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Confirmation of Glucose Transporters through Targeted Mutagenesis and Transcriptional Analysis in *Clostridium acetobutylicum*

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Abstract: The solvent-producing bacterium *Clostridium acetobutylicum* is able to grow on a variety of carbohydrates. The main hexose transport system is the phosphoenolpyruvate-dependent phosphotransferase system (PTS). When the gene *glcG* that encodes the glucose transporter was inactivated, the resulting mutant *glcG*::int(1224) grew as well as the wild type, yet its glucose consumption was reduced by 17% in a batch fermentation. Transcriptomics analysis of the phosphate-limited continuous cultures showed that the cellobiose transporter GlcCE was highly up-regulated in the mutant *glcG*::int(1224). The *glcCE* mutation did not affect growth and even consumed slightly more glucose during solventogenesis growth compared to wild type, indicating that GlcG is the primary glucose-specific PTS. Poor growth of the double mutant *glcG*::int(1224)-*glcCE*::int(193) further revealed that GlcCE was the secondary glucose PTS and that there must be other PTSs capable of glucose uptake. The observations obtained in this study provided a promising foundation to understand glucose transport in *C. acetobutylicum*.

1. Introduction

Clostridium acetobutylicum is able to grow on a variety of carbohydrates and thus encodes a diverse set of metabolic enzymes and transporters for hexoses and pentoses uptake. Transcriptional analysis of differential carbohydrate utilization by *C. acetobutylicum* unveiled that the hexoses were primarily taken up by the phosphoenolpyruvate phosphotransferase systems (PTS) [1,2]. A typical PTS contains two soluble components, enzyme I (EI), the histidine-containing protein (HPr) and a membrane-bound protein enzyme II (EII).

The EII complex incorporates three domains, termed IIA, IIB and IIC, in which the IIC is integral membrane protein to translocate carbohydrate into cytoplasm [3]. They are mainly responsible for the absorption of a large number of carbohydrates (such as hexose, 6-deoxyhexose, amino sugar, N-acetylamino sugar, gluconic acid, pentitol, ascorbic acid and disaccharides) in the process of energy transportation and catalyze their conversion into their respective phosphate esters.

Nevertheless, the diffusion of other carbohydrates, mainly pentose, is driven by ATP hydrolysis or ion gradients [4,5]. Non-PTS could also be used to transport a small amount of glucose if all PTS were deficient [6]. PTS is not only a sugar transport system (which mediates the intake and phosphorylation of carbohydrates) but also has a very strong

regulatory capacity. PTS participates in the regulation of the central carbon and nitrogen metabolism [7–12], the regulation of metal ion homeostasis [13–15] and the regulation of bacterial virulence [16–18]. Increasingly, studies show that PTS is closely related to the stress response [19–22].

Bioinformatic analysis of the genome sequence of *C. acetobutylicum* ATCC 824, which was published in 2001, identified 13 putative PTS EII enzymes in this strain [1,23]. Although the family of these 13 putative PTS EII domains were assigned by phylogenetic analysis and the probable substrates were inferred, a considerable amount of functional characters were still required. For glucose uptake, it was predicted that the *glcG* gene (CAC0570) coded for a glucose transporter in *C. acetobutylicum*. However, after *glcG* was inactivated, the mutant fermented glucose as efficiently as the parent strain in a batch culture [6,24]. This result suggested that an additional glucose transporter exists in this organism.

Despite its importance, the lack of basic information of glucose uptake systems in *C. acetobutylicum* has limited the understanding of glucose assimilation in this strain. In contrast, glucose uptake in *Escherichia coli* had been studied extensively. There is a main glucose transporter that is very efficient for glucose uptake, and the maltose, mannose and galactose transport systems are also able to transport glucose into the cytoplasm [25–27]. Therefore, it of interest to investigate whether a similar glucose transport mechanism exists in *C. acetobutylicum*.

Moreover, a more detailed physiological analysis will further increase our appreciation of the importance of the PTS in this organism [28]. The complex metabolism of *C. acetobutylicum* is characterized by two sequential phases in a batch culture [29,30]. This culture grows fast during acidogenesis, and the main products are acetate and butyrate. Then, solventogenesis occurs as the growth rate slows down, the acids are re-assimilated, and the solvents (butanol, acetone and ethanol) become main products. The physiological process and metabolic change have been investigated extensively in batch fermentations [29,31–33].

However, some details cannot be observed easily due to the unstable growth rate, pH and glucose consumption of a batch culture. Phosphate-limited continuous fermentation (chemostat cultivation) of *C. acetobutylicum* makes it possible to keep the cells in a steady acidogenesis or solventogenesis simply by controlling the pH [34–36]. Since all the cells are identical in continuous culture, this is preferred to be used for 'omics' studies.

DNA microarray is a powerful tool to monitor the expression of all the genes of bacteria. It is commonly used to investigate bacterial metabolism and gene function [37]. In the present work, the ClosTron technology was used to inactivate the genes of glucose transporter candidates. The mutants and wild type strain were cultivated in batch and continuous cultures. Then, the growth phenotype and transcriptome analysis of the mutants were compared to the wild type, revealing a series of PTSs and their preference order in *C. acetobutylicum*.

