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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Adaptive Evolution for Fast Growth on Glucose and the Effects on the Regulation of Glucose Transport System in *Clostridium tyrobutyricum*

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ABSTRACT: Laboratory adaptive evolution of microorganisms offers the possibility of relating acquired mutations to increased fitness of the organism under the conditions used. By combining a fibrous-bed bioreactor, we successfully developed a simple and valuable adaptive evolution strategy in repeated-batch fermentation mode with high initial substrate concentration and evolved *Clostridium tyrobutyricum* mutant with significantly improved butyric acid volumetric productivity up to 2.25 g/(L h), which is the highest value in batch fermentation reported so far. Further experiments were conducted to pay attention to glucose transport system in consideration of the high glucose consumption rate resulted from evolution. Complete characterization and comparison of the glucose phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) were carried out in the form of toluene-treated cells and cell-free extracts derived from both *C. tyrobutyricum* wide-type and mutant, while an alternative glucose transport route that requires glucokinase was confirmed by the phenomena of resistance to the glucose analogue 2-deoxyglucose and ATP-dependent glucose phosphorylation. Our results suggest that *C. tyrobutyricum* mutant is defective in PTS activity and compensates for this defect with enhanced glucokinase activity, resulting in the efficient uptake and consumption of glucose during the whole metabolism.

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KEYWORDS: *Clostridium tyrobutyricum*; adaptive evolution; glucose transport; PEP-dependent PTS; ATP-dependent glucokinase

Introduction

Because of the upward trend in the price of oil, public concerns about the environmental pollution caused by the petrochemical industry, and consumers' preference for bio-based natural ingredients in foods, cosmetics, and pharmaceuticals, the production of renewable chemicals by microbial and enzymatic routes has been strongly stimulated, which leads to a new manufacturing concept, generally referred to as the biorefinery (Tan et al., 2010; Timothy et al., 2010). Microbially produced butyric acid, one of the four-carbon short chain organic acids, represents potential building block molecule for this new and expanding chemical industry (Sauer et al., 2008). In fact, production of butyric acid by saccharolytic clostridia species has been known for a long time (Péaud-Lenoël, 1952; Playne, 1985), but recent studies indicate a renewed interest in this fermentation due to advances in our understanding of the genetics and physiology of acid production by these microorganisms, as well as the improvement and innovation of culture technologies (He et al., 2005; Jiang et al., 2010a; Liu et al., 2006; Mitchell et al., 2009; Zhu et al., 2005). Furthermore, it has been more recently disclosed that butanol regarded as one of the most promising biofuels can be synthesized via a catalytic reaction of butyric acid and hydrogen, both of which can be obtained by microbial fermentation (Song et al., 2010).

Since fed-batch fermentation can minimize the loss of remaining substrate, but maximize the final concentration and yield of a target product, it is often favored over batch or

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continuous fermentation. To date, the most promising culture technology for the biological production of butyric acid in industry-scale employs a fibrous-bed bioreactor (FBB) in a fed-batch cultivation mode with cells immobilized in a fibrous matrix packed in the reactor, which has been used to produce butyric acid with significantly improved volumetric productivity, product yield, and final product concentration (Huang et al., 2002; Jiang et al., 2009; Wu and Yang, 2003; Zhu et al., 2002). Our previous study was further successful in producing butyric acid as high final concentration as 86.9 ± 2.17 g/L via fed-batch fermentation mode in FBB (Jiang et al., 2011). However, this fed-batch mode can also present several shortcomings. Namely, as a consequence of low substrate consumption rate, the duration of the culture is usually long (more than 250 h) in a fed-batch process, which can decrease the overall volumetric productivity (0.35 ± 0.01 g/(L h)). Running proper fed-batch cultures in industry-scale often requires additional control schemes, supplementary equipment, and added operational effort during longer periods. Besides, when a glucose-concentrated solution is fed to the bioreactor, glucose gradients due to insufficient mixing are very likely to occur at large scales, which trigger a battery of physiological responses with negative impacts for the process (Lara et al., 2006). These drawbacks could be overcomed by culturing the cells in a simpler batch mode as long as the substrate inhibition at high concentration is effectively prevented, which unfortunately is rarely feasible in practical applications.

In this article, we firstly explored a simple and valuable culture strategy to improve cell performance at high glucose and cell concentrations combining immobilization and adaptive evolution. Herein the operation was consistently carried out in the FBB in a repeated-batch mode feeding with high initial glucose concentration (up to 150 g/L), as an advantageous alternative for quickly adapting and spontaneously evolving of mutant with high glucose consumption rate. We also interested in further characterization of *Clostridium tyrobutyricum* with respect to understanding the basis of elevated levels of glucose utilization. In a biotechnological context, information with regard to sugar transport systems may be crucial when considering strategies to improve productivity of desired metabolite. In most gram-negative and gram-positive prokaryotes, the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) is the predominant transport mechanism for sugar substrates, for which there is evidence that, in addition to have a role in mediating the uptake and phosphorylation of numerous carbohydrates, it is also involved in the regulation of gene expression (Tangney et al., 2003). The PTS is a group translocation process in which the transfer of the phosphate moiety of PEP to carbohydrates is catalyzed by two general cytosolic proteins, called Enzyme I and phosphocattier protein (HPr), as well as a substrate specific enzyme complex called Enzyme II (Barabote and Saier, 2005). Nevertheless, not all carbohydrates are accumulated in this way, and in common with mechanisms of other bacteria, uptake of some carbohydrates appears to be driven by ATP hydrolysis (ATP-binding cassette transporters) or by ion gradients (H^+ or Na^+ symporters) (Lee et al., 2005). It is important to know how transport is energized and regulated, whether there is an alternative transport system in *C. tyrobutyricum*, and how they change in the presence of adaptive evolution. This article represents an initial approach to these questions and the findings herein will be helpful for further understanding of the control mechanism of glucose assimilation in this organism.

