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Increasing succinic acid production using the PTS-independent glucose transport system in a *Corynebacterium glutamicum* PTS-defective mutant

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Abstract Succinic acid synthesized from glucose shows potential as a bio-based platform chemical. However, the need for a high glucose concentration, and the accompanying low yields, limit its industrial applications. Despite efficient glucose uptake by the phosphotransferase system (PTS), 1 mol of phosphoenolpyruvate is required for each mole of internalized glucose. Therefore, a PTS-defective *Corynebacterium glutamicum* mutant was constructed to increase phosphoenolpyruvate availability for succinic acid synthesis, resulting in a lower glucose utilization rate and slower growth. The transcriptional regulator *iolR* was also deleted to enable the PTS-defective mutant to utilize glucose via *iolT*-mediated glucose transport. Deletion of *iolR* and overexpression of *iolT1* and *ppgk* (polyphosphate glucokinase) in the PTS-deficient *C. glutamicum* strain completely restored glucose utilization, increasing production by 11.6 % and yield by 32.4 % compared with the control. This study revealed for the first time that *iolR* represses the expression of the two glucokinase genes (*glk* and *ppgk*).

Keywords Phosphoenolpyruvic acid · *Corynebacterium glutamicum* · PTS-independent glucose uptake genes · Transcriptional regulator · Succinic acid

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Introduction

As a member of the C4-dicarboxylic acid family, succinic acid is a platform chemical with potential for bio-based synthesis. It is a precursor for the synthesis of various important chemicals, including 1,4-butanediol, tetrahydrofuran, and adipic acid [30], and demand for succinic acid is increasing [22]. Succinic acid is currently produced from fossil fuels by a synthetic process. However, the high environmental cost of this process motivates the development of a cleaner and more economical biological routes to succinic acid production.

The bio-based succinic acid industry is mainly focused on succinic acid-producing organisms, including *Corynebacterium glutamicum* [18, 25], *Anaerobiospirillum succiniciproducens* [27], *Actinobacillus succinogenes* [5], *Escherichia coli* [16], and *Mannheimia succiniciproducens* [17]. In particular, *C. glutamicum*, a rapidly growing Gram-positive bacterium, is commonly used to produce various amino and organic acids [23, 26, 34]. Under anaerobic conditions, *C. glutamicum* can ferment glucose, to give lactic acid, succinic acid, and acetic acid [8, 23].

Theoretically, 1.71 mol of succinic acid can be produced per mole of glucose (plus CO_2) depending on the electron availability. This theoretical yield can be increased to 2.00 mol of succinic acid per mol of glucose if CO_2 and additional reducing power are supplied [22]. Wild-type *C. glutamicum* contains a metabolic pathway for succinic acid production that yields only 0.29 mol per mole of glucose. However, metabolic engineering can increase succinic acid yields in this species. For example, a *C. glutamicum* Δ*ldhA* (lactate dehydrogenase) mutant with enhanced pyruvate carboxylase activity showed an increased succinic acid yield of 1.4 mol/mol [24], while a BOL-1 strain overexpressing *pyc*, along with an NAD^+-coupled formate dehydrogenase, accumulated succinic acid with a yield of 1.7 mol/mol [18].

In *C. glutamicum*, metabolite interconversion at the phosphoenolpyruvate (PEP)–pyruvate–oxaloacetate node (PPON) involves a set of reactions that connects the major pathways of carbon metabolism. These reactions are responsible for the distribution of the carbon flux among catabolism, anabolism, and the cellular energy supply (Fig. 1). Sauer et al. [28] showed that the PPON is a good target for metabolic engineering to achieve optimal metabolite production. Under anaerobic conditions, succinic acid is primarily synthesized from PEP, which is converted to oxaloacetate by phosphoenolpyruvate carboxylase (encoded by *ppc*). Oxaloacetate is then converted to succinic acid via the reductive tricarboxylic acid cycle (TAC) branch [8].

