the protein sample preparation and enzyme assay were all carried out in the anaerobic chamber. The cultured cells were suspended in 1 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), lysed with 100 μg/mL of mutanolysin (Sigma-Aldrich, St. Louis, MO) at 37 °C for 30 min, and centrifuged to remove the cell debris. Standard Bradford protocol (Bio-Rad, Hercules, CA) was used to measure protein content in the cell extract sample.

The activities of AK and BK were assayed by the method of Rose using potassium acetate and sodium butyrate (Fisher Scientific) as the substrate (*17*), respectively. One unit of AK or BK was defined as the amount of enzyme producing 1 μmol of hydroxamic acid (Sigma-Aldrich) per minute. The PTA and PTB activities were measured by monitoring the liberation of CoA from acetyl-CoA and butyryl-CoA (Sigma-Aldrich) at 405

nm (*18*). One unit of PTA or PTB activity was defined as the amount of enzyme converting 1 μmol of acetyl-CoA or butyryl-CoA per minute. Hydrogenase activity was detected using the procedure developed by Drake (*19*). One unit of hydrogenase activity is defined as 2 μmol of methyl viologen (Sigma-Aldrich) reduced (equivalent to 1 μmol of H_2 oxidized) per minute. Specific enzyme activity (U/mg) was calculated as the units of activity per mg of total protein. The relative enzyme activity (%) was reported by comparing the specific enzyme activities in the mutant with the corresponding specific enzyme activities in the wild type.

Protein samples for SDS−PAGE electrophoresis were prepared following standard protocol (Bio-Rad). Total protein samples (24 μg each) were loaded into wells and 12.5% SDS−PAGE gel was run at 100 V for 2.5 h with PROTEAN II xi Cell (Bio-Rad). The gel was stained and distained following the manufacturer instruction.

Butyric Acid Tolerance Study. To evaluate the inhibition effect of butyrate on cell growth, various concentrations of butyrate (0−15 g/L) were applied to *C. tyrobutyricum* culture in serum tubes containing 10 mL of media. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer (Sequoia-Turner, model 340), and the specific growth rates at various initial butyrate concentrations were estimated from the OD_{600} data.

Fermentation Kinetic Studies. Unless otherwise noted, the fermentation kinetics of *C. tyrobutyricum* was studied in a 5-L stirred-tank fermentor (Marubishi MD-300; BE Marubishi Co., Tokyo, Japan) containing 2 L of the medium with glucose as the substrate. The fermentation was operated at a fed-batch mode, agitated at 150 rpm, and controlled at pH 6.0 and 37 °C. The fermentation kinetics was also studied with cells immobilized in a fibrous-bed bioreactor (FBB), which was made of a glass column packed with spiral wound cotton towel and had a working volume of ~500 mL. The FBB was connected to the fermentor and operated as a well mixed reactor with media recirculation (*5*, *20*). The anaerobiosis was achieved by sparging the fermentor medium and the FBB with N_2. The 100 mL of cell suspension prepared in a serum bottle was inoculated into the fermentor. After 3 days of growth, the cells with media in the fermentor were circulated through the fibrous bed and immobilized in the fibrous matrix. After most of the cells had been immobilized in ~2 days, as indicated by the OD value in the fermentor broth, the spent medium was replaced with fresh medium. The fed-batch mode was then operated by pulse feeding concentrated substrate solution when the sugar level in the fermentation broth was close to zero. The feeding was continued until the fermentation ceased to consume the sugar substrate due to product inhibition. The FBB fermentation was also studied with xylose as the substrate.

Analytical Methods. Gas (H_2 and CO_2) production in the fermentation was monitored using an online respirometer system equipped with both H_2 and CO_2 sensors (Micro-oxymax system, Columbus Instrument, Columbus, OH). Liquid samples were taken at regular intervals from the fermentor for the analyses of cell density, substrate, and products. Cell density was analyzed by measuring the optical density of the cell suspension at 600 nm (OD_{600}) with a spectrophotometer (Sequoia-Turner, model 340). One unit of OD_{600} corresponded to 0.68 g/L of cell dry weight. A high performance liquid chromatograph (HPLC) was used to analyze the organic compounds, including glucose, xylose, butyrate, and acetate in the liquid samples. Details of the HPLC method can be found elsewhere (*5*).

Results and Discussion

Cloning of *ack* Deleted Mutant. The partial *ack* gene fragment in *C. tyrobutyricum* was successfully amplified by PCR using degenerate primers. The obtained partial *ack* gene had 564 nucleotides encoding for 188 amino acids (a.a.), which can be found in GenBank (GenBank accession no. AY706093). The deduced partial amino acid sequence of AK was then compared with the known sequences of complete AK from other microorganisms by homologous alignment. As shown in Figure 3, high degrees of identities of amino acid sequences were found between the partial AK of *C. tyrobutyricum* and the AK of *E. coli* (52%, 189 a.a.), *M. thermophila* (53%, 189 a.a.), *B. subtilis* (48%, 189 a.a.), *H. influenzae* (47%, 192 a.a.), *C. acetobutylicum* (44%, 190 a.a.), and *M. genitalium* (40%, 184 a.a.).