Materials and Methods

Organism and Culture Conditions

C. tyrobutyricum wide-type (ATCC 25755) was purchased from Guangdong culture collection center (Collection number: GIM 1.262), and stored in reinforced clostridial medium (Huang and Yang, 1998). A detailed description of the working cultures has been given elsewhere (Jiang et al., 2010b), except for the initial glucose concentration being 150 g/L. The samples were incubated at 37°C in serum bottles.

Bioreactor Setup and Evolution Culture

The fermentation system consisted of a 5-L stirred-tank fermentor (B. Braun, B. Braun Biotech International, Melsungen, Germany) connected to a 0.5-L fibrous bed bioreactor. Details about the setup of the bioreactor have been given elsewhere (Jiang et al., 2009). To adapt the culture to tolerate high concentrations of glucose and butyric acid, and to improve butyric acid volumetric productivity, the reactor was operated at repeated-batch fermentation mode whenever the sugar level in the fermentation broth was close to zero. After nearly 130-day fermentation, the adapted cells in the FBB were removed from the fibrous matrix by vortexing the matrix in sterile distilled water under anaerobic conditions and subcultured in serum bottles for further analysis.

Preparation of Toluene-Treated Cells

Cells were harvested (40 mL) during the logarithmic phase (optical density, approximately 2.0) by centrifugation (12,000*g*, 10 min, 4°C) and washed once with 100 mM sodium–potassium phosphate buffer (pH 7.2) containing 5 mM $MgCl_2$. The cells were suspended in 10 mL of the same buffer and stored on ice. An aliquot (1 mL) of each cell suspension was treated with 30 μL of a toluene–ethanol mixture (1:9, v/v), as previously described (Kornberg and Reeves, 1972).

Preparation of Cell-Free Extracts

Cells from 400 mL of culture, harvested as described above, were sonicated for 30 min (Ningbo Scientz Biotech Co., Ningbo, China; JY92-II; microtip; 30% duty cycle; 0°C). The

unbroken cell debris was eliminated by centrifugation (37,000*g*, 20 min, 4°C), and the supernatant was used as the source of the crude extract. The crude extracts were prepared and fractionated into the soluble and membrane components by ultracentrifugation, as described previously by Mitchell and Booth (1984). The membrane fractions were washed with buffer and concentrated by dialysis using dialysis bags. The soluble extracts were fractionated on a Sephadex G-100 column (2.5 cm × 80 cm) at 4°C at a flow rate of 18 mL/h. The fractions (5 mL) were collected and assayed for the presence of PTS components, as described below. Both the soluble and membrane fractions were used immediately or stored at −80°C. The protein concentrations in the active pools were measured with a Bio-Rad Protein Assay Kit (micro-assay) with bovine gamma globulin as the standard.

Partially purified HPr for SDS–PAGE electrophoresis was prepared following standard protocol (Bio-Rad, Shanghai, China), and a blot of the gel on a polyvinylidene difluoride membrane was used for N-terminal sequencing of 18 amino acids in His-15 section at Sangon Biotech (Shanghai, China) Co., Ltd. ATP-dependent phosphorylation of partially purified HPr (Ser-46) was carried out in the presence of [γ-^{32}P]ATP and *Bacillus* HPr kinase as described by Galinier et al. (1997). A reaction mixture without HPr was used as a negative control, and *Bacillus* HPr was used as a positive control.

Glucose Phosphorylation Assay

The method of Kornberg and Reeves was used to measure PEP- and ATP-dependent glucose phosphorylation in *C. tyrobutyricum* (1972). The reaction mixture (1.0 mL) contained 100 mM sodium–potassium phosphate buffer (pH 7.2), 0.5 mM $MgCl_2$, 0.5 mM PEP or 0.5 mM ATP, 0.1 mM dithiothreitol (DTT), 0.04 mM β-NADH, 0.3 μg (2 U) of lactate dehydrogenase, and 200 μL of toluene-treated cells. The reaction was initiated by the addition of 0.5 mM sugar substrate, and the decrease in A_{340} was monitored for 4–5 min at 37°C with a recording spectrophotometer equipped with a thermostatically controlled cell compartment and an automatic sample changer. A similar cuvette containing the reaction mixture to which the sugar substrate was not added was used as the control. Endogenous phosphorylation was estimated from a control lacking PEP or ATP. The molar extinction coefficient of NADH was taken to be 6.22×10^3 L/(mol cm).

The glucokinase activity in the cell-free extracts of *C. tyrobutyricum* was assayed by the spectrophotometric method of Martin and Russell (1986). The formation of glucose-6-phosphate was coupled to $NADP^+$ reduction by glucose-6-phosphate dehydrogenase. The reaction mixture (1.0 mL) contained 100 mM sodium–potassium phosphate buffer (pH 7.2), 0.5 mM $MgCl_2$, 0.1 mM DTT, 1 mM ATP, 0.5 mM D-glucose, 0.08 mM $NADP^+$, and 6.4 U of glucose-6-phosphate dehydrogenase. The inhibitory effects of 2-deoxyglucose (2-DG) and mannose on PTS and glucokinase activity were also measured. All incubations and experiments were performed in triplicate and the variance is indicated by standard deviations.

Analysis Methods

Cell density was analyzed by measuring the optical density of the cell suspension at a wavelength of 600 nm (OD_{600}) with a spectrophotometer. One unit of OD_{600} corresponded to 0.683 g/L cell dry weight for cells grown in the glucose medium. Quantitative analysis of butyric acid and acetic acid was performed by gas chromatography (Agilent 6820 GE, Agilent Technologies, Shanghai, China). Osmolality of properly diluted, cell-free media samples was measured in a vapor pressure osmometer (VPO, Kanuer K-7000, Kanuer Instrument, Berlin, Germany). The specific rate of glucose consumption was estimated from changes in glucose concentration as a function of time and the biomass concentration.