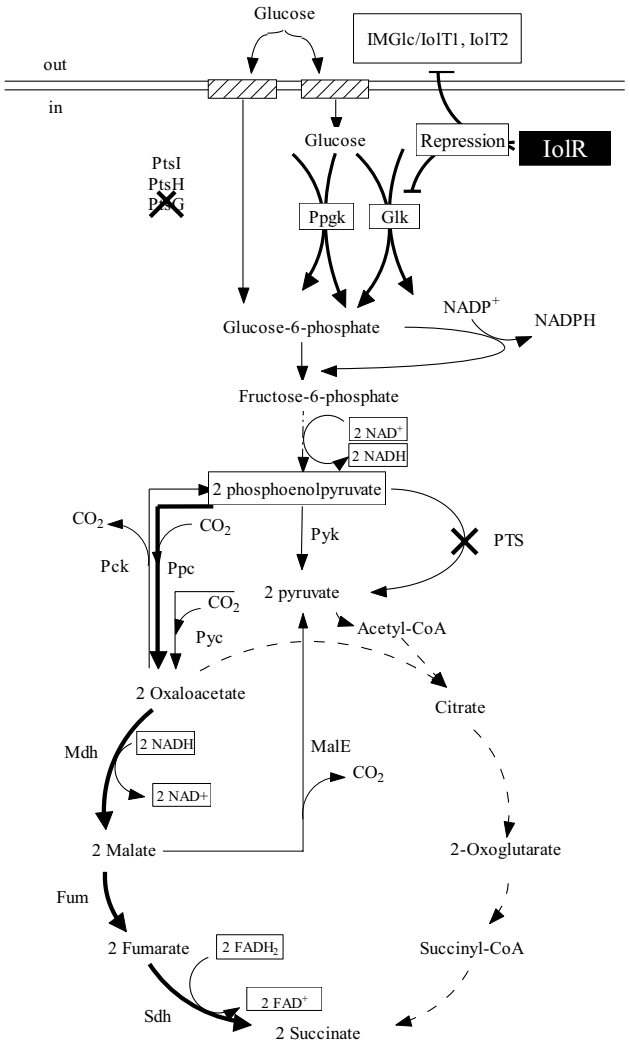

Fig. 1 Schematic representation of the metabolic pathways relevant for succinic acid production by *C. glutamicum* under anaerobic condition. *Bold line* predominant pathways under oxygen deprivation. *Dashed lines* the oxidative branch of TCA pathway where the enzymes of are repressed by glucose and hypoxia [2]. *IMGlc* glucose import system, *IolT1*, *IolT2* inositol transporters, *Glk*, *Ppgk* glucokinases, *IolR* transcriptional regulator, *PtsI*, *PtsH*, *PtsG* phosphoenolpyruvate:sugar phosphotransferase system (*PTS*), *Pck*: *PEP* carboxykinase, *Ppc*:*PEP* carboxylase, *Pyc* pyruvate carboxylase, *Pyk* pyruvate kinase, *Mdh* malate dehydrogenase, *Fum* fumarase, *Sdh* succinodehydrogenase, *MalE* malic enzyme

Under normal conditions, half of the available PEP in the cell is used for glucose uptake and phosphorylation, which reduces the amount of PEP available for succinic acid production [3]. Wittmann et al. [37] investigated the metabolic flux distribution in a *pts*$^-$ *C. glutamicum* strain versus the control strain and showed that the PTS-defective mutant had increased PEP available for the synthesis of aromatic compounds. However, the slow growth and low glucose utilization rates of the PTS-defective mutant meant that it was not suitable for industrial applications [3, 20, 21, 33, 36]. *C. glutamicum* contains two glucokinases: *glk* (cg2399), and *ppgk* (cg2091). Improved glucose utilization and L-lysine production were achieved by overexpressing *iolT1* and *ppgk* in the PTS$^-$ strain [19]. In an *E. coli* PTS$^-$ mutant, overexpression of *galP* (encoding galactose permease) and *glk* (encoding glucokinase) restored glucose transport and increased the glycolytic flux to fermentation products [6]. Two *myo*-inositol transporters capable of mediating glucose uptake, encoded by *iolT1* (Cgl0181) and *iolT2* (Cgl3058), have been identified in *C. glutamicum* [9]. These transporters are responsible for *myo*-inositol utilization and can be induced by a specific concentration of inositol [15]. Glucose phosphorylation is not involved in PTS-independent glucose uptake; thus, glucokinase activity may be required.

In this study, two strategies were attempted to restore the glucose utilization rate of a PTS-defective *C. glutamicum* strain. Ikeda et al. [9] previously identified a suppressor mutant derived from a PTS-defective *C. glutamicum* strain on glucose agar plates, which was later confirmed to have an *iolR* mutation. The promoter operator region of *iolT1* could be repressed in this suppressor mutant, and real-time polymerase chain reaction (RT-PCR) analysis was used to investigate the effects of *iolR* on the expression of PTS-independent glucose utilization genes. However, little is known about these genes in the *iolR*-deficient mutant, particularly the effects of this mutation on the expression of *ppgk* and *glk* [9, 11]. Therefore, our first strategy was to experimentally inactivate *iolR* and observe the effects on gene expression. The second strategy was to increase the transcriptional levels of *iolT1* and compensate for the deletion of *ppgk* in the PTS$^-$ strain through overexpression.