2. Materials and Methods

2.1. Bacterial Strains and Generation of ClosTron Mutants

All bacterial strains and plasmids in this study are listed in Table S1. *C. acetobutylicum* ATCC 824 was stored as spore suspensions at 4 °C. Cultures were inoculated with spores in clostridial growth medium (CGM) [38] and heat-shocked at 80 °C for 10 min, and then the strain was cultured at 37 °C overnight as a pre-culture. Procedures requiring anaerobic conditions were performed in an anaerobic chamber (COY system, Grass Lake, MI, USA). Hungate tubes (Ochs GmbH, Bovenden, Germany), and serum bottles (Müller and Krempel AG, Bülach, Switzerland) were used for liquid cultivation in CGM.

Chromosomal genes of *C. acetobutylicum* were inactivated using ClosTron technology. The targeted genes in present study were *glcG* (encoding PTS EII and CAC0570) and *glcCE* (encoding PTS cellobios-specific component IIC and CAC0386). The *glcG* gene encodes three typical domains, IIA, IIB and IIC. The mutant *C. acetobutylicum glcG*::int(1224) was obtained using the ClosTron technology, and the insertion site was targeted to domain C (IIC). The gene *glcCE* is located in a putative cellobiose transporter operon (CAC0383-

CAC0386), and CAC0386 is annotated as domain C (IIC) in this operon. As the integral membrane protein, IIC takes charge of translocating the substrate to cytoplasm, which is the targeted insertion to make the mutant.

The procedure of construction and electroporation of the retargeted plasmid pMTL007C-E2 was as described previously [39–41]. For the double mutant (*glcG*::int(1224)-*glcCE*::int(193)), the mutant *glcG* was used as the parent strain. The retargeted plasmid pMTL007C-E2 of *glcCE* and the plasmid pAN2 were transformed to the mutant *C. acetobutylicum glcG*::int(1224), and the transformants were selected using the procedure described for ClosTron technology. The ClosTron mutants were verified by gene check primers listed in Table S1, and the correct insertion sites of intron were validated by Southern hybridization.

Southern hybridization was performed by digesting chromosomal DNA overnight with the *Hin*dIII and running on a 0.8% agarose gel. Transfer and hybridization was performed according to standard protocols as described previously with an intron-specific probe, which was generated from the PCR product using the primers intron II-F and intron II-R listed in Table S1 with pMTL007C-E2 plasmid DNA as the template [42]. In addition, the ClosTron mutants were stored at $-80\,^{\circ}$C in CGM containing 30% glycerol.

2.2. Batch and Continuous Fermentation Experiments

Batch fermentations were performed in minimal medium at $37\,^{\circ}$C using 50 g/L glucose or cellobiose as the carbon source. Two independent cultivations were performed for each strain (wild type or mutants), and the average value of the OD_{600} was used for the growth curve. The minimal medium was prepared as described previously in 1 L distilled water containing the following ingredients: glucose, 50 g; $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 1 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; NaCl, 0.01 g; $NaMoO_4 \cdot 2H_2O$, 0.01 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; $MnSO_4 \cdot H_2O$, 0.015 g; $FeSO_4 \cdot 7H_2O$, 0.015 g; biotin, 0.1 mg; thiamine hydrochloride, 2 mg; and *p*-aminobenzoic acid, 2 mg [35].

The phosphate-limited minimal medium is a modified minimal medium used in phosphate-limited continuous fermentation. Phosphate is the limiting factor; thus, there is no K_2HPO_4 and only 0.07 g/L KH_2PO_4 (0.5 mM) in the phosphate-limited minimal medium. The pH of the medium was adjusted to 2 using H_2SO_4, and the glucose concentration in this medium was 40 g/L [43]. The continuous fermentation experiment was performed in a one-liter Biostat B plus fermenter system (Sartorius BBI Systems, Milsungen, Germany) with 0.75 liter working volume.

For the continuous fermentation, the stains (wild type and mutants) were cultured in minimal medium for about 12 h in the fermenter, and then the fermenter was connected to the phosphate-limited minimal medium to start the continuous fermentation. The dilution rate was 0.075 h^{-1}, and the pH was adjusted automatically to 5.7 for acidogenic growth and 4.5 for solventogenic growth by adding 2 M KOH [36,44]. Cell pellets, for RNA isolation, were collected by centrifugation at $-20\,^{\circ}$C and 9000 rpm (Hettich Universal 320 R, Tuttlingen, Germany) for 15 min. Then, the pellets without supernatant were quickly frozen in liquid nitrogen and stored at $-80\,^{\circ}$C until RNA extraction.

2.3. Analytical Methods

Cell growth was determined by measuring the optical density at 600 nm in an Ultraspec 3300 *pro* UV/vis spectrophotometer (GE-Healthcare, Munich, Germany). The glucose in culture was determined by D-Glucose (GOPOD Format) Kit (Megazyme, Wicklow, Ireland).

2.4. DNA Microarray Experiments

The array contained 3840 oligonucleotides representing 99.8% of all annotated open reading frames (ORFs) in *C. acetobutylicum*, including all ORFs located on the megaplasmid (*pSOL*). Moreover, 13 putative PTS EII enzymes are encoded by 26 genes, which are all included in the array [1]. RNA isolation and DNAse treatment procedures were performed as previously described in detail [36]. For a microarray experiment in the present study,

RNA was isolated from wild type and the mutant cultivated at the same fermentation condition and labeled with different fluorescent dyes by reverse transcription.

Therefore, one RNA (DNA-free) sample of wild type was normally labeled with Cy-3 (GE Healthcare, Munich, Germany), and the other RNA sample of the mutant was labeled with Cy-5 (GE Healthcare, Munich, Germany). The labeled samples were then mixed and hybridized with the array. The cDNA labeling, hybridization, DNA chips preparation, data normalization (GenePix Pro 6.0 software, Axon Instruments, Seattle, WA, USA) and data analysis were described in detail previously [36,37]. The transcriptional data of expression ratios of all the 26 genes in PTS were reproduced in two independent biological experiments. The average of two expression ratios of one gene was the final ratio of this gene in the present study.