Materials

[γ-^{32}P]ATP and Sephadex G-100 were purchased from Amersham Phamacia Biotech (Shanghai, China; Shanghai Ruiqi Biological Technology Co., Ltd., Shanghai, China). *Bacillus* HPr and HPr kinase were obtained from Zeigler DR (*Bacillus* Genetic Stock Center, USA). 2-DG, ATP, PEP, β-NADH, $NADP^+$, glucose-6-phosphate dehydrogenase, and other reagents were obtained from Sigma Chemical Co. (Sigma-Aldrich. Inc., Berks, PA).

Results and Discussion

Adaptive Evolution in Repeated-Batch Fermentation

The selection of mode of fermentation may vary with respect to different processes. Batch fermentation was superior in most respects but the volumetric productivity. The purpose of this experiment was to allow bacteria to gradually adapt to the considerable amount of substrate microenvironment (~150 g/L glucose) to select for mutants with higher butyric acid volumetric productivity in repeated-batch FBB fermentation. As can be seen in Figure 1, the immobilized-cell fermentation in the FBB exhibited a higher productivity 0.64 g/(L h) even in the first batch mode (Fig. 1b), which was 1.8-fold (vs. 0.35 ± 0.01 g/(L h)) greater than that from the fed-batch fermentation with the highest butyric acid concentration as mentioned before (also see Jiang et al., 2011). Batch fermentation experiments were by repeated when the glucose in the fermentation broth was exhausted. After the 60 batches fermentation lasting for 130 days, the butyric acid volumetric productivity finally reached 2.25 g/(L h), together with a butyric acid concentration of 67.5 g/L and a yield of 0.45 g/g glucose (Fig. 1a, see detail in Fig. 1c), which to the best of our knowledge is the highest butyric acid volumetric productivity at the

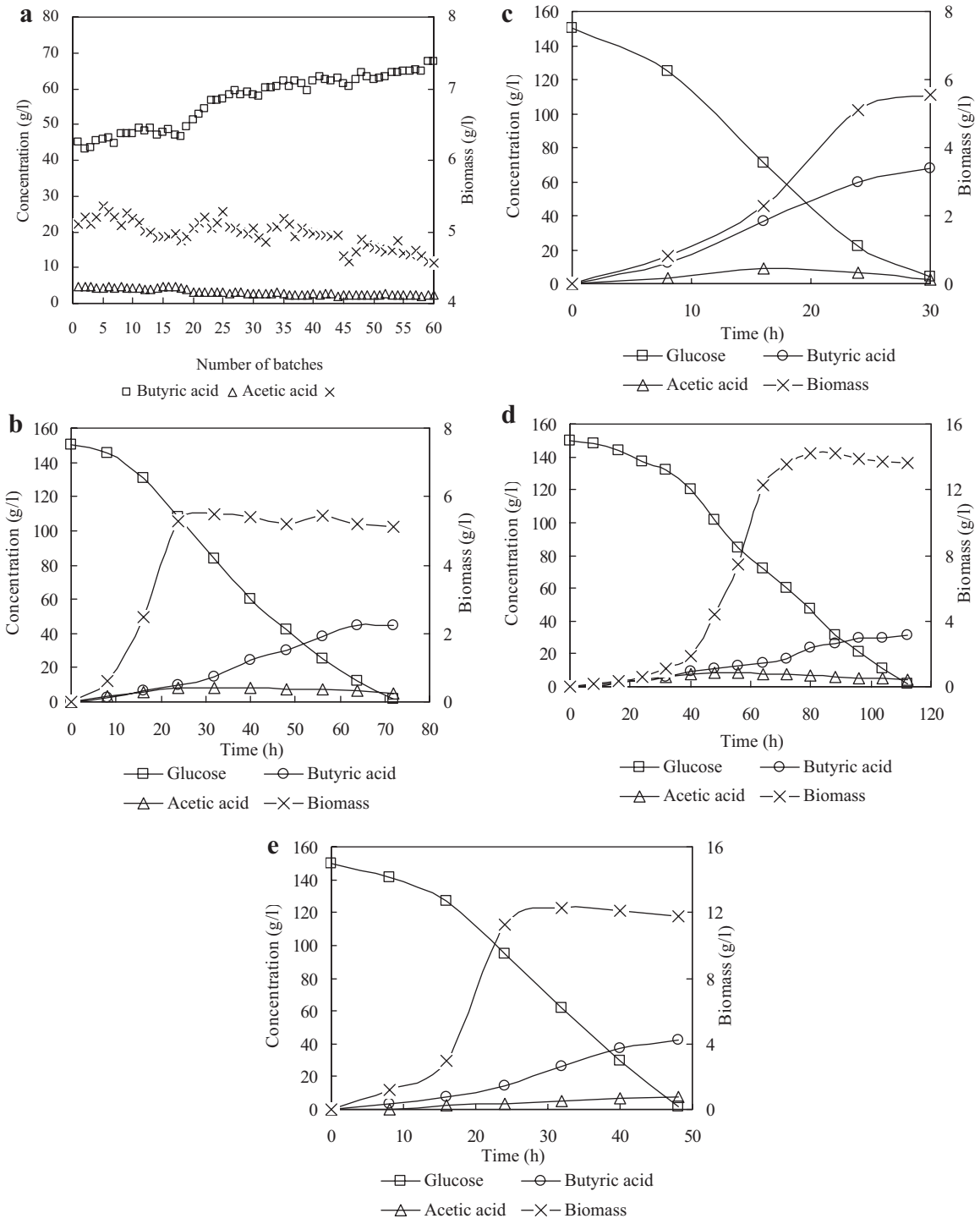

Figure 1. Summary of fermentation products and cell growth of *C. tyrobutyricum* in repeated-batch FBB fermentation during the adaptive evolution (**a**). Kinetic behaviors of butyric acid fermentation were also list in the order of the first batch (**b**) and the last batch (**c**) of repeated-batch FBB immobilized fermentation, and the wide-type strain (**d**) and mutant strain (**e**) in free-cell fermentation. Note that the biomass (g/L) in the figure represented the cell density in the fermentation broth.