After electroporation, a selective medium containing Em was used to grow mutant cells containing the non-replicative plasmids. A total of ~10 Em-resistant colonies were obtained after electroporation with a transformation efficiency of 1 colony per μg of DNA, which was similar to those obtained for the integrational plasmids in *C. acetobutylicum* ATCC 824 (*12*). As a negative control, the non-replicative plasmid with only the Em^r cassette but without the *ack* fragment was also used to transform *C. tyrobutyricum* cells; no transformant was obtained since the plasmid cannot be replicated in the cells without integrating into the chromosome via homologous recombination. These results suggested that pAK-Em was integrated into the chromosome by homologous recombination in the transformed cells. Since the homologous region in pAK-Em is the internal DNA sequence of *ack*, the transformed cells was mutagenic and the original *ack* gene on the chromosome should have been disrupted, which was indirectly confirmed by the reduced AK activity and altered fermentation kinetics discussed in the following sections.

Protein Expression and Enzyme Activities. Protein expression was studied by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) to better understand the effect of *ack* gene disruption in the mutant cell. The SDS−PAGE gel map (Figure 4) clearly showed that the highly expressed protein with ~32 kDa molecular mass in the wild type diminished in PAK-Em. This 32-kDa protein was likely the deleted acetate kinase (*11*), although its identity cannot be confirmed because neither antibody to this AK protein nor proteomic data for this bacterium is available. It was also noted that the expression of the protein with ~70 kDa molecular mass was higher in PAK-Em. The hydrogenase in a similar species, *C. acetobutyricum*, has been isolated, and its molecular mass was reported to be around 70 kDa (*21*). We thus speculated that this 70-kDa protein was the hydrogenase, whose activity increased significantly in the mutant as found in the enzyme activity assay, which is discussed below. Further identification of these proteins may be carried out with non-denaturing PAGE, followed with on-gel enzyme activity assay. However, more work would be needed to develop the method, especially for hydrogenase, which is highly sensitive to oxygen and unstable in the purified form.

The specific enzyme activities for acid forming enzymes (PTA, AK, PTB, and BK) and the hydrogen-catalyzing enzyme (hydrogenase) in the PAK-Em mutant were measured and compared with those of the wild type. As shown in Figure 5, the mutant's AK activity was reduced by 50% (from 14.99 U/mg in the wild type to 6.96 U/mg in PAK-Em) while its PTA activity was increased by 42% (from 0.260 U/mg in the wild type to 0.34 U/mg in PAK-Em). It is noted that *pta* gene lies upstream from *ack* gene in the same operon, and the disruption of *ack* would not negatively affect the expression of *pta*.