Materials and methods

Microorganisms and medium

Strain genotypes and plasmids are shown in Table 1. For plasmid construction, *E. coli* JM109 was cultured at 37 °C

Table 1 Bacterial strains and plasmids used in this study

Strain, plasmids	Genotypes, properties	Sources or references
Strains		
C. glutamicum NC-3	*C. glutamicum* ATCC 13032 derivative with an in-frame deletion of the *ldhA*, with integration of xylose metabolic gene (*xylA* ,*xylB*) and *gapA*	[35]
C. glutamicum NC-3b	*C. glutamicum* NC-3 derivative with inactivation of *ptsG*	This study
C. glutamicum NC-3b-1	*C. glutamicum* NC-3b derivative with in-frame deletion of *iolR*	This study
C. glutamicum NC-3b-*iolT1*	*C. glutamicum* NC-3b derivative with chromosomal integration of *gapA* promoter and *iolT1* gene into the *ptsG* locus	This study
C. glutamicum NC-3b-*iolT1*(pXMJ19-*ppgk*)	*C. glutamicum* NC-3b-*iolT1* derivative with pXMJ19-*ppgk*	This study
C. glutamicum NC-3b-2	*C. glutamicum* NC-3b-1 derivative with chromosomal integration of *gapA* promoter and *iolT1* gene into the *ptsG* locus	This study
C. glutamicum NC-3b-3	*C. glutamicum* NC-3b-2 derivative with chromosomal integration of *ppgk* gene under the control of the *gapA* promoter into the *iolR* locus	This study
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′ [*traD36 proAB* + *lacI*^{q} *lacZ*ΔM15]	Takara
Plasmids		
pXMJ19	Cm^r; *E. coli-C. glutamicum* shuttle vector, *tac* promoter	[12]
pXMJ19-*ppgk*	Cm^r, pXMJ19 derivative containing the *ppgk* gene under the control of an IPTG-inducible tac promoter	This study
pk18*mobsacB*	Kan^r; vector for allelic exchange in *C. glutamicum*	[32]
pk18*mobsacBΔptsG*	Derived from pk18*mobsacB*, containing up- and downstream regions of *ptsG*	This study
pk18*mobsacBΔiolR*	Derived from pk18*mobsacB*, containing up- and downstream regions of *iolR*	This study
pk18*mobsacBΔptsG*::P*gapA-iolT1*	Derived from pk18*mobsacBΔptsG* with 2.8-kb *Eco*RV-*Sph*I DNA fragment containing P*gapA-iolT1* gene	This study
pk18*mobsacBΔiolR*::P*gapA-ppgk*	Derived from pk18*mobsacBΔiolR* with 1.7-kb *Xba*I-*Sph*I DNA fragment containing P*gapA-ppgk* gene	This study

in lysogeny broth complex medium (LB) [29]. *C. glutamicum* strains were routinely cultivated at 30 °C in LB medium. For selection of pK18mobsacB and its derivatives, 50 and 25 μg/ml kanamycin was added to *E. coli* and *C. glutamicum* cultures, respectively. Nutrient-rich medium (A medium) was used for aerobic growth and the mineral salts medium (BT medium) was used for anaerobic fermentation [38].

Anaerobic succinic acid production in a lidded bottle

A single colony of the appropriate *C. glutamicum* strain was inoculated into 5 ml of LB medium from a fresh LB agar plate, and the culture was incubated on a rotary shaker for 12 h at 30 °C. A 2-ml aliquot of this starter culture was then inoculated into a 500-ml baffled shake flask containing 50 ml of A medium supplemented with 110 mM glucose for 18 h, 200 rpm at 30 °C. The cells were then harvested by centrifugation (8000×*g*, 4 °C, 10 min), resuspended in 25 ml of BT medium, and transferred into a 100-ml lidded bottle. The cell suspension was incubated for 18 h, 150 rpm at 30 °C. Oxygen deprivation (dissolved oxygen concentration <0.01 ppm) was achieved using a gassing manifold with oxygen-free CO_2 for 30 s according to the manufacturer's instructions.