comparative final concentration under identical fermentation conditions. In contrast, a productivity of 1.25 g/(L h) was obtained with a butyric acid concentration of 62.8 g/L by *C. tyrobutyricum* CIP I-776 in fed-batch fermentation feeding with a non-limiting supply of substrate (Fayolle et al., 1990). Song et al. (2010) also reported that 0.78 g/(L h) of butyric acid with the final concentration of 62.5 g/L was produced with glucose as the carbon source by

C. tyrobutyricum ATCC 25755 in batch fermentation. Furthermore, control experiments with both wide-type strain originally inoculated the fermentor and mutant strain selected from the last batch of repeated-batch FBB fermentation were also carried out in free-cell fermentation mode. From the fermentation kinetics of butyric acid production shown in Figure 1d and e, the mutant strain not only produced more concentration (42.0 g/L vs. 31.5 g/L) but also exhibited a much higher volumetric productivity of 0.88 g/(L h), which was three times more compared to the wide-type strain.

The possibility accounted for the higher productivity after the adaptation culture could be attributed to the high cell density in the FBB as well as immobilization-induced cellular or genetic modifications (Doran and Bailey, 2009; Suwannakham and Yang, 2005; Zhu and Yang, 2003). The total cell biomass in the FBB system herein harvested at the end of the sixtieth batch fermentation study was 33.5 g, which was equivalent to a high cell density of 67 g/L cells immobilized in the fibrous matrix. Similar high cell density of immobilization effect was also observed by our previous work (Jiang et al., 2011), with more than 72.8 g/L cell biomass maintained in the FBB at the end of the last fed-batch fermentation. The decreased value of cell biomass (67 g/L vs. 72.8 g/L) would attribute to the substrate inhibition on cell growth. A relevant characteristic of the media formulated with high initial glucose concentration was the intrinsic high permeability due to the elevated amount of media components. Osmolality of cultures at initial glucose concentration as high as 150 g/L was very high, reaching values of closed to 2.0 Osm/kg in the most concentrated media, which have been reported to inhibit growth of saccharolytic clostridia (Walter et al., 1987) and can therefore be a cause for the decreased growth rate observed here. Nevertheless, the liquid environment near immobilized cells was considered to be sufficiently different from that experienced by suspended cells to account for any changes in observed cell properties. Cells grown in the fibrous matrix are more robust and have capacity to tolerate higher substrate and product inhibition, which otherwise could be dramatically affected by the high concentration of inhibitory products, such as organic acids, amino acids, alcohols, in suspension culture (Lee et al., 2008; Wu and Yang, 2003; Zhang and Yang, 2009). Immobilization mimics what occurs in nature when cells adsorb and grow on surfaces or in natural structures. It is spontaneous for cells to modify their pattern of growth and replication as a result of direct physical and chemical interactions with support surface and other cells. Immobilization also results in the change of physicochemical properties of the microenvironment, including the presence of ionic charges, reduced osmotic pressure, altered water activity, modified surface tension, and cell confinement (Patil et al., 2006). Growth under these situations can induce evolutional responses at the metabolic even genetic level that cause fundamental changes in bacteria, which could not be achieved in free-cell fermentation.

However, the significantly improved productivity did not only result from a simple increase of the biomass, but also implied the enhanced glucose assimilation after adaptive evolution. It is reported that immobilized cells and suspended cells show different fermentation behaviors, such as lag time (Huang et al., 1998), growth rate (Farmakis et al., 2007), and cell metabolism (Hilge-Rotmann and Rehm, 1990), which by all means have the subtle relationship with the sugar uptake and consumption. As shown in Figure 2, the glucose consumption rate was increased in the proportion of butyric acid volumetric productivity as well as the decrease of residual sugar concentration with the long-term adaptation process. During the initial 20 batches of the culture, the glucose consumption rate remained constant before increasing progressively, which was in correspond with the sharp increase of butyric acid production (Fig. 1a). Although several phenomena could potentially explain this metabolic profile, the results obtained above prompted us to examine whether this increased activity could be correlated to the enhanced glucose transport system, which is probably a key rate-limiting reaction in an earlier report (Gourdon et al., 2003). Moreover, the adapted cells from the FBB maintained their distinct phenotype even after being successively subculture in serum bottles as suspension cultures, indicating a mutant with improved glucose tolerance and fermentative ability resulted from the adaptive evolution. Further experiments were thus conducted to study the underlying changes or causes with respect to the substrate uptake system between wide-type of *C. tyrobutyricum* and mutant strain.

Glucose Uptake and Consumption

Since the PEP-dependent sugar PTS is employed to transport sugars in clostridia (Mitchell et al., 2007;

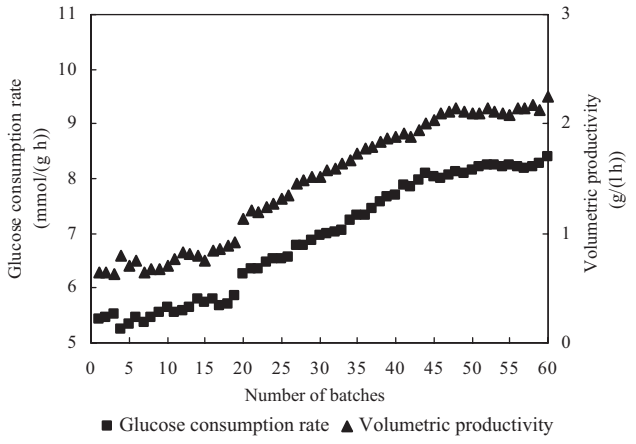

Figure 2. Summary of glucose consumption rate and butyric acid volumetric productivity of *C. tyrobutyricum* in repeated-batch FBB fermentation during the adaptive evolution.