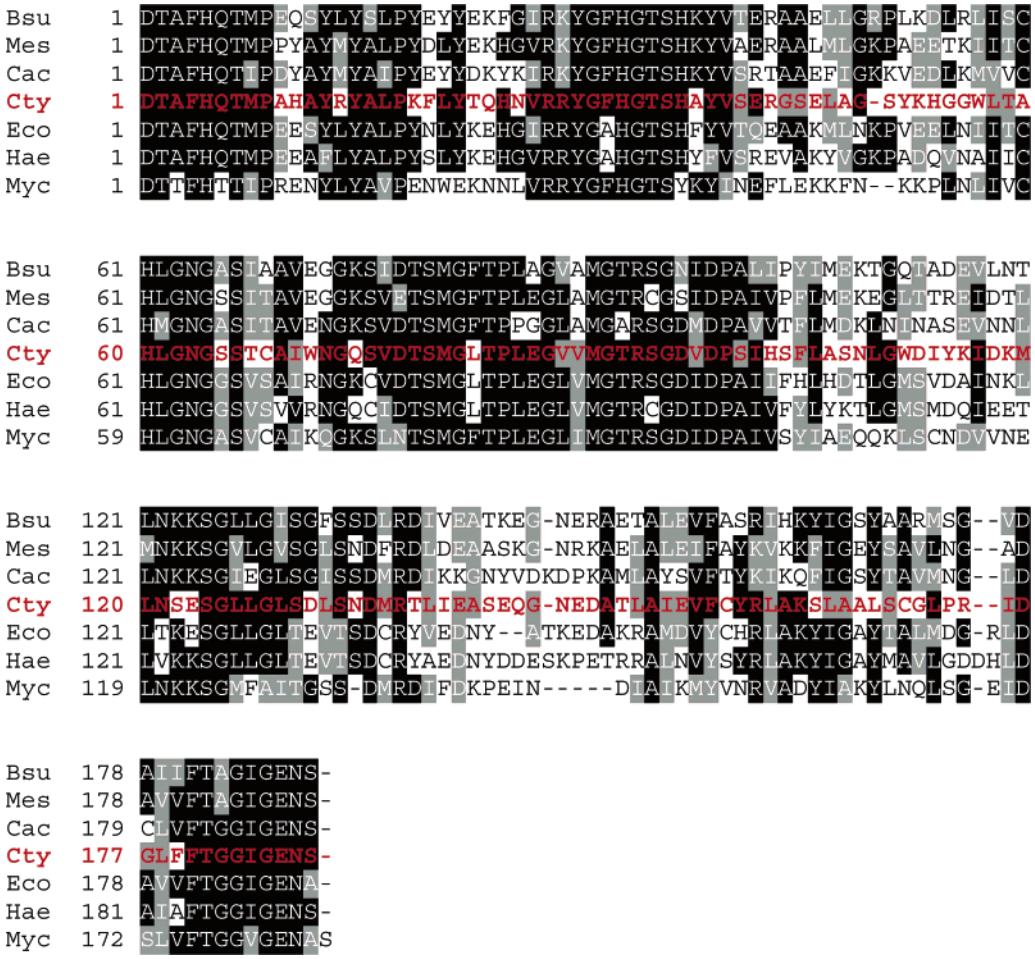

Figure 3. Alignment of partial amino acid sequences of AK from *B. subtilis* (Bsu; GenBank accession no. L17320), *Methanosarcina thermophila* (Mes; GenBank accession no. L23147), *C. acetobutylicum* (Cac; GenBank accession no. U38234), *C. tyrobutyricum* (Cty, GenBank accession no. AY706093), *E. coli* (Eco; GenBank accession no. M22956), *H. influenzae* (Hae; GenBank accession no. L45839), and *Mycoplasma genitalium* (Myc; GenBank accession no. L43967).

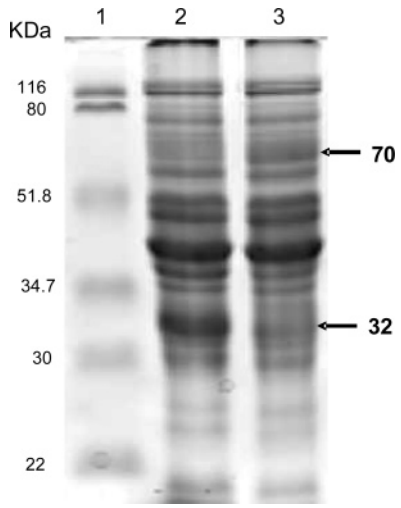

Figure 4. SDS polyacrylamide gel electrophoresis of cellular proteins from *C. tyrobutyricum*. Lane 1, molecular marker; Lane 2, wild type; Lane 3, PAK-Em mutant.

However, the accumulation of acetyl-CoA resulted from the (partial) inactivation of the PTA-AK pathway could have a positive control effect on the expression level of PTA. It is not clear why the mutant still showed significant AK activity even though *ack* should have been disrupted. The apparent AK activity probably was from other enzymes that also can use the same substrate to produce acetic acid (*8*, *11*). For example, CoA transferase has been reported to catalyze the formation of acetate from acetyl-CoA in some clostridia species (*10*). Also, some AK isozymes also might be present in *C. tyrobutyricum*. It was also found that the disruption of *ack* gene had no effect on the activities of butyrate-forming enzymes, PTB (~0.0095 U/mg) and BK (~0.15 U/mg). Interestingly, the hydrogenase activity in the mutant increased 40%, resulting in increased hydrogen production by PAK-Em as found in the fermentation kinetic studies. More work would be needed in order to fully understand the mutation and its effects on gene regulation and the metabolism. However, this may require us to undertake a genomic approach, which is difficult to do at present as the genomic sequence of this bacterium is still unknown.

Butyric Acid Tolerance. End product inhibition is one of the key factors limiting the organic acid production by fermentation. To determine the butyric acid tolerance in the mutant, cells were grown as free-cell suspension cultures at different initial butyric acid concentrations (0−15 g/L). The specific growth rates determined from the growth data are shown as the relative growth rate with the rate at zero initial butyrate concentration being 100%. As shown in Figure 6, the *ack*-deleted mutant had a much higher tolerance to butyric acid than the wild type. At 15 g/L of butyric acid, the mutant retained more than 30% of its maximum growth rate as compared to less than 10% in the wild type. The growth inhibition by butyric acid followed the noncompetitive inhibition kinetics with different inhibition rate constants K_P, 1.59 g/L for the wild type

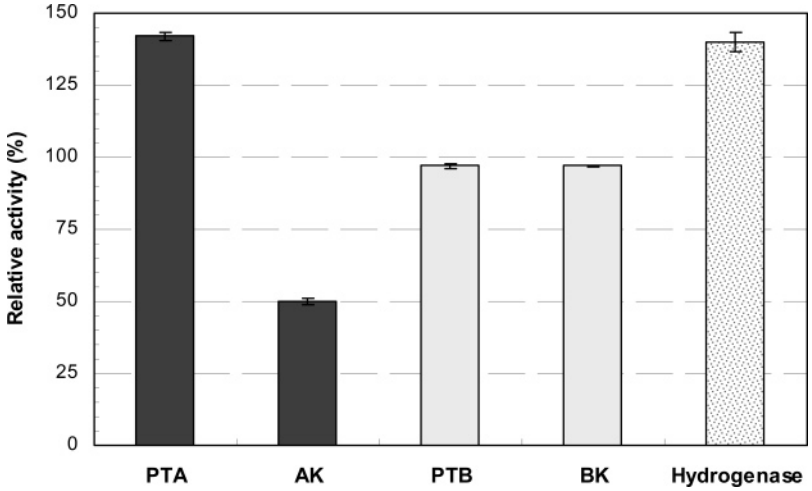

Figure 5. Relative activities of key enzymes in the acetate-forming and hydrogen production pathways in the PAK-Em mutant as compared with the wild type. Abbreviations: AK, acetate kinase; BK, butyrate kinase; PTA, phosphotransacetylase; PTB, phosphotransbutyrylase.