3. Results and Discussion

3.1. Generation and Verification of the Mutants C. acetobutylicum glcG::int(1224), glcCE::int(193) and glcG::int(1224)-glcCE::int(193)

To investigate the glucose transporters in *C. acetobutylicum*, *glcG* and *glcCE* were targeted for insertion inactivation using ClosTron mutagenesis as described previously [39,40]. ClosTron technology is a proven system to construct single gene knockouts in clostridia, especially in *C. acetobutylicum* [41,45]. To allow the construction of multiple ClosTron mutants in *C. acetobutylicum*, the *ermB* gene is used as a selection marker. It can be deliberately excised by FLP-recombinase, and thus the mutants lose the erythromycin resistance [39].

The length of *glcG* was 1998 bp, and the target site was designed to be located at 1224/1225 bp. The length of *glcCE* was 1353 bp, and the target site was designed to be located at 193/194 bp. The strains, plasmids and primers used are listed in Table S1. Putative mutants (*glcG*::int(1224) and *glcCE*::int(193)) were obtained by screening the CGM plates. Erythromycin-resistant clones (putative mutants) were verified by gene check primers (CAC0570F, CAC0570R and CAC0386F, CAC0386R) as listed in Table S1. The results showed that wild type genome DNA gave a band of ~300 bp, while the correct mutants exhibited products of ~2.1 kb, which were 1.8 kb longer than the wild type (Figure S1).

For the double mutant (*glcG*::int(1224)-*glcCE*::int(193)), the correct retargeted plasmids, pMTL007C-E2-*glcCE* (Table S1) was transformed into the mutant *C. acetobutylicum glcG*::int(1224). Since the parent strain, *C. acetobutylicum glcG*::int(1224) already contained the erythromycin marker, the concentration of erythromycin used for selection increased from 5 to 15 μg/μL. The colonies obtained on the selection plates that grew faster were inoculated into fresh CGM medium and cultured overnight at 37 °C. Then, these colonies were verified by PCR using gene check primers (CAC0570F, CAC0570R and CAC0386F, CAC0386R) as listed in Table S1. The correct mutants exhibited products of ~2.1 kb, which were 1.8 kb longer than those of the wild type (Figure S1).

To further investigate the insertion sites of the intron, the two 2.1 kb PCR products from genomic DNA of clones containing the insertion in *glcG* and *glcCE*, respectively, were sequenced. Analysis of the sequences proved that the intron inserted the *glcG* at 1224/1225 bp and the *glcCE* at 193/194 bp, respectively.

These results verified that *glcG* and *glcCE* were inactivated by insertion of the ClosTron-derived group II intron into the desired positions and the correct *glcG* and *glcCE* insertion mutants were obtained. To further confirm that only one copy of the intron existed in the chromosomal DNA of the mutants *C. acetobutylicum glcG*::int(1224) and *glcCE*::int(193), Southern hybridization was performed with an intron-specific probe (Table S1). For the double mutant (*glcG*::int(1224)-*glcCE*::int(193)), two copies of the intron were inserted into the chromosomal DNA, showing two bands on the nylon membrane (Figure S2).

No hybridization signal was observed for wild type genomic DNA, and genomic DNA of the *glcG*::int(1224) and *glcCE*::int(193) mutants both exhibited a single band, demonstrating that strains *glcG*::int(1224) and *glcCE*::int(193) possessed only one copy of the intron on the chromosome. Two bands appeared in the lane of the double mutant, with one the

same as that of the mutant *glcG*::int(1224) and the other identical to that of the mutant *glcCE*::int(193) (Figure S2), which verified that two copies of intron were found in the chromosomal DNA of the mutant *glcG*::int(1224)-*glcCE*::int(193) and that the two introns were inserted into *glcG* and *glcCE* genes, respectively.

3.2. The Phenotypes of Wild Type and the Mutant C. acetobutylicum glcG::int(1224) in Batch and Phosphate-Limited Continuous Fermentations

The mutant *C. acetobutylicum glcG*::int(1224) cultured in minimal medium with 60 g/L glucose exhibited similar growth phenotypes; however, the glucose consumption was reduced by ~17% compared to wild type (Figure 1), which led to an ~25% reduction in butanol production (Figure S3A,B). Under the same conditions, wild type and the mutant *glcG*::int(1224) achieved steady-state growth during acidogenic and solventogenic growth in the continuous fermentation (Figure 2). The optical density of the wild type was almost identical to that observed for the mutant *glcG*::int(1224) during acidogenic and solventogenic growth.
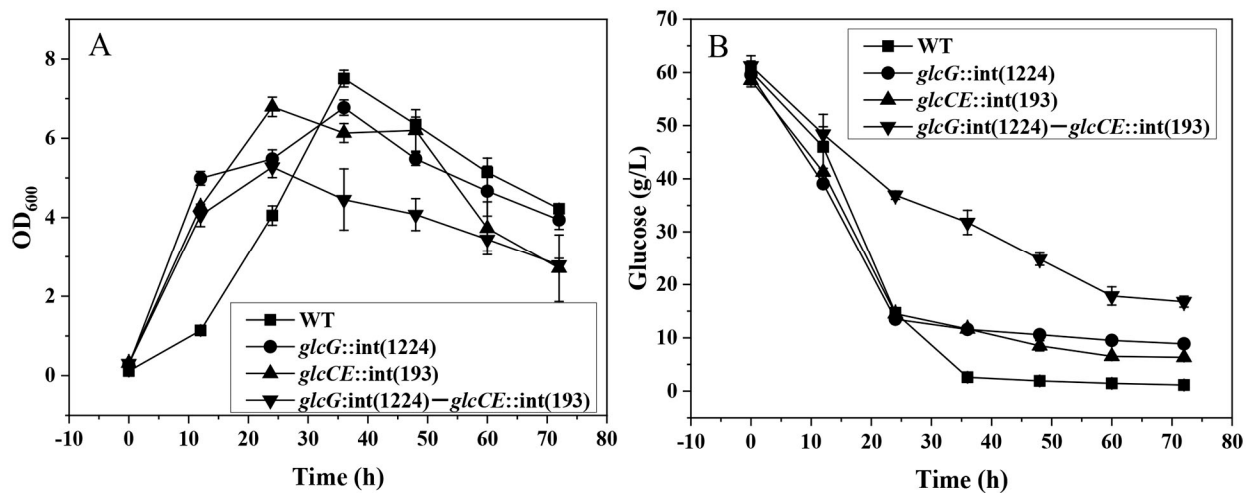