Tangney and Mitchell, 2007), and the sucrose PTS in *C. tyrobutyricum* has been reported by our previous study (Jiang et al., 2010b), the effects of mannose and 2-DG on the glucose uptake and consumption by intact cells of both strains were first investigated and the results were listed in Table I. The specific rate of glucose consumption for both cells was inhibited in the presence of mannose or 2-DG, which are known PTS substrates (Mitchell, 1998). The inhibition of glucose uptake and consumption in the presence of 2-DG suggests the involvement of glucose PTS in glucose transport in both *C. tyrobutyricum* wide-type and mutant. However, glucose consumption in wide-type strain was strongly inhibited by non-metabolizable glucose analogue 2-DG, whereas the inhibition was less severe in mutant, which indicated that the glucose may be transported via an alternative transport mechanism in this strain.

In fact, the presence of multiple glucose transport systems is a common feature in many organisms. For instance, the Gram-negative model bacterium *Escherichia coli* possesses at least a glucose-PTS, a mannose-PTS, a proton symporter and an ABC transporter to transport glucose (Gosset, 2005), and expression of the genes encoding these transporters is influenced by several factors, such as the nature and the concentration of sugar (Vanderpool and Gottesman, 2004). The low-GC Gram-positive *Lactococcus lactis* internalizes glucose by a mannose-PTS, a cellobiose-PTS, and the novel GlcU during vegetative growth (Castro et al., 2009).

PEP-Dependent Glucose PTS

The following experiments were therefore carried out to ascertain the general glucose PTS in *C. tyrobutyricum* and to investigate the difference between *C. tyrobutyricum* wide-type and mutant. Toluene-treated cells of *C. tyrobutyricum* were used to determine the inducibility of the glucose PTS. As shown in Table II, the phosphorylation of glucose was detected with PEP as the phosphoryl donor when the cells were grown on glucose, fructose, and glucitol, respectively, implying that glucose phosphorylation is constitutive. In addition, the PEP-dependent PTS activity for glucose phosphorylation of glucose-grown *C. tyrobutyricum* mutant was 70% of that observed for glucose-grown wide-type strain. Defective glucose PTS activity in the mutant is consistent with the decreased inhibition of glucose consumption in the presence of 2-DG compared with the wide-type (Table I).

Because the sucrose PTS in *C. tyrobutyricum* is composed of both soluble and membrane-bound substrate-specific proteins, the architecture of the glucose PTS in this bacterium was investigated with a similar methodology (Jiang et al., 2010b). Crude extracts were prepared from cultures grown on glucose as the sole carbon source, and the membrane and soluble fractions of crude extracts were separated and used in glucose phosphorylation assays. The effect of different combinations of soluble extracts and membrane fractions on glucose PTS activity is shown in Figure 3. The separation of the extracts into membrane and soluble fractions resulted in the loss of activity, which was restored by recombining the two preparations, clearly demonstrating that the PEP-dependent phosphorylation of glucose is dependent on both fractions of the extracts in *C. tyrobutyricum*. The recovered PTS activity from a combination of membrane and soluble fractions derived from *C. tyrobutyricum* was lower (Fig. 3) than the PTS activity observed for cell extracts shown in Table II. This may be due to the loss of enzyme activity during the separation of membrane fractions and soluble extracts. However, a combination of membrane fractions and soluble extracts derived from mutant strain demonstrated lower PTS activity (~70%) than membrane and soluble fractions derived from wide-type. This result is consistent with the results obtained from the glucose PTS assay with cell extracts (Table II). Four different combinations of soluble extracts and membrane fractions derived from *C. tyrobutyricum* were used for the glucose phosphorylation assay (Fig. 3). The combination of soluble extracts and membrane fractions derived from the wide-type strain demonstrated the highest

Table I. Effects of mannose and 2-DG on the specific rate of glucose consumption by intact cells of *C. tyrobutyricum* wide-type and mutant.*

	Glucose consumption rate (mmol/(g h))		
Strain	Control[a]	Mannose	2-DG
Wide-type	5.44 ± 0.12	4.08 ± 0.10	3.38 ± 0.07
Mutant	8.41 ± 0.24	8.11 ± 0.13	6.82 ± 0.11

*The specific rate of glucose consumption was measured by adding 60 g/L glucose in the presence of 1 M mannose and 2-DG.
[a]The control experiment is defined as the glucose consumption rate in the absence of other sugars.

Table II. Glucose phosphorylation with toluene-treated cells derived from *C. tyrobutyricum*.

	Glucose phosphorylated (nmol of NADH oxidized/mg protein per min)			
	With PEP as the phosphoryl donor		With ATP as the phosphoryl donor	
Growth substrate	Wide-type	Mutant	Wide-type	Mutant
Glucose	5.51 ± 0.52	3.86 ± 0.67	3.35 ± 0.65	8.32 ± 0.97
Fructose	4.53 ± 0.47	2.42 ± 0.39	1.89 ± 0.43	4.21 ± 0.73
Mannose	3.97 ± 0.42	1.56 ± 0.57	1.51 ± 0.54	3.62 ± 0.68
Glucitol	3.06 ± 0.31	1.25 ± 0.29	1.18 ± 0.33	2.52 ± 0.42