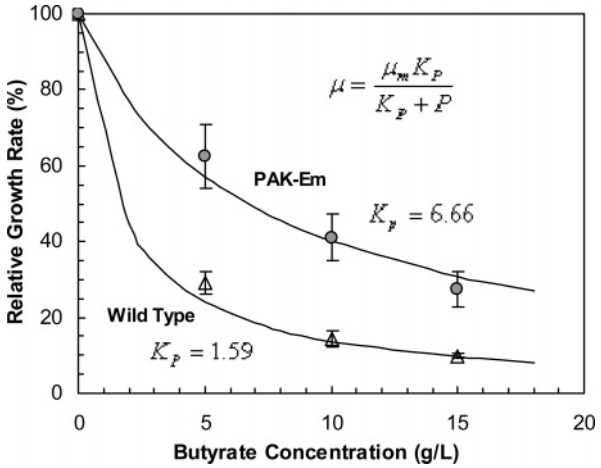

Figure 6. Noncompetitive inhibition of butyric acid on cell growth of *C. tyrobutyricum* wild type (△) and mutant PAK-Em (□).

and 6.66 g/L for the mutant. It is clear that butyric acid strongly inhibited cell growth of the wild type but not as strongly that of the mutant. The growth inhibition by butyric acid can be partially attributed to the inhibition effect on the key enzymes in the metabolic pathway (*22*). The enzymes in the PTA-AK pathway in *C. tyrobutyricum* was found to be more sensitive to butyric acid inhibition than those in the PTB-BK pathway (*22*). Therefore, with *ack* gene disruption and partially impaired PTA-AK pathway, the mutant generated ATP mainly from the PTB-BK pathway and became less sensitive to butyrate inhibition. Consequently, the improved butyric acid tolerance allowed the mutant to produce more butyric acid at a higher final concentration as found in the fermentation kinetic study discussed below. Although the wild type also can generate ATP from the PTB-BK pathway, cells would require more time to adapt and respond to higher butyrate concentrations when they had largely relied on the PTA-AK pathway for energy supply. Further study will be needed in order to fully understand the underlying reasons for the mutant's improved butyrate tolerance.

Fermentation Kinetics. Figure 7 shows the kinetics of fed-batch fermentations of glucose at pH 6.0 and 37 °C by the wild-type and mutant PAK-Em. In general, cells grew exponentially in the first fed-batch and then entered the stationary phase. They continued to produce butyrate until the fermentation completely stopped at a high butyrate concentration that inhibited the cells. It was found that the mutant had a specific growth rate lower than that of the wild type. However, the mutant produced butyrate at a much higher final concentration (41.65 g/L) than that from the wild-type fermentation (19.98 g/L). Besides the greatly increased final butyrate concentration produced by the mutant, the butyrate yield was also increased, from 0.34 g/g glucose (3.4 g/g cell) by the wild type to 0.42 g/g glucose (7.0 g/g cell) by the mutant (Table 1). The higher butyrate concentration produced by the mutant was consistent with the finding that the mutant had a higher tolerance to butyric acid inhibition.

When the fermentation is carried out with cells immobilized in the fibrous-bed bioreactor (FBB), much more butyric acid can be produced as a result of the adaptation benefit of the FBB (*22*). At pH 6.0, the mutant in the FBB produced a high final butyric acid concentration of 50.1 g/L (Figure 8), with a butyrate yield of 0.45 g/g glucose. The wild type in the FBB fermentation produced 44 g/L of butyric acid with a yield of 0.42 g/g glucose. The final butyric acid concentration and yield from the immobilized-cell fermentations were significantly enhanced as a result of the reduced cell growth and improved butyrate tolerance resulting from cell adaptation in the FBB (*22*). It is clear that *ack* deletion resulted in higher butyrate yield and final butyrate concentration obtained in the fed-batch fermentations with either free cells or immobilized cells.

However, inactivation of the *ack* gene did not seem to significantly affect acetate formation in the glucose fed-batch fermentation at pH 6.0. As can be seen in Table 1, acetate yield from glucose was not significantly different between the wild type and the mutant. The seemingly unchanged acetate formation was somewhat inconsistent with the observed lower AK activity in the mutant. This point will be further discussed later in this paper. It is noted, however, that more butyrate was produced in the mutant and the butyrate/acetate ratio (B/A) increased from 4.5 g/g for the wild type to 5.4 g/g for PAK-Em mutant in free-cell fermentations and to 5.99 g/g for PAK-Em in the FBB fermentation. Clearly, the mutation on the *ack* gene increased the carbon flux toward the PTB-BK pathway.