Figure 1. Growth curves (**A**) and residual glucose concentrations (**B**) of *C. acetobutylicum* wild type, mutants *glcG*::int(1224), *glcCE*::int(193) and *glcG*::int(1224)-*glcCE*::int(193) in minimal medium with glucose as the sole carbon source. (■), Wild type; (●), *glcG*::int(1224); (▲), *glcCE*::int(193); and (▼), *glcG*::int(1224)-*glcCE*::int(193).
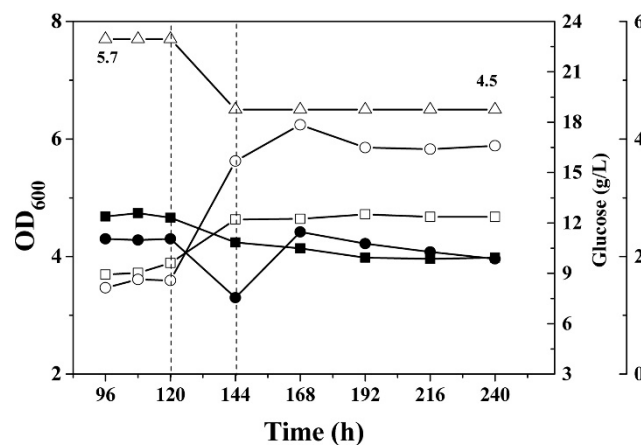

Figure 2. Growth curves and residual glucose concentration in phosphate-limited continuous culture of *C. acetobutylicum* wild type and the mutant *glcG*::int(1224). (△), pH; (■), OD_{600} of wild type; (●) OD_{600} of *glcG*::int(1224); (□), residual glucose during wild type cultivation; and (○), residue glucose during cultivation of *glcG*::int(1224). The dashed line indicates the initiation of dynamic pH shift from 5.7 to 4.5 in continuous fermentation.

There was 40 g/L glucose in the phosphate-limited continuous medium. Assays of the residual glucose concentration in the continuous culture indicated approximately 9 g/L glucose during acidogenic growth for the wild type and the mutant *glcG*::int(1224), while approximately 12 g/L glucose was determined for the wild type and 17.5 g/L glucose for the mutant *glcG*::int(1224) in the continuous culture during solventogenic growth.

Due to inactivation of the *glcG* gene, the rate of glucose uptake by the mutant *glcG*::int(1224) was lower than that of wild type; additionally, some previous studies showed the key role of GlcG in glucose transport [6,24,46], which suggested that GlcG (encoded by *glcG*, CAC0570) indeed acted as EII of PTS for glucose transport, that an alternative PTS can compensate the loss of *glcG* in *C. acetobutylicum*, and that some of them may be bifunctional, such as those in *E. coli*, *S. cerevisiae* and other microorganisms [26,27,47].

Moreover, the fermentation of *C. acetobutylicum* can be divided into two distinctive growth phases [30,33]. In most studies, the characterization of mutants is done in batch cultures [41,45]. However, this does not allow characterization of differences between wild type and mutants specific for acidogenic or solventogenic growth. Phosphate-limited continuous fermentations make it possible to compare the phenotypes of wild type and the mutants during two-phase growth [34–36].

In present work, the results showed that there was a great advantage in studying the glucose transporters in *C. acetobutylicum* using phosphate-limited continuous fermentation. The glucose consumption of wild type and the mutant *glcG*::int(1224) were identical in batch fermentations (Figure 1A), whereas they were clearly different during the solventogenic growth in phosphate-limited continuous fermentation (Figure 2). Due to the same growth rate of all the cells in the continuous culture, it was also important to analyze the expression profile differences caused by the mutation between the wild type and the mutant.

3.3. Transcription Analysis of the Mutant C. acetobutylicum glcG::int(1224) as Compared to Wild Type

To test whether another PTS can compensate for the inactivation of GlcG, DNA microarray experiments were conducted to detect changes in the transcription level of all the 13 PTS EII protein genes caused by the *glcG* mutation compared to wild type. Cells of steady-state acidogenic growth (t = 120 h) and solventogenic growth (t = 240 h) of the chemostat culture of the mutant *glcG*::int(1224) and wild type were used for microarray experiments (Figure 2). The microarray data of all the 13 PTS EII protein genes are listed in Table 1.