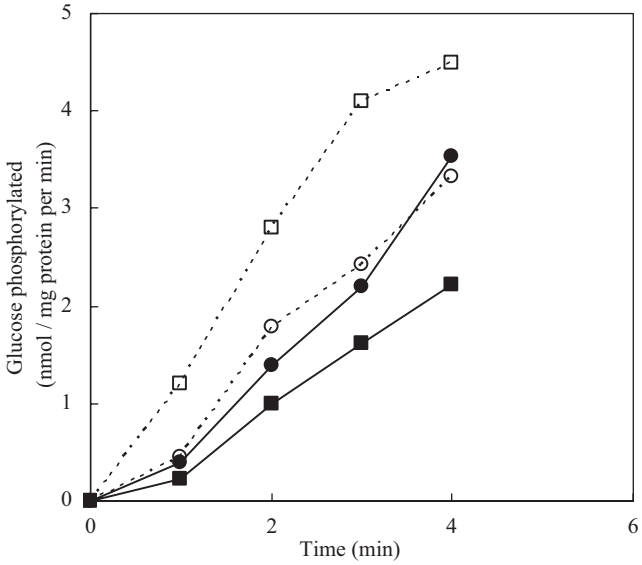

Figure 3. Effect of different combinations of membrane fractions and soluble extracts derived from *C. tyrobutyricum* wide-type and mutant on glucose PTS activity. Symbols: □, soluble extract and membrane fraction of wide-type (combination 1); ○, soluble extract of wide-type and membrane fraction of mutant (combination 2); ●, membrane fraction of wide-type and soluble extract of mutant (combination 3); ■, soluble extract and membrane fraction of mutant (combination 4).

PTS activity (combination 1), while the lowest PTS activity was observed for the combination of membrane and solution fractions derived from the mutant strain (combination 4). PTS activities observed for combinations 2 and 3 (extracts and membrane fractions from wide-type and mutant) were intermediate with respect to the PTS activities observed for combinations 1 and 4. These results suggest that soluble extracts and membrane fractions from both *C. tyrobutyricum* strains are complementary to each other and that extracts derived from the mutant contain PTS components whose activities are lower than those associated with wide-type. It is likely that the decreased activity of PTS components present in both the soluble extracts and membrane fractions in *C. tyrobutyricum* mutant is responsible for the decreased PTS activity.

Properties of PTS Component HPr

To further examine whether the soluble extracts from *C. tyrobutyricum* contained the typical soluble PTS protein HPr, which may be a key element in regulation of carbohydrate metabolism in clostridia as it is in other bacteria (Barabote and Saier, 2005), the soluble protein fraction from glucose-grown cells was concentrated and subjected to gel chromatography on a Sephadex G-100 column, which effectively separates proteins according to large differences in their molecular weights. The protein fractionation patterns of soluble extracts from *C. tyrobutyricum* wide-type and mutant are nearly identical. When the eluate was tested for its ability to complement membranes in a PEP-dependent glucose phosphorylation assay, a single peak of activity was observed in both wide-type and mutant at the similar positions (Fig. 4). Calibration of the column with bovine serum albumin (68,000), ovalbumin (43,000), and carbonic anhydrase (29,000) as markers allowed us to estimate the molecular weights of the protein to be ~12,500, a value very similar to those of the corresponding soluble HPr proteins of *C. beijerinckii* and *C. acetobutyricum* (Lee and Blaschek, 2001; Martin and

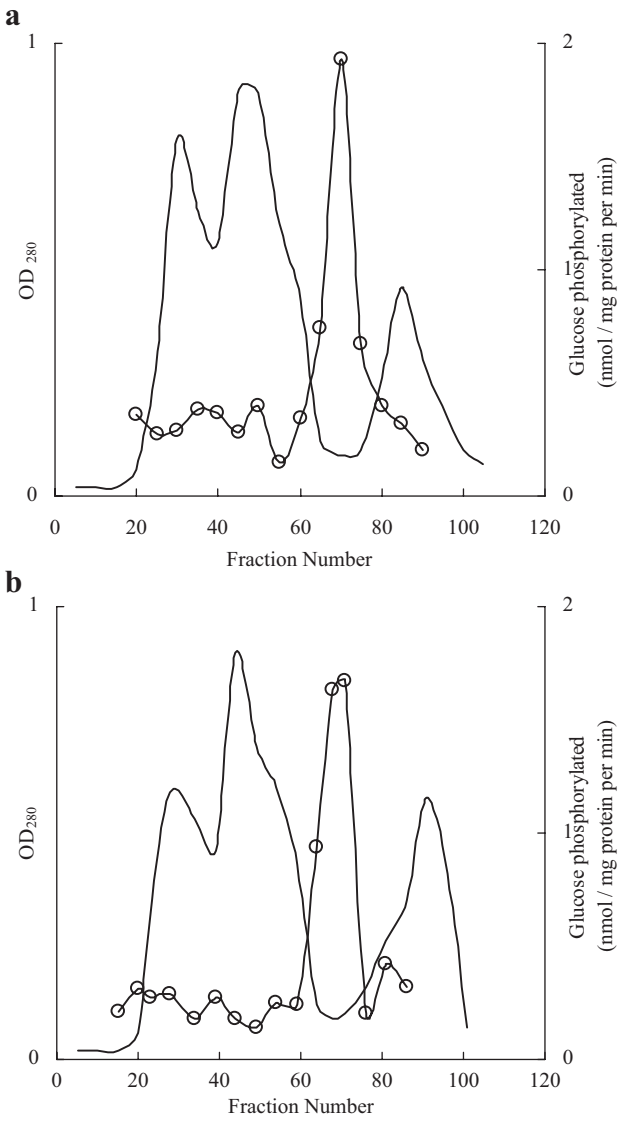

Figure 4. Fractionation of *C. tyrobutyricum* soluble proteins of wide-type (**a**) and mutant (**b**) by Sephadex G-100 gel chromatography. A 70-mg portion of soluble protein in a volume of 5 mL was applied to the column, and samples (500 μL) of eluate fractions were assayed for sugar phosphorylation activity in a volume of 1.0 mL in the presence of *C. tyrobutyricum* membranes (0.2 mg of protein; glucose as substrate) over a 10 min period. No PTS activity was observed in either soluble extracts or membrane fraction itself. Symbol: ○, glucose phosphorylated.

Russell, 1986; Mitchell et al., 1991). Therefore, at least superficially, the *C. tyrobutyricum* PTS appears to have a molecular architecture similar to that of previously characterized PTSs.