Although the wild type produced similar amounts of hydrogen and CO_2, the amount of hydrogen produced by PAK-Em was significantly higher than that of CO_2. Consequently, the mutant produced 50% more hydrogen from glucose than did the wild type (0.024 g/g vs. 0.016 g/g). Apparently, more electrons were transferred to H^+, and thus, more hydrogen and NADH were produced in the mutant, which was consistent with the finding that the mutant had a 40% higher hydrogenase activity as compared to the wild type (see Figure 5). It is also noted that

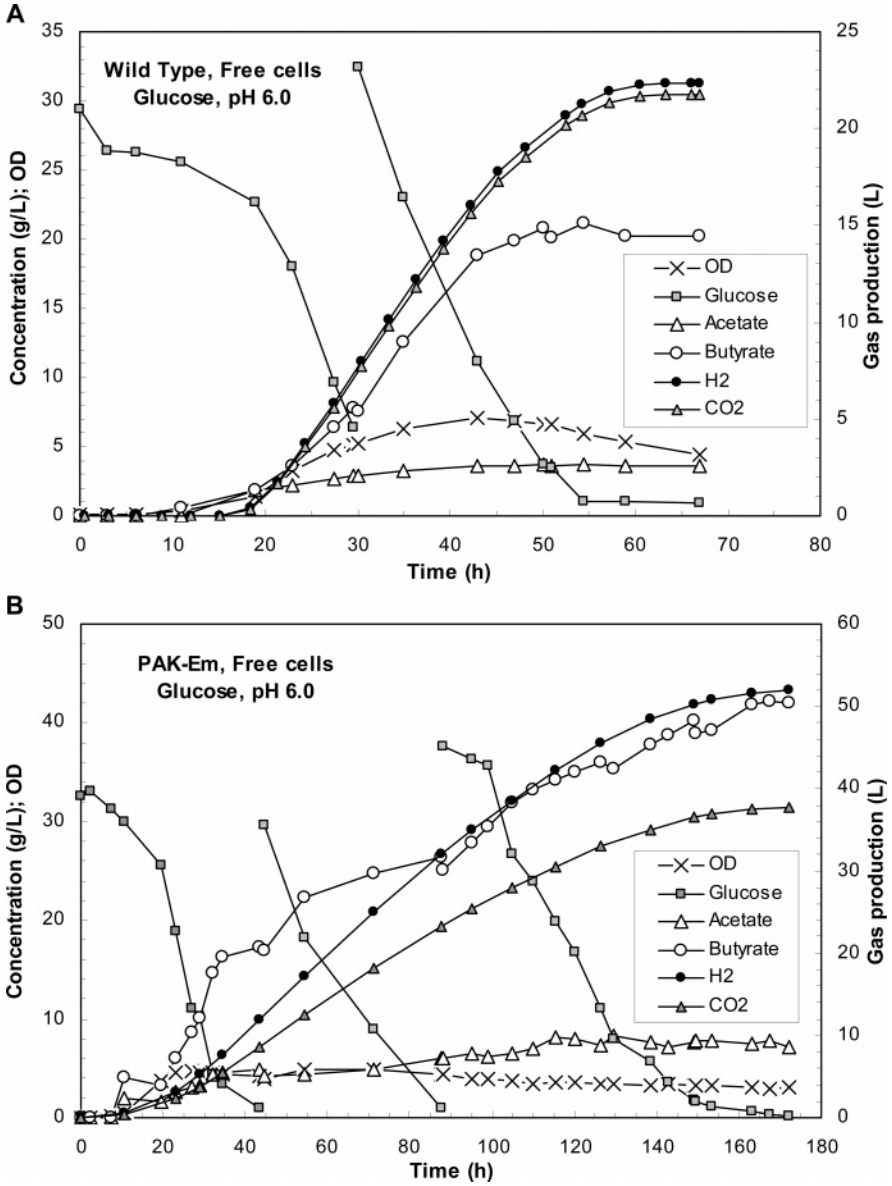

Figure 7. Fed-batch fermentations of glucose by free cells of *C. tyrobutyricum* at pH 6.0 and 37 °C. (A) Fermentation by the wild type. (B) Fermentation by mutant PAK-Em. Symbols: OD_{600} (×), sugar concentration (■), butyrate concentration (○), acetate concentration (△), hydrogen (●) and carbon dioxide (▲).

Table 1. Comparison of Fed-Batch Fermentations of Glucose by *C. tyrobutyricum* Wild Type and *ack* Deleted Mutant, PAK-Em, at 37 °C and pH 6.0[a]

	wild type		PAK-Em	
	free cells	immobilized cells	free cells	immobilized cells
		Cell Growth		
specific growth rate (h^{-1})	0.21 ± 0.03	0.11 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
biomass yield (g/g glucose)	0.10 ± 0.01	0.06 ± 0.001	0.06 ± 0.002	0.04 ± 0.01
		Acid Production		
final butyric acid concn (g/L)	19.98 ± 3.07	44.1 ± 0.1	41.65 ± 0.63	50.11 ± 2.42
butyric acid yield (g/g glucose)	0.34 ± 0.02	0.42 ± 0.01	0.42 ± 0.01	0.45 ± 0.02
final acetic acid concn (g/L)	4.42 ± 0.55	8.6 ± 0.9	7.75 ± 0.76	8.38 ± 0.04
acetic acid yield (g/g glucose)	0.07 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
B/A ratio (g/g)	4.52 ± 0.85	5.1 ± 0.5	5.41 ± 0.61	5.99 ± 0.55
		Gas Production		
H_2 yield (g/g glucose)	0.016 ± 0.001	NA	0.024 ± 0.001	0.023 ± 0.004
CO_2 yield (g/g glucose)	0.32 ± 0.02	NA	0.37 ± 0.02	0.34 ± 0.01
H_2/CO_2 ratio (mol/mol)	1.04 ± 0.001	NA	1.44 ± 0.06	1.59 ± 0.01
carbon balance[b]	0.95 ± 0.04	NA	0.95 ± 0.01	0.96 ± 0.02

[a] All fermentations were duplicated and the average values ± standard errors are reported from the duplicated fermentation runs. NA: data not available. [b] Carbon balance = total carbons recovered in the cell biomass and products (butyric acid, acetic acid, and CO_2)/total carbons in the substrate glucose. The carbon content in the cell biomass was assumed to be 46.7% of the total cell biomass, which has been reported for *Clostridia* (*10*).