Table 1. Transcriptional profiles of all the PTS enzyme II protein genes of mutants *C. acetobutylicum glcG*::int(1224) and *glcCE*::int(193) compared to the wild type.

ORF	Protein	*glcG*::int(1224)		*glcCE*::int(193)	
		pH 5.7 t = 120 h	pH 4.5 t = 240 h	pH 5.7 t = 120 h	pH 4.5 t = 240 h
CA_P0066	PTS, mannose-specific IIAB component	—	—	—	—
CA_P0067	PTS, mannose/fructose-specific IIC component	—	—	—	—
CA_P0068	PTS, mannose-specific IID component	—	—	—	—
CAC0154	PTS, mannitol-specific IIBC component	1.34	1.10	1.01	1.05
CAC0156	PTS, mannitol-specific IIA domain	n.d.	n.d.	n.d.	n.d.
CAC0233	PTS, fructose-specific IIA component	4.99	3.74	−1.11	n.d.
CAC0234	PTS, fructose-specific IIBC component	2.06	1.88	−1.43	n.d.
CAC0383	PTS, cellobiose-specific IIA component	13.89	23.18	n.a.	n.a.
CAC0384	PTS, cellobiose-specific IIB component	28.11	7.17	n.a.	n.a.
CAC0386	PTS, cellobiose-specific IIC component	40.23	2.83	n.a.	n.a.
CAC0423	Fusion PTS, beta-glucosides specific IIABC component	—	—	—	—

Table 1. *Cont.*

ORF	Protein	glcG::int(1224)		glcCE::int(193)	
		pH 5.7 t = 120 h	pH 4.5 t = 240 h	pH 5.7 t = 120 h	pH 4.5 t = 240 h
CAC0532	PTS, arbutin-like IIBC component	—	—	—	—
CAC0570	PTS enzyme II, ABC component	n.a.	n.a.	−1.01	1.02
CAC1353	PTS, N-acetylglucosamine-specific IIBC component,	1.06	n.d.	−1.49	n.d.
CAC1354	PTS, N-acetylglucosamine-specific IIA component	1.08	1.54	−1.58	−1.81
CAC1407	PTS, beta-glucosides-specific IIABC component	—	—	—	—
CAC1457	PTS, fructose(mannose)-specific IIA component	—	—	—	—
CAC1458	PTS, fructose(mannose)-specific IIB	—	—	—	—
CAC1459	PTS, fructose(mannose)-specific IIC	—	—	—	—
CAC2956	PTS, galactitol/fructose specific IIC component	—	—	—	—
CAC2957	PTS, galactitol/fructose specific IIB component	—	—	—	—
CAC2958	PTS, galactitol/fructose specific IIA component	—	—	—	—
CAC2964	PTS, lactose-specific enzyme IIBC component	—	—	—	—
CAC2965	PTS, lactose-specific enzyme IIA component	—	—	—	—
CAC3425	PTS, possibly glucose-specific IIBC component	n.d.	n.d.	n.d.	n.d.
CAC3427	PTS, possibly glucose-specific IIA component	1.15	n.d.	n.d.	−1.40

The numbers show expression ratios in which the positive value was considered as up-regulated and the negative value was considered as down-regulated. Protein name based on the whole genome sequence by Nölling et al. (2001) [23] Genes are listed in order of ORFs. (—) indicates the genes were not transcribed both in wild type and mutants; n.d.: No ratio was calculated due to used filter criteria of the microarray (Not detected); n.a.: the ratio was not analyzed due to the inactivation of the genes (Not analyzed); and ORF: Open reading frame.

Assays of the transcription profiles of all the PTS EII protein genes of the mutant *glcG*::int(1224) compared to the wild type indicated that the gene expression of five PTSs were up-regulated (Table 1). Among these five PTSs, a cluster of genes (CAC0383-CAC0386), encoding a putative cellobiose-specific transporter exhibited the most pronounced elevated transcription in the mutant *glcG*::int(1224) during acidogenic and solventogenic growth in continuous culture.

In addition, the transcription of genes encoding a fructose-specific transporter (CAC0233-CAC0234) also increased compared to the wild type. Furthermore, slight changes in the expression of the other three PTSs occurred, including a putative mannitol-specific transporter (CAC0154-CAC0156), a putative N-acetylglucosamine-specific transporter (CAC1353-CAC1354) and another putative glucose-specific transporter (CAC3425-CAC3427). These results suggested that the putative cellobiose transporter is able to transport glucose in the cell in the case of a GlcG inactivation.

3.4. The Phenotypes and Transcription Analysis of Wild Type and the Mutant C. acetobutylicum glcCE::int(193) in Batch and Phosphate-Limited Continuous Fermentations