Anaerobic succinic acid production in fed-batch mode

A single colony was picked from plates and inoculated into 5 ml LB medium and cultivated at 30 °C for 12 h. Aliquots (2 ml) were inoculated into three 500-ml baffled shake flasks containing 100 ml of A medium supplemented with 110 mM glucose. After approximately 16 h of incubation at 200 rpm and 30 °C, or when cultures reached an optical density at 600 nm (OD_{600}) of 10, a 5-l fermentation bioreactor (BIOTECH-5JG, China) containing 3 l of A medium supplemented with 185 mM glucose was inoculated with the starter culture and run for 16 h, to a final OD_{600} of 35. The cells were then harvested by centrifugation (8000×*g*, 4 °C, 10 min), resuspended in 1 l of BT medium containing 367 mM glucose and 300 mM $NaHCO_3$, and transferred into a 1-l bioreactor (Biotech-5JG-9000A, China). Oxygen deprivation of oxygen (dissolved oxygen concentration <0.01 ppm) was achieved using a gassing manifold with oxygen-free CO_2 for 5 min. Under anaerobic conditions, the cell suspension was incubated at 30 °C and 150 rpm

Table 2 Sequences of primers

Name	Sequence (5′–3′)	Function and restriction site
ppgk-F	GATAAGCTT**AAAGGAGGA**CAACCATGACTGAGACTGGATTT	Expression of *ppgk*; *Hin*dIII
ppgk-R	ACAGAATTCTTATGGGGTGAGGTGTTG	Expression of *ppgk*; *Eco*RI
ΔptsG-F1	TCCCCCGGGATGGCGTCCAAACTG	*ptsG* deletion; *Sma*I
ΔptsG-R1	*ACATGCATGCTTAGGATATCCGG*TGGCAGGAAGTAGAA	*ptsG* deletion; *Sph*I, *Eco*RV
ΔptsG-F2	*CCGGATATCCTAAGCATGCATGT*ACCAGGCATTGCAAT	*ptsG* deletion; *Eco*RV, *Sph*I
ΔptsG-R2	GATAAGCTTTACTCGTTCTTGCCG	*ptsG* deletion; *Hin*dIII
ΔiolR-F1	TCCCCCGGGACTTCGTGAGTGCTC	*iolR* deletion; *Sma*I
ΔiolR-R1	*ACATGCATGCTTAGTCTAGACTAG*GGGAGCTTCGGTGGTCAT	*iolR* deletion; *Sph*I, *Xba*I
ΔiolR-F2	*CTAGTCTAGACTAAGCATGCATGT*CGGTGCGCGCCGAGCAGT	*iolR* deletion; *Xba*I, *Sph*I
ΔiolR-R2	GATAAGCTTGAAACCAGCCCATGT	*iolR* deletion; *Hin*dIII
P*gapA-iolT1*-F1	CCGGATATCCGGCGAAAACGAAA	Overexpression of *iolT1* under the control of the *gapA* promoter; *Eco*RV
P*gapA-iolT1*-R1	*CGGCCTGAATGAAGGTACTAGCCATGTTGTGTCTCCTCTAAAGATTGTAGG*	
P*gapA-iolT1*-F2	*CCTACAATCTTTAGAGGAGACACAACATGGCTAGTACCTTCATTCAGGCCG*	
P*gapA-iolT1*-R2	ACATGCATGCGCTGTGATCACACCATG	Overexpression of *iolT1* under the control of the *gapA* promoter; *Sph*I
P*gapA-ppgk*-F1	CTAGTCTAGACGGCGAAAACGAAA	Overexpression of *ppgk* under the control of the *gapA* promoter; *Xba*I
P*gapA-ppgk*-R1	*CAATTCCAAATCCAGTCTCAGTCATGTTGTGTCTCCTCTAAAGATTGTAGG*	
P*gapA-ppgk*-F2	*CCTACAATCTTTAGAGGAGACACAACATGACTGAGACTGGATTTGGAATTG*	
P*gapA-ppgk*-R2	ACATGCATGCTTATGGGGTGAGGTGTTG	Overexpression of *ppgk* under the control of the *gapA* promoter; *Sph*I

Restriction sites are underlined and linker sequences for crossover PCR are shown in italics. The bold letters mean the Shine–Dalgarno sequence

for 48 h. In two independent fermentations, the pH was maintained at 7.2 by automated addition of 20 % (v/v) $NH_3 \cdot H_2O$.