It is well-known that HPr is conserved among bacteria and processes two phosphorylation sites: His-15 and Ser-46 (Barabote and Saier, 2005). Additional characterization of partially purified HPr from both *C. tyrobutyricum* wide-type and mutant was then carried out by SDS–PAGE. In the denatured SDS–gel, HPr proteins derived from both *C. tyrobutyricum* wide-type and mutant were the same size. A blot of the gel was used for the N-terminal sequence analysis of HPr. It can be concluded that the sequences of His-15 region (HARP) of HPr derived from *C. tyrobutyricum* are highly homologous compared with other clostridial HPrs (Fig. 5a). The amino acid sequences of HPr protein purified from *C. tyrobutyricum* wide-type and mutant were found to be identical. This indicates that there is no mutation in the catalytic region (His-15) of the HPr protein in *C. tyrobutyricum* mutant so that phosphate transfer between PTS components may not be changed. In vitro phosphorylation of partially purified HPr was used to examine an alteration of another phosphorylation site (Ser-46) by using HPr kinase purified from *Bacillus* and ^{32}P-labeled ATP as described by Galinier et al. (1997). As can be seen in Figure 5b, essentially the same levels of phosphorylation by [γ-^{32}P]ATP in Ser-46 section were observed for *Bacillus* HPr and partially purified clostridial HPrs. HPr from *C. tyrobutyricum* mutant was phosphorylated by the ATP-dependent HPr kinase at the same level as HPr derived from *C. tyrobutyricum* wide-type. HPr protein derived from the mutant did not demonstrate any difference in either amino acid sequence of the catalytic site (His-15) or in activity of the regulatory site (Ser-46) relative to HPr protein derived from wide-type strain. These results suggest that either the aforesaid two phosphorylation residues of HPr from the mutant can be phosphorylated or dephosphorylated for PTS activity and regulation at the same rate as the HPr from the wide-type. Based on the observations, it is likely that *C. tyrobutyricum* mutant may have a mutation upstream of the *pts* gene or in a regulatory region for *pts* gene expression, possibly at the transcriptional level similar to other bacterial HPrs, which results in the defective properties of glucose PTS (Barabote and Saier, 2005).

ATP-Dependent Glucose Transport System

Although a PTS defect in *C. tyrobutyricum* mutant is in accordance with a 2-DG-resistant phenotype as well as the

a

C. tyrobutyricum Wide-type	M	I	Q	K	E	F	V	V	K	N	S	T	G	L	H	A	R	P
C. tyrobutyricum mutant	M	I	Q	K	E	F	V	V	K	N	S	T	G	L	H	A	R	P
C. botulinum ATCC 3502	M	L	E	R	L	E	T	V	N	S	S	T	G	L	H	A	R	P
C. cellulovorans 743B	M	V	N	K	E	V	V	V	V	S	E	T	G	L	H	A	R	P
C. saccharolyticum WM1	M	V	S	A	K	V	V	I	K	N	P	T	G	I	H	L	R	P
C. acetobutylicum ATCC824	M	V	T	K	S	V	V	V	K	S	S	T	G	L	H	A	R	P
C. perfringens	M	V	T	K	E	V	I	V	K	N	S	T	G	L	H	A	R	P
C. beijerinckii 8052	M	I	A	K	E	V	T	V	K	N	S	S	G	L	H	A	R	P
C. phytofermentans ISDg	M	I	S	K	K	I	V	I	K	I	P	T	G	L	F	A	R	P

b

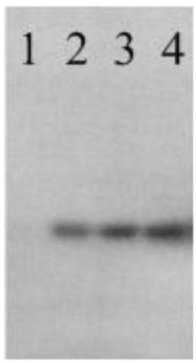

Figure 5. Analysis of two conserved regions in the HPr protein. **a**: Alignment of amino acid sequences around the His-15 residue in HPr of *C. tyrobutyricum* and other clostridia. **b**: In vitro phosphorylation of HPr (Ser-46). [γ-^{32}P]ATP and *Bacillus* HPr kinase were incubated with semipurified HPr from *C. tyrobutyricum* wide-type and mutant. Lane 1, negative control without HPr; lanes 2, partially purified HPr from *C. tyrobutyricum* wide-type; lanes 3, semipurified HPr from *C. tyrobutyricum* mutant; lanes 4, *Bacillus* HPr.

decreased inhibition of glucose uptake in the presence of 2-DG compared to the wide-type, a PTS defect in mutant could not readily explain why this strain carries out a much more rapid glucose uptake and consumption after adaptive evolution. In other words, *C. tyrobutyricum* cells possess more glucose transport capacity than that indicated by the in vitro glucose PTS assay alone. As a matter of fact, the similar observation has been made for solvent-hyperproducing mutant *C. beijerinckii* BA101 generated by using 2-DG, which resulted in improved glucose utilization and involved in an ATP-dependent glucose uptake system (Annous and Blaschek, 1991). From Table II, it was observed that glucose phosphorylation by toluene-treated cells of *C. tyrobutyricum* wide-type and mutant harvested in the logarithmic phase was also dependent on the presence of ATP. Because it was impossible to exclude the possibility of ATP production, the phosphorylation of the 2-DG was examined with cells grown on glucose. *C. tyrobutyricum* displayed higher activity with PEP than with ATP in both strains (2.06 vs. 0.18 for wide-type strain and 1.45 vs. 0.41 for mutant strain, respectively, unit: nmol of NADH oxidized/mg protein per min), and the values were much higher than the endogenous activity (no PEP or ATP added). Furthermore, when ATP was added to the soluble fractions of cell-free extracts derived from *C. tyrobutyricum* wide-type and mutant, the 2-DG was phosphorylated (11.58 pmol vs. 19.32 pmol of NADH oxidized/mg protein per min, respectively), while it was negligible with PEP (0.25 pmol vs. 0.15 pmol of NADH oxidized/mg protein per min, respectively). The endogenous phosphorylation by the cell extracts was minimal (<10%). Therefore, it was concluded that *C. tyrobutyricum* contains a soluble ATP-dependent kinase capable of phosphorylating 2-DG.