Sequence analysis suggested that the operon (CAC0383-CAC0386) encodes a cellobiose transporter in *C. acetobutylicum* [1]. However, there was no experimental evidence to prove this in vivo until now. The gene *glcCE* (CAC0386) encoding a PTS EIIC in this operon was inactivated by ClosTron insertion and the resulting mutant *glcCE*::int(193) showed poor growth in the batch fermentation with cellobiose as the sole carbon source (Figure 3). The highest optical density the mutant *glcCE*::int(193) achieved was approximately 4, which was significantly lower than that of wild type with the highest optical density of 9 in the batch culture with cellobiose as the sole carbon source.
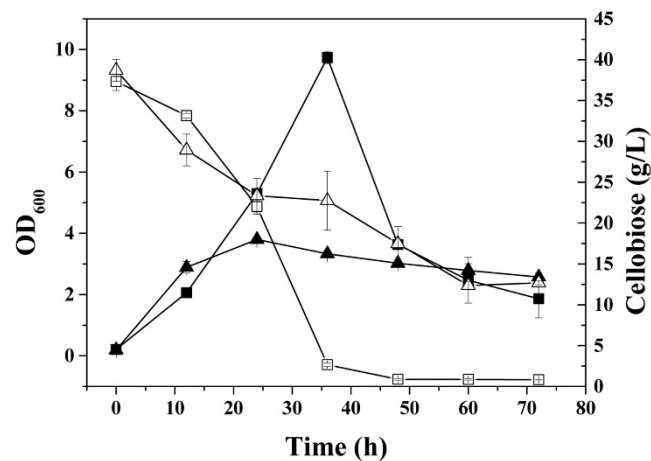
Figure 3. Growth curves and residual cellobiose concentrations of the *C. acetobutylicum* wild type and the mutant *glcCE*::int(193) cultured in minimal medium with cellobiose as the sole carbon source. (■), OD$_{600}$ of wild type; (▲), OD$_{600}$ of *glcCE*::int(193); (□), residual cellobiose during cultivation of wild type; and (△), residual cellobiose during cultivation of *glcCE*::int(193).

There was still 30% of cellobiose left in the *glcCE*::int(193) culture, which suggested that the transporter encoded by the operon (CAC0383-CAC0386) was indeed a cellobiose-specific transporter (Figure 3). Whereas the weak growth of *glcCE*::int(193) on cellobiose and the 70% cellobiose consumption suggested there is a secondary cellobiose transporter in *C. acetobutylicum*, which needs to be identified in a future study.

To test whether this cellobiose-specific transporter is able to transport glucose, the mutant *glcCE*::int(193) was cultured in minimal medium in the presence of 60 g/L glucose and in a phosphate-limited continuous culture. The results illustrated that the mutant *glcCE*::int(193) and wild type exhibited similar growth phenotypes in both batch and continuous fermentation (Figures 1 and 4) and that the concentration of butanol was nearly identical (Figure S3C), suggesting that the *glcCE* mutation did not affect the growth and glucose uptake of the strain. The mutant *glcCE*::int(193) even consumed slightly more glucose and produced more butanol during solventogenesis growth compared to that of wild type in the continuous fermentation (Figure 4 and Table S2), indicating that energy consumption of each glucose transporter was different.
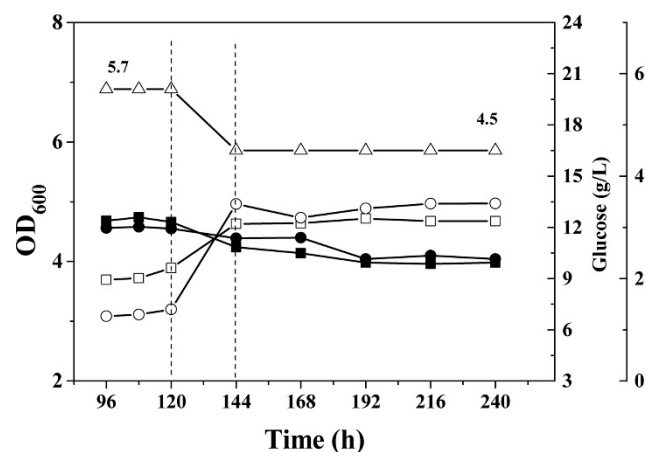

Figure 4. Growth curves and residual glucose concentration in phosphate-limited continuous culture of the *C. acetobutylicum* wild type and the mutant *glcCE*::int(193). (△), pH; (■), OD$_{600}$ of wild type; (●) OD$_{600}$ of *glcCE*::int(193); (□), residual glucose during cultivation of wild type; and (○), residual glucose during cultivation of *glcCE*::int(193). The dashed line indicates the initiation of dynamic pH shift from 5.7 to 4.5 in the continuous fermentation.

On the other side, the growth and sugar consumption of the mutant *glcCE*::int(193) both decreased in the batch culture with cellobiose as the sole carbon source (Figure 3) and the concentration of acetone and butanol were significantly less as compared to those of wild type (Figure S4), indicating that one of the PTSs of *C. acetobutylicum*, GlcCE, played a key role in cellobiose transport. To further investigate the effects the of *glcCE* mutation, DNA microarray experiments were conducted to detect transcription level changes of all 13 PTS EII protein genes compared to the wild type.

During acidogenic and solventogenic stead-state growth of the mutant *glcCE*::int(193) on glucose, the transcriptional levels of genes for the glucose transporter (*glcG*, CAC0570) and a putative mannitol-specific transporter (CAC0154-CAC0156) were identical to that of wild type (Table 1). The genes coding for a putative fructose-specific transporter (CAC0233-CAC0234), a putative N-acetylglucosamine-specific transporter (CAC1353-CAC1354) and a putative glucose-specific transporter (CAC3425-CAC3427) were even slightly repressed compared to the wild type.

These results confirmed that GlcG (CAC0570) is the predominate glucose transporter in *C. acetobutylicum*. When it worked normally in the culture with glucose as carbon source, all of the other PTSs retired to the second line. To prove that the cellobiose transporter can also transport glucose, it was necessary to construct a double mutant in which both of the *glcG* and *glcCE* genes were inactivated. In addition, the genes for GlcCE (CAC0383-CAC0386) in wild type grown on cellobiose were up-regulated compared to those grown on glucose, indicating that GlcCE is the primary cellobiose transporter of *C. acetobutylicum*. The operon (CAC2951-2956) in mutant *glcCE*::int(193) grown on cellobiose were up-regulated compared to the wild type, implying that CAC2956 may encode the secondary cellobiose transporter of *C. acetobutylicum* (Table S3).