Recombinant DNA techniques

Standard protocols were used for construction, purification, and analysis of plasmid DNA, and for *E. coli* transformation. Extraction of *C. glutamicum* chromosomal DNA and transformation of *C. glutamicum* by electroporation were performed as described previously [9]. PCR was performed using a DNA thermal cycler (2720 Thermal cycler, Applied Biosystems, USA) using Prime STARHS DNA Polymerase (Takara Bio, Japan). The restriction endonuclease, ligase, and other materials for the polymerase chain reaction (PCR) were purchased from Takara. PCR was performed in a 50-µl reaction system containing 5 µl $MgCl_2$ (20 mmol/l), 5 µl $dNTP_S$ (20 mmol/l), 3 µl chromosomal DNA (400 µg/ml), 1 µl DNA polymerase, and 5 µl oligonucleotide primers, made up to the final volume with ddH_2O. The thermal cycler conditions were 30 cycles of 95 °C for 30 s, 62 °C for 15 s, and 72 °C for 2 min 30 s. All primers used in this study are shown in Table 2 and were synthesized by Genscript Biotechnology (Nanjing, China).

Construction of plasmids and *C. glutamicum* mutant strains

A *C. glutamicum* mutant with an in-frame deletion of *ptsG*, which encodes Glc, a membrane-bound, glucose-specific permease of the phosphotransferase system, was constructed in a two-step homologous recombination procedure as described previously [10]. Briefly, regions flanking *ptsG* were amplified by PCR using the primer pairs Δ*ptsG*-F1/Δ*ptsG*-R1 and Δ*ptsG*-F2/Δ*ptsG*-R2. The resulting amplicons were then joined using a crossover PCR protocol using the primer pair Δ*ptsG*-F1/Δ*ptsG*-R2. The resulting 906-bp product was digested with *Sma*I/*Hin*dIII and cloned into pK18mobsacB digested with the same restriction enzymes, generating pK18mobsacBΔ*ptsG*. pK18mobsacBΔ*ptsG* was electroporated into *C. glutamicum* NC-3, generating *C. glutamicum* NC-3b. The deletion of *ptsG* was verified by PCR using the primers Δ*ptsG*-F1 and Δ*ptsG*-R2. Primers used are listed in Table 2.

iolR was inactivated by a crossover PCR. Flanking regions of *iolR* were amplified using the primer pairs Δ*iolR*-F1/Δ*iolR*-R1 and Δ*iolR*-F2/Δ*iolR*-R2. The two amplicons were then joined by crossover PCR using the primers Δ*iolR*-F1 and Δ*iolR*-R2. The resulting 1-kb product was digested with *Sma*I/*Hin*dIII and cloned

Table 3 Oligonucleotides used in RT-PCR

Gene	Sequence (5′–3′)
iolT1	F: GTTGCACTAGTTGCGACGTT R: TTAGTCCGAGCTCACGTGTC
iolT2	F: CTCCATGCAGACTTTCCTCA R: CGATACCCTTCATTCGGACT
glk	F: CTGGAAGATTTCAGCGAGTG R: GGTCAAGGACATCAGCAATG
ppgk	F: CCAACACAGAACTCGGTCAC R: TTCTCGTATTCGCTCAGCAC

into pK18mobsacB digested with the same restriction enzymes, generating pK18mobsacBΔ*iolR*. The resulting plasmid was isolated and used for gene disruption. Integration into the genome in the resulting strain *C. glutamicum* NC-3b-1 was verified by PCR using primers Δ*iolR*-F1 and Δ*iolR*-R2.

Plasmid pK18mobsacBΔ*ptsG*::P_{gapA}-*iolT1* was used to express *iolT1* by inserting *iolT1* into the *ptsG* locus. To construct pK18mobsacBΔ*ptsG*::P_{gapA}-*iolT1*, the coding region of *iolT1* was amplified using primers P_{gapA}-*iolT1*-F2 and P_{gapA}-*iolT1*-R2, with *C. glutamicum* genomic DNA as a template. The upstream region of *gapA* (from −1 to −927 bp), containing the promoter, was amplified using primers P_{gapA}-*iolT1*-F1 and P_{gapA}-*iolT1*-R2. The two fragments were fused by PCR using primers P_{gapA}-*iolT1*-F1 and P_{gapA}-*iolT1*-R2. The resulting 2.8-kb fragment was digested with *Eco*RV/*Sph*I, and then ligated into the corresponding sites of pK18mobsacBΔ*ptsG* to yield pK18mobsacBΔ*ptsG*::P_{gapA}-*iolT1*. Plasmid pK18mobsacBΔ*ptsG*::P_{gapA}-*iolT1* was electroporated into *C. glutamicum* NC-3b and *C. glutamicum* NC-3b-1, resulting in *C. glutamicum* NC-3b-*iolT1* and *C. glutamicum* NC-3b-2, respectively.