To ascertain whether glucokinase (50,000 Da) or hexokinase (100,000 Da) is involved in this glucose phosphorylation with ATP as a phosphoryl donor (Lunin et al., 2004), cell-free extracts of *C. tyrobutyricum* wide-type and mutant were separated on a Sephadex G-100 column previously calibrated with standard molecular weight protein markers, and the kinases of both organisms were found in the similar ~48,500-Da fraction. The addition of glucose-6-phosphate, a known inhibitor of hexokinases, did not inhibit glucose phosphorylation, which further indicated the presence of a glucokinase rather than a hexokinase in *C. tyrobutyricum* (data not shown).

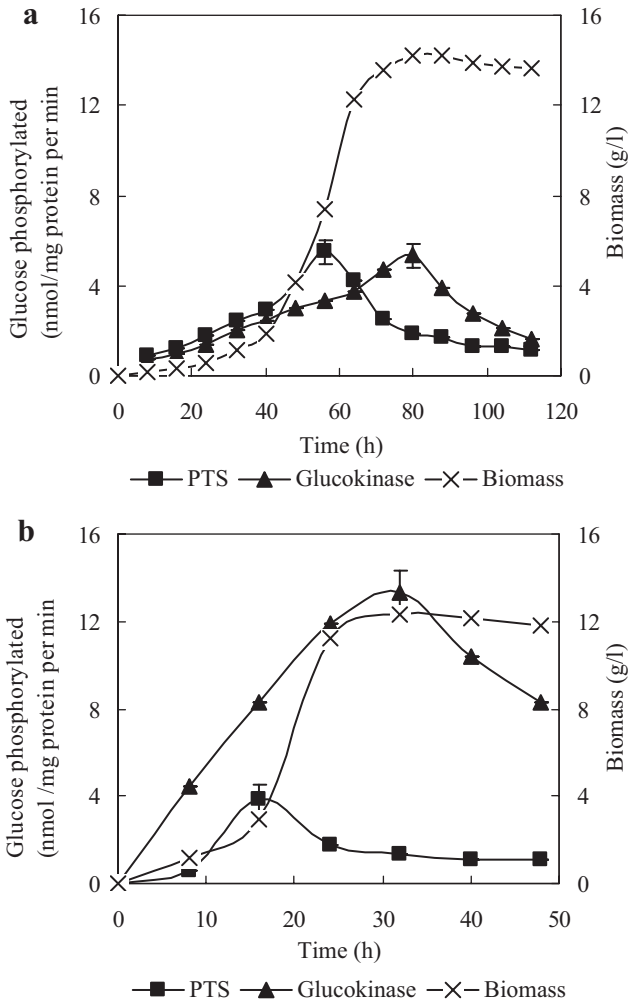

Figure 6. Glucose phosphorylation by toluene-treated cells of *C. tyrobutyricum* wide-type (**a**) and mutant (**b**) in different growth stages. Phosphorylation rates in the presence of PEP (■) and ATP (▲) are shown relative to the time at which samples were removed from the culture.

Expression of PTS and Glucokinase Activity

In order to investigate the increased utilization of glucose in *C. tyrobutyricum* more carefully, glucose phosphorylation in toluene-treated cells harvested at different point during the free-cell fermentation in a 5-L fermentor with the initial glucose concentration of 150 g/L was measured. Cell density in the culture was measured in order to identify the times of onset of cell multiplication until the glucose was exhausted. As shown in Figure 6, mutant strain grown much faster than the wide-type with a bit lower maximum biomass. PTS activities associated with toluene-treated cells of both *C. tyrobutyricum* wide-type and mutant were highest in the middle of the logarithmic phase and decreased thereafter, and the activity of mutant strain was ~30% lower than that of wide-type, consistent with aforesaid results in Table II. On the other hand, glucokinase activity, as measured by ATP-dependent phosphorylation, increased with cells grown, reaching a peak in the stationary phase, and subsequently declined. It is interesting that in both *C. tyrobutyricum* wide-type and mutant, an increased level of glucokinase activity was detected during the growth stage when the PTS activity had decreased. This implies that glucose transported into the cell is phosphorylation by either the PTS or glucokinase, with the contribution of each being dependent upon the physiological state of the cells. The

contribution of the PTS to glucose uptake is most significant in the growth-phase cells of both strains, while cells in stationary phase display lowered PTS activity and therefore show greater reliance on a non-PTS route of glucose uptake and phosphorylation. This inverse relationship between PTS activity and glucokinase activity at various growth stages implies that PTS may be involved in the regulation of glucokinase and an alternative glucose transport system.

Conclusions

In this study, a process engineering approach was developed to obtain a mutant with significantly improved volumetric productivity of butyric acid at high initial substrate concentration. It is possible that the microenvironment in the FBB with high glucose and butyric acid concentrations during long-time adaptive evolution has caused the observed physiological changes of the wild-type *C. tyrobutyricum* and eventually resulted in a mutant with improved glucose tolerance and fermentative ability. We have also presented evidence of the glucose uptake and metabolism by a PTS and an alternative transport route that requires glucokinase in *C. tyrobutyricum* and defined their general properties by taking the enzymatic and biochemical approach, which to the best of our knowledge have not been previously reported for acidogenic clostridia. As regards the greater acid formation and more efficient glucose utilization by *C. tyrobutyricum* mutant relative to the wide-type, it is tempting to conclude that these characteristics may be due to differences in cellular metabolism as well as the gene regulation, resulting in an enhanced glucokinase activity which more than compensates for the PTS defect of this strain. To obtain a deeper profile of the glucose assimilation in *C. tyrobutyricum*, a series of genetic experiments should be performed in further trials.

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