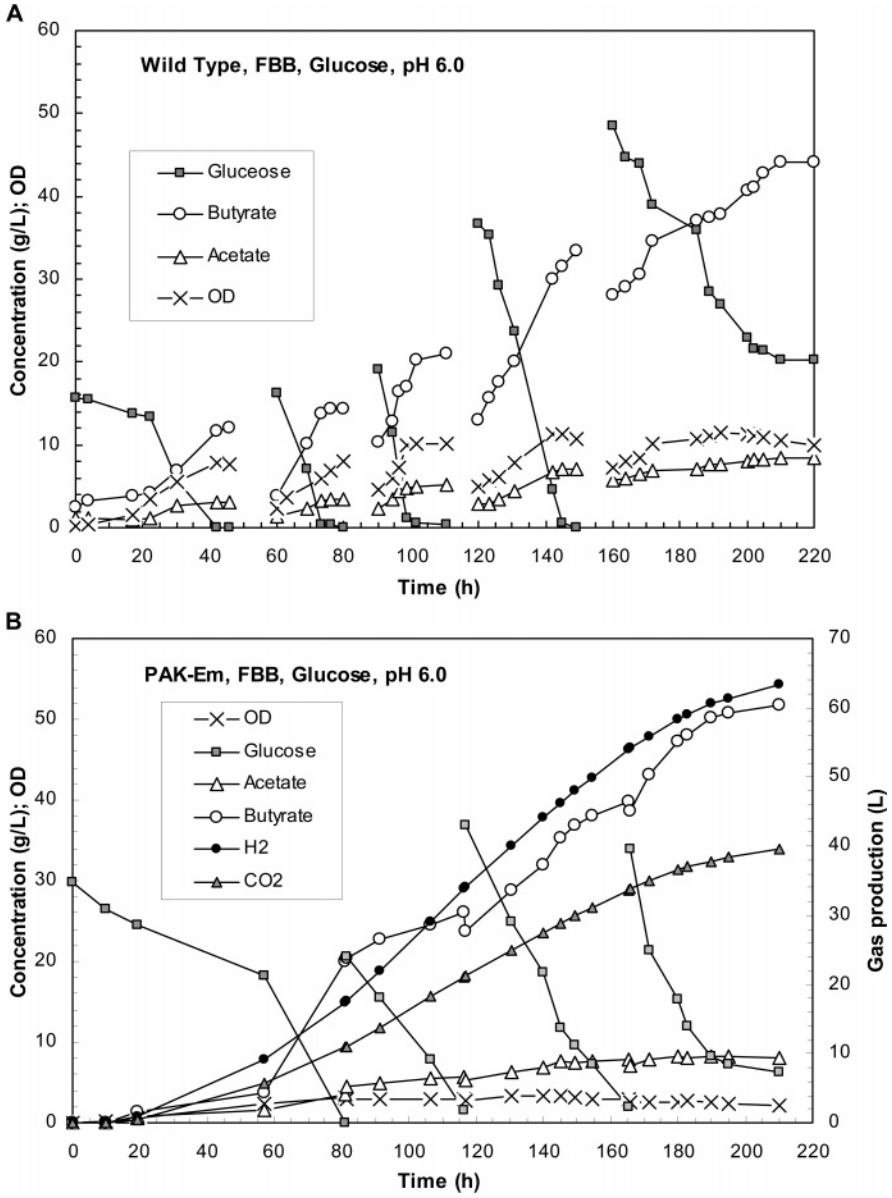

Figure 8. Fed-batch fermentations of glucose by immobilized cells of *C. tyrobutyricum* in fibrous bed bioreactor at pH 6.0 and 37 °C. (A) Fermentation by the wild type. (B) Fermentation by mutant PAK-Em. Symbols: OD_{600} (×), sugar concentration (■), butyrate concentration (○), acetate concentration (△), hydrogen (●) and carbon dioxide (▲).

the carbon balance in the fed-batch fermentations was close to 100% for both the wild type and the mutant (see Table 1), indicating a complete recovery of carbon in the fermentation products and cells from the substrate, glucose.