Vector pXMJ19 was used for IPTG-inducible overexpression. *ppgk* was amplified from *C. glutamicum* ATCC13032 genomic DNA using the primers listed in Table 2. To construct pXMJ19-*ppgk*, the PCR product was digested with *Hin*dIII/*Eco*RI and cloned into pXMJ19 digested with the same restriction enzymes. pXMJ19-*ppgk* was electroporated into *C. glutamicum* NC-3b-*iolT1*, resulting in *C. glutamicum* NC-3b-*iolT1*(pXMJ19-*ppgk*).

To construct pK18mobsacBΔ*iolR*::P_{gapA}-*ppgk*, the coding region of *ppgk* was amplified using primers P_{gapA}-*ppgk*-F2 and P_{gapA}-*ppgk*-R2, while the *gapA* promoter region was amplified using primers P_{gapA}-*ppgk*-F1 and P_{gapA}-*ppgk*-R1. The two fragments were fused by PCR using the primers P_{gapA}-*ppgk*-F1 and P_{gapA}-*ppgk*-R2. The resulting 1.7-kb fragment was digested with *Xba*I/*Sph*I and then ligated to the corresponding sites of pK18mobsacBΔ*iolR*, yielding pK18mobsacBΔ*iolR*::P_{gapA}-*ppgk*. This plasmid was then electroporated into *C. glutamicum* NC-3b-2, resulting in *C. glutamicum* NC-3b-3. All primers were designed based on the genome sequences of *C. glutamicum* ATCC13032.

RNA extraction, cDNA synthesis, and RT-PCR

Exponential growth-phase cells (1.5 ml) cultured in A medium were harvested by centrifugation at 8000×*g* for 1 min at 4 °C. Total RNA was extracted from *C. glutamicum* strains and purified as described previously [7]. cDNA was synthesized from 500 ng of RNA and was analyzed by RT-PCR, as previously described [13, 14], with gene expression levels standardized to 16S rRNA expression and calculated by the comparative cycle threshold method [31]. Oligonucleotides used in RT-PCR are listed in Table 3.

Analytical methods

Cell growth was monitored by measuring the cell density of cultures at OD_{600} using a UV–Visible spectroscopy system (Mapada Co., China). Glucose concentration was determined using a glucose analyzer (SBA-40E, Biology Institute of Shandong Academy of Sciences, China) containing glucose oxidase according to the manufacturer's instructions. The concentrations of succinic acid and its by-products (acetic acid and pyruvic acid) were determined using a high-performance liquid chromatography system (Agilent, USA) equipped with a UV detector and a conductivity meter, and a Grace Prevail column (length, 250 nm; internal diameter, 4.6 nm). The mobile phase consisted of 25 mM KH_2PO_4 (pH 2.5) at a flow rate of 1.0 ml/min, and the column was operated at 25 °C, with detection at OD_{215}. The mass yields of the products were defined as the amount of product generated per mole of glucose consumed, and expressed in mol/mol. Dry cell weight (DCW) was estimated by correlating OD_{600} values with 0.3 g DCW/l as described previously [1].

Results

PtsG-deficient *C. glutamicum* exhibits higher succinic acid yield and lower glucose utilization

To increase the metabolic flux of PEP to oxaloacetate, a *C. glutamicum ptsG* mutant (NC-3b) was constructed by two-step homologous recombination. The growth of *C. glutamicum* NC-3b in minimal medium with 110 mM glucose as the sole carbon source was severely impaired compared with that of the wild type (growth rates of 0.17 ± 0.03 and 0.5 ± 0.02 h^{-1}, respectively) (Fig. 2a). The glucose consumption was also lower than that of *C. glutamicum* NC-3, with glucose consumption rates of 1.49 ± 0.3 and

3.94 ± 0.2 mM/h, respectively (Fig. 2b). However, the succinic acid yield of *C. glutamicum* NC-3b was lower than that of *C. glutamicum* NC-3, with respective succinic acid yields of 1.30 ± 0.1 and 0.95 ± 0.08 mol/mol. The *C. glutamicum* NC-3b by-product yields were significantly lower than those of the wild type, with pyruvic acid yields of 0.11 ± 0.02 and 0.18 ± 0.01 mol/mol, and acetic acid yields of 0.24 ± 0.04 and 0.40 ± 0.08 mol/mol, respectively (Table 4).