3.5. The Phenotype of the Mutant C. acetobutylicum glcG::int(1224)-glcCE::int(193) in Batch and Phosphate-Limited Continuous Fermentations

The mutant *C. acetobutylicum glcG*::int(1224)-*glcCE*::int(193) was grown in a fermenter with 0.75 L minimal medium as the working volume, and then the pH was set to 5.7. After overnight cultivation, the OD_{600} of the culture achieved 4.5, and then continuous fermentation started with phosphate-limited minimal medium at the dilution rate of 0.075 h^{-1}. The growth result demonstrated that the mutant *glcG*::int(1224)-*glcCE*::int(193) was unable to achieve steady-state growth during continuous fermentation (Figure 5).
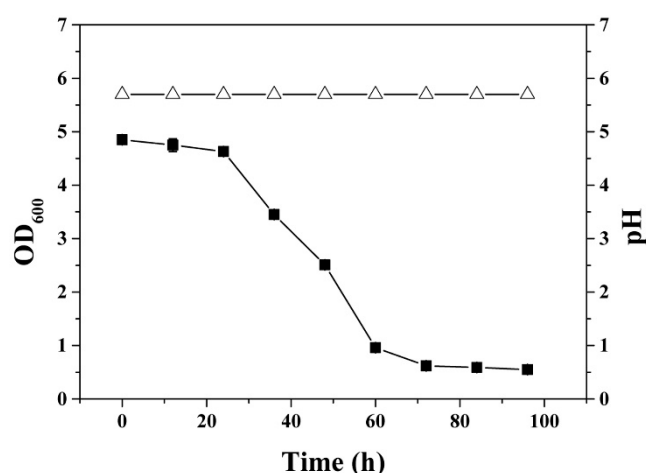

Figure 5. Growth curve of the mutant *C. acetobutylicum glcG*::int(1224)-*glcCE*::int(193) in phosphate-limited continuous culture. (△), pH; and (■), OD_{600} of *glcG*::int(1224)-*glcCE*::int(193).

Under the same fermentation condition, wild type, mutants *glcG*::int(1224) and *glcCE*::int(193) consumed approximately 32 g/L glucose (the total glucose concentration was 40 g/L) and exhibited comparable steady-state growth during acidogenic grow in continuous fermentation (Figures 2 and 4). We speculated that the double mutant is not able to

assimilate enough glucose to support steady-state growth in continuous fermentation due to the inactivation of *glcG* and *glcCE*. Therefore, the cells were washed out of the fermenter in continuous culture.

Despite that the double mutant was not able to grow in the continuous fermentation, it was able to grow in batch culture with 60 g/L glucose as the sole carbon source. The highest optical density that the double mutant reached was approximately an OD_{600} of 5, which was lower than those of the wild type, mutants *glcG*::int(1224) and *glcCE*::int(193) with a highest OD_{600} of 7. At the end of batch fermentation, the glucose consumption and butanol production were 30% and 44% lower than the wild type, respectively, and the output of other products also decreased to different degrees (Figure 1 and Figure S3D).

These results also showed that the simultaneous inactivation of the primary glucose transporter and the cellobiose transporter impaired the growth of the strain on glucose. Importantly, the mutant *glcG*::int(1224)-*glcCE*::int(193) was still able to grow in the batch fermentation, implying that yet another PTS could transport glucose into cytoplasm except the glucose- and cellobiose-specific transporters.

3.6. Transcription Analysis of the Phosphotransferase Systems of the Mutant C. acetobutylicum glcG::int(1224)-glcCE::int(193) Compared to Wild Type

The simultaneous inactivation of gene *glcG* and *glcCE* genes resulted in weak growth of the double mutant, suggesting that a third glucose transporter exists in *C. acetobutylicum*. To test which PTS was able to transport glucose except for GlcG and GlcCE, a DNA microarray experiment was conducted to detect changes of the transcription level of all 13 PTS EII protein genes compared to the wild type in batch culture. Cells of the mutant *C. acetobutylicum glcG*::int(1224)-*glcCE*::int(193) and wild type were harvested after 12 h of batch culture and were used for RNA isolation and subsequent microarray experiments (Figure 1) to identify any compensatory adjustment of gene expression of PTS caused by the mutations of the glucose transporter (encoded by CAC0570) and the cellobiose transporter (encoded by CAC0383-CAC0386) in the double mutant.

The microarray data of all the 13 PTS genes is shown in Table 2. The result indicated that only three PTSs showed changes in the expression patterns of the double mutant as compared to wild type. Among these three PTSs, the expression of genes encoding a mannitol-specific transporter (CAC0154-CAC0156) was up-regulated by 2.8-fold, while the expression of genes encoding a putative fructose-specific transporter (CAC0233-CAC0234) and a putative N-acetylglucosamine-specific transporter (CAC1353-CAC1354) were down-regulated compared to those of the wild type (Table 2). This result suggested that the mannitol-specific transporter is possibly able to transport glucose, which has to be further studied.

Table 2. Transcriptional profiles of all the PTS enzyme II protein genes of mutant *C. acetobutylicum glcG*::int(1224)-*glcCE*::int(193) compared to the wild type in the batch culture.