Effect of pH on Metabolic Pathway Shift. Table 2 summarizes the FBB fermentation results at pH 5.0 by the wild type and PAK-Em grown on glucose and xylose as the substrate, respectively. The fermentation kinetics was quite different for cell growth on glucose and xylose. Compared with glucose fermentation, xylose fermentation usually gives a lower energy efficiency and, consequently, lower specific growth rate and biomass yield. Interestingly, *C. tyrobutyricum* grown on xylose shifted its metabolic pathway from being a major butyric acid producer at pH 6.0 to producing mainly lactic and acetic acids at pH 5.0 (*23*). This metabolic pathway shift did not happen when glucose was the substrate. With xylose as the sugar source at pH 5.0, the wild type produced 33.5 g/L of lactic acid and 25.5 g/L of acetic acid but only a small amount of butyric acid (5.3 g/L) in the fed-batch fermentation. However, there was no lactic acid or acetic acid production from xylose and butyric acid was the only acid product produced by the PAK-Em mutant at pH 5.0 (Figure 9). Butyric acid reached a final concentration of 13.6 g/L with a yield of 0.44 g/g xylose in the FBB fermentation at pH 5.0. In contrast, at pH 5.0 the glucose fermentation by PAK-Em showed kinetics similar to that of the wild type except for the lowered acetic acid production.

Effects of *ack* Disruption on Butyric Acid Fermentation. With the *ack* disruption, PAK-Em produced more butyrate at a higher concentration from both glucose and xylose as compared with the wild type. This improvement can be attributed to mainly the enhanced tolerance to butyrate inhibition and to some extent the reduced carbon flux through the PTA-AK pathway as evidenced by the increased butyrate-to-acetate ratio (B/A ratio) in the mutant. However, at pH 6.0 acetic acid production by the mutant was not significantly affected in the fermentation, although the mutant did have a much lower AK activity and the PTA-AK pathway should have been largely impaired. Apparently, in addition to AK, there are other enzymes or pathways in *C. tyrobutyricum* that can also produce acetate from acetyl-CoA and perhaps other substrates as well (*8*, *11*).

Table 2. Immobilized Cell Fermentation of Glucose and Xylose by *C. tyrobutyricum* Wild Type and *ack* Deleted Mutant PAK-Em at 37 °C and pH 5.0[a]

	wild type		PAK-Em	
	glucose	xylose	glucose	xylose
		Cell Growth		
specific growth rate (h^{-1})	NA	0.04 ± 0.006	0.08 ± 0.004	0.05 ± 0.01
biomass yield (g/g)[b]	NA	NA	0.11 ± 0.03	0.04 ± 0.003
		Final Acid Concentration		
butyric acid (g/L)	14.4	5.3	14.8 ± 1.0	13.6 ± 1.2
acetic acid (g/L)	5.43	25.5	2.2 ± 1.3	0
lactic acid (g/L)	0	33.5	0	0
		Product Yield[b]		
butyric yield (g/g)	0.365	0.05 ± 0.01	0.42 ± 0.03	0.44 ± 0.01
acetic yield (g/g)	0.14	0.43 ± 0.03	0.06 ± 0.004	0
lactic yield (g/g)	0	0.61 ± 0.03	0	0
hydrogen (g/g)	NA	NA	0.023 ± 0.001	0.021 ± 0.001
carbon dioxide (g/g)	NA	NA	0.037 ± 0.007	0.30 ± 0.002
metabolic shift[c]	no	yes	no	no

[a] All fermentations were duplicated and the average values ± standard errors are reported from the duplicated fermentation runs. NA: data not available. [b] Cell biomass and product yields are based on substrate consumption [c] Metabolic shift from producing butyrate as the main product to producing lactate and acetate as the main products when the fermentation pH was reduced from 6.0 to 5.0.