Derepression of the transcriptional regulator *iolR* has positive effects on PTS-independent glucose transport

To restore the glucose uptake rate in the *ptsG* mutant *C. glutamicum* NC-3b, an *iolR*-deficient mutant, *C. glutamicum* NC-3b-1, was constructed by homologous recombination. The growth rate of *C. glutamicum* NC-3b-1 (Δ*iolR*) in minimal medium with 110 mM glucose as the sole carbon source was higher than that of *C. glutamicum* NC-3b (Δ*ptsG*), with observed growth rates of 0.44 ± 0.07 and 0.17 ± 0.03 h^{-1}, respectively (Fig. 2a). The glucose consumption rate was also higher than that of *C. glutamicum* NC-3b (Δ*ptsG*), with rates of 2.68 and 1.49 ± 0.3 mM/h, respectively (Fig. 2b). Accordingly, we used RT-PCR analysis to investigate the transcript levels of the PTS-independent glucose transport genes *iolT1*, *iolT2*, *ppgk*, and *glk* in *C. glutamicum* NC-3b (Δ*ptsG*). As shown in Fig. 3, inactivation of *iolR* resulted in a 5.11 ± 0.22-fold increase in *iolT1* transcript, and a 3.25 ± 0.29-fold increase in the transcription of *iolT2*, while *ppgk* and *glk* transcript levels were increased by 2.23 ± 0.29- and 2.14 ± 0.23-fold, respectively.

Expression of either *iolT1* or *ppgk* partly restores glucose uptake in *C. glutamicum* NC-3b

To compare the effects of *iolT1* and *ppgk* overexpression with the effects of *iolR* deletion in the PTS inactivation mutant (NC-3b), *C. glutamicum* NC-3b-*iolT1* and *C. glutamicum* NC-3b-*iolT1*(pXMJ19-*ppgk*) were constructed and analyzed for their glucose uptake capabilities. The growth of *C. glutamicum* NC-3b was slightly increased following expression of *iolT1*, with growth rates of 0.28 ± 0.07 and 0.17 ± 0.03 h^{-1} for strains NC-3b-*iolT1* and NC-3b, respectively. The glucose consumption rate of strain NC-3b-*iolT1* was also higher than that of *C. glutamicum* NC-3b (2.17 ± 0.5 and 1.49 ± 0.3 mM/h, respectively). Overexpression of *ppgk* in *C. glutamicum* NC-3b-*iolT1* further increased the growth rate and glucose uptake of the mutant to levels closer to those of wild-type strain *C. glutamicum* NC-3 (growth rate, 0.5 ± 0.02 h^{-1}; glucose consumption rate, 3.94 ± 0.2 mM/h), with strain NC-3b-*iolT1*(pXMJ19-*ppgk*) demonstrating a growth rate of 0.36 ± 0.03 h^{-1} and a glucose consumption rate of 2.76 ± 0.3 mM/h. However, these results showed that expression of *iolT1* and *ppgk* only partly restored glucose uptake in *C. glutamicum* NC-3b; thus, a combination of the two strategies was examined in an attempt to completely restore glucose utilization in the PTS-deficient strain.