ORF	Protein	*glcG*::int(1224)-*glcCE*::int(193)
		t = 12 h
CA_P0066	PTS, mannose-specific IIAB component	—
CA_P0067	PTS, mannose/fructose-specific IIC component	—
CA_P0068	PTS, mannose-specific IID component	—
CAC0154	PTS, mannitol-specific IIBC component	2.80
CAC0156	PTS system, mannitol-specific IIA domain	n.d.
CAC0233	PTS, fructose-specific IIA component	−1.46
CAC0234	PTS, fructose-specific IIBC component	n.d.
CAC0383	PTS, cellobiose-specific IIA component	n.a.
CAC0384	PTS, cellobiose-specific BII component	n.a.
CAC0386	PTS, cellobiose-specific IIC component	n.a.
CAC0423	Fusion PTS, beta-glucosides specific IIABC component	—

Table 2. *Cont.*

ORF	Protein	*glcG*::int(1224)-*glcCE*::int(193)
		t = 12 h
CAC0532	PTS, arbutin-like IIBC component	—
CAC0570	PTS enzyme II, ABC component	n.a.
CAC1353	PTS, N-acetylglucosamine-specific IIBC component,	−1.34
CAC1354	PTS, N-acetylglucosamine-specific IIA component	−2.22
CAC1407	PTS, beta-glucosides-specific IIABC component	—
CAC1457	PTS, fructose(mannose)-specific IIA component	—
CAC1458	PTS, fructose(mannose)-specific IIB	—
CAC1459	PTS, fructose(mannose)-specific IIC	—
CAC2956	PTS, galactitol/fructose specific IIC component	—
CAC2957	PTS, galactitol/fructose specific IIB component	—
CAC2958	PTS, galactitol/fructose specific IIA component	—
CAC2964	PTS, lactose-specific enzyme IIBC component	—
CAC2965	PTS, lactose-specific enzyme IIA component	—
CAC3425	PTS, possibly glucose-specific IIBC component	—
CAC3427	PTS, possibly glucose-specific IIA component	—

The numbers showed expression ratios in which the positive value was considered as up-regulated changed folds and in which the negative value was considered as down-regulated changed folds. Protein name based on the whole genome sequence by Nölling et al. (2001) [23] Genes are listed in order of ORFs. (—) indicates the genes were not transcribed both in wild type and mutant; n.d.: No ratio was calculated due to used filter criteria of the microarray (Not detected); n.a.: the ratio was not analyzed due to the inactivation of the genes (Not analyzed); and ORF: Open reading frame.

4. Conclusions

GlcG is the primary glucose transporter of *C. acetobutylicum*, whereas the cellobiose-specific transporter GlcCE is capable of transporting glucose into cytoplasm when GlcG is inactivated, revealing that it is a secondary glucose transporter. Each PTS is responsible for transporting a certain carbohydrate but may be multifunctional. Transcription analysis suggested that the PTSs encoded by CAC0154 and CAC2956 may be a tertiary glucose and secondary cellobiose transporter in *C. acetobutylicum*, respectively. On the basis of the data, we propose a model for glucose uptake in *C. acetobutylicum* (Figure 6), which provides a foundation to study the PTSs in this microorganism.
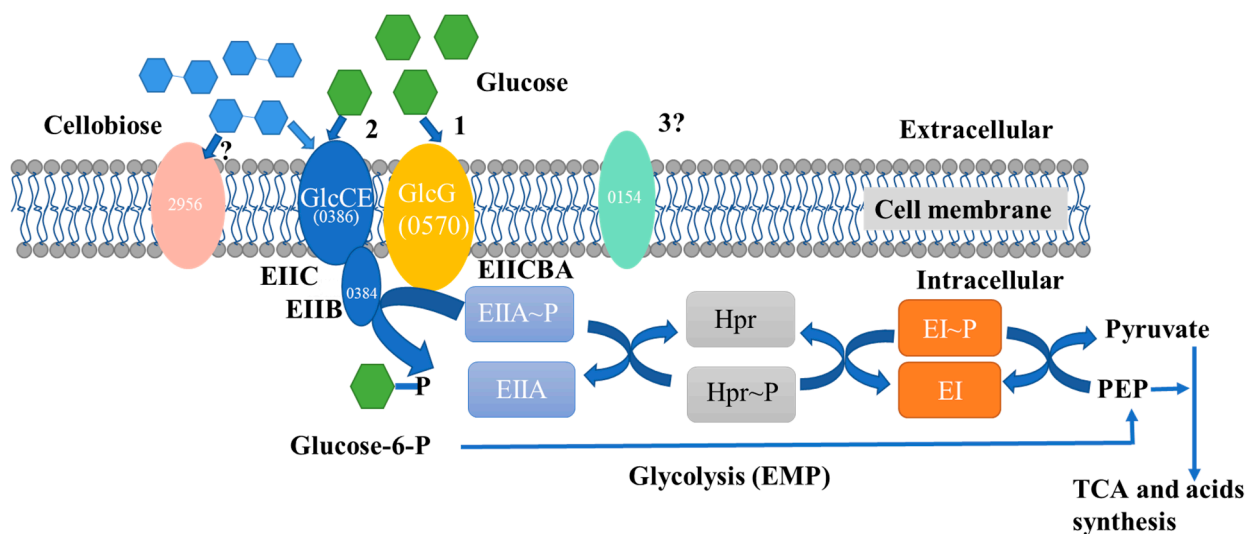

Figure 6. Glucose uptake model for *C. acetobutylicum*. 1, 2 and 3: work order of the glucose transporters; PEP: phosphoenolpyruvate; EI: enzyme I; EII: enzyme II; Hpr: histidine-containing protein; and 0154, 0384, 0386, 0570 and 2956: the gene ID of *C. acetobutylicum*.

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