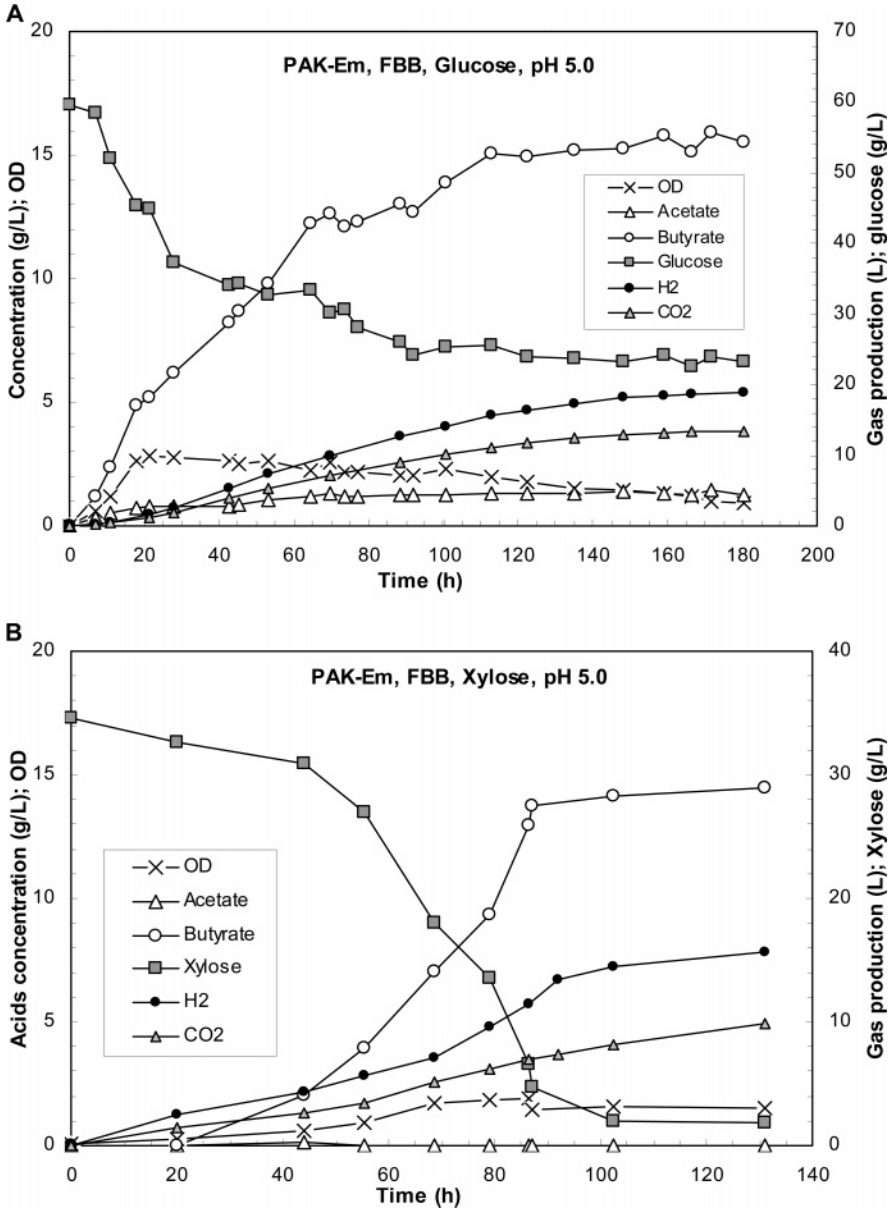

Figure 9. Fed-batch fermentations of glucose and xylose by *C. tyrobutyricum* mutant PAK-Em immobilized in the fibrous bed bioreactor at pH 5.0 and 37 °C. (A) Glucose fermentation. (B) Xylose fermentation. Symbols: OD_{600} (×), sugar concentration (■), butyrate concentration (○), acetate concentration (△), hydrogen (●) and carbon dioxide (▲).

Nevertheless, *ack* disruption did effectively shut down acetate production from xylose at pH 5.0, proving that the PTA-AK pathway has been greatly impaired in the mutant. The observed different effects on glucose and xylose fermentations may be related to the control of balancing the redox potential or NADH and NAD^+ in cell metabolism. It has been reported that regulation of pyruvate metabolism is dependent on the imbalance between catabolism and anabolism (*24*, *25*). Lactic acid production can be induced when lactate dehydrogenase (LDH) is activated by a high NADH/NAD^+ ratio (*24*), which is less likely to happen when the PTA-AK pathway is impaired and acetyl-CoA is directed mostly through the PTB-BK pathway, in which NADH is oxidized to NAD^+ (see Figure 1). Therefore, there was no metabolic shift to over-produce lactate (and acetate), and butyrate was the only acid product from xylose at pH 5.0 by PAK-Em. The production of butyric acid as a sole acid product in the fermentation gives additional process advantage for its reduced separation and purification costs. It is also noted that at pH 5.0, which is close to the pK_a value of butyric acid (4.89), most of the butyric acid is present in the form of free acid that is relatively easy to be recovered by solvent extraction (*26*).

The hydrogen production of the mutant was also increased by the enhanced hydrogenase activity. Although this result was not anticipated from the original *ack* disruption experimental design, this is the first time the hydrogen production of *C. tyrobutyricum* has been improved by genetic engineering. More hydrogen was produced in the mutant perhaps because the mutant needed to maintain the redox balance by producing more NADH and to compensate the lost energy efficiency due to the reduced flux through the PTA-AK pathway. In summary, the *ack* disruption resulted in global metabolic flux changes through various metabolites, including acetic acid, lactic acid, butyric acid and hydrogen, due to the need for redox balance and redistribution of carbon and energy.

The *ack* mutant was very stable. After growing the mutant in the medium without Em for ~10 generations (48 h), no differences in the key enzyme activities were detected as compared to the mutant in the antibiotics-containing medium, indicating no revertants (data not shown). Also, there was no obvious change in cell growth and acid production kinetics in repeated batch fermentations even in the absence of the antibiotics. It thus can be concluded that the mutant with gene mutation resulted from the homologous recombination on the chromosome is stable and can be maintained without using the antibiotics in long-term fermentation.

Conclusions

In this work, the *ack* gene fragment was cloned from *C. tyrobutyricum* and used to inactivate the parental gene to develop the metabolically engineered mutant strain for enhanced butyrate and hydrogen production from glucose and xylose. The mutant's higher tolerance to butyrate inhibition and reduced flux through the PTA-AK pathway resulted in the higher butyric acid production. Also, the enhanced hydrogenase activity in the mutant increased hydrogen production in the fermentation, adding an additional benefit of producing a clean energy product from low-cost renewable resources. The fermentation can be improved by cell immobilization and adaptation in the FBB, which further increased the butyrate yield to 0.45 g/g glucose and the final butyrate concentration to 50.1 g/L at pH 6.0. The FBB fermentation with the mutant thus should have a good potential for economically producing butyric acid and hydrogen from biomass.

Acknowledgment

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