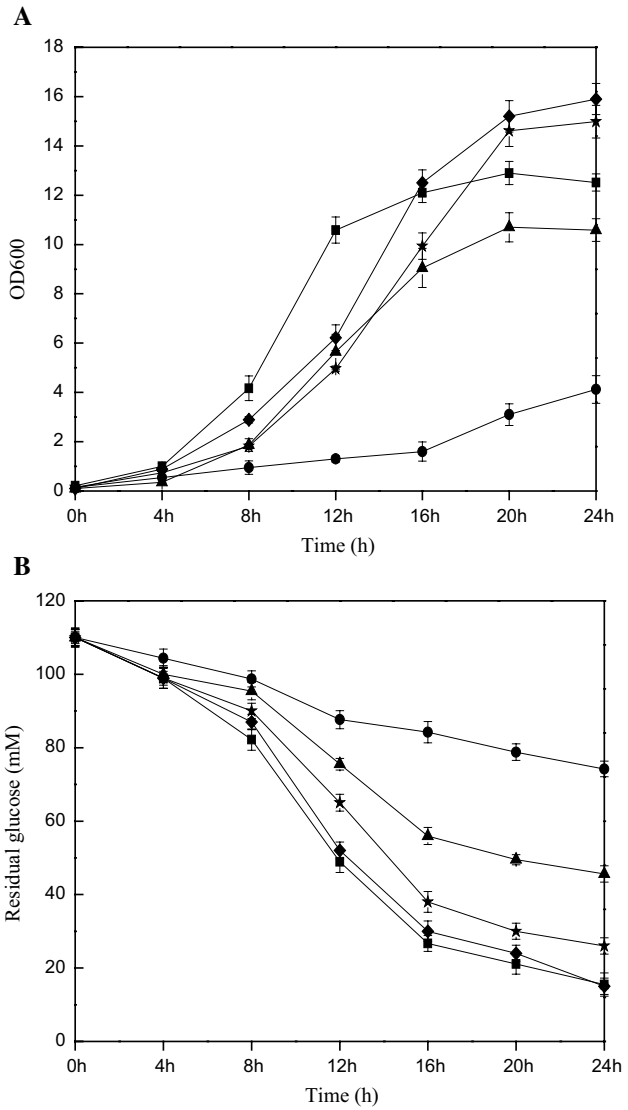

Fig. 2 Growth (**a**) and glucose consumption (**b**) of *C. glutamicum* NC-3, *C. glutamicum* NC-3b, *C. glutamicum* NC-3b-1, *C. glutamicum* NC-3b-2 and *C. glutamicum* NC-3b-3 in minimal medium with 110 mM glucose as the sole carbon source under aerobic condition. *Filled squares*, *C. glutamicum* NC-3; *filled circles*, *C. glutamicum* NC-3b; *filled triangles*, *C. glutamicum* NC-3b-1; *filled stars*, *C. glutamicum* NC-3b-2; *filled diamonds*, *C. glutamicum* NC-3b-3. Arithmetic means and absolute *errors* from at least three independent cultivation are given

Table 4 Succinic acid production of *C. glutamicum* NC-3 and NC-3b and derivatives from 183 mM glucose

Strain	Anaerobic biomass (g L^{-1})	Succinic acid (mM g^{-1} DCW)	Succinic acid yield (mol/mol)	Pyruvate caid yield (mol/mol)	Acetate acid (mol/mol)
C.glutamicum NC-3	8.19 ± 0.2	15.73 ± 0.4	0.95 ± 0.03	0.18 ± 0.01	0.40 ± 0.08
C.glutamicum NC-3b	3.78 ± 0.1	11.68 ± 0.3	1.30 ± 0.15	0.11 ± 0.02	0.24 ± 0.04

Results of sealed bottles experiments using 183 mM glucose in anaerobic medium after 18 h for incubation, and the values shown are averages of triplicate experiments ± standard deviations

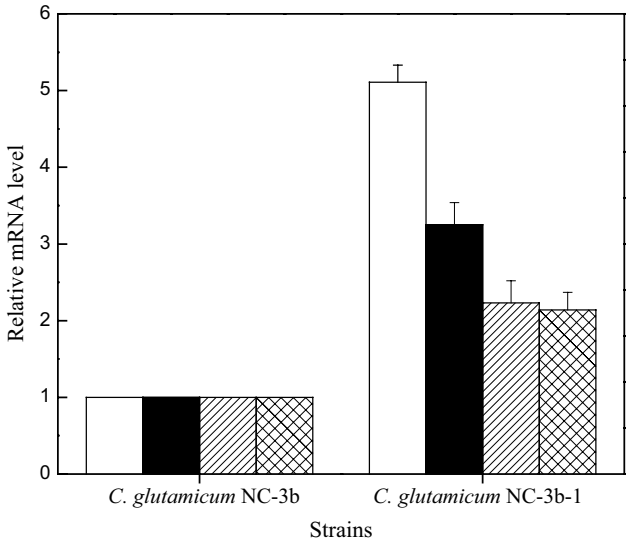

Fig. 3 Relative mRNA levels of the PTS-independent glucose PTS-independent glucose transport genes in *C. glutamicum* NC-3b-1 carrying the mutation of *iolR*. Total RNAs were prepared from cells grown to the exponential phase in A medium. Aliquots of RNAs were reverse transcribed and subjected to qPCR. The transcript levels of *iolT1* (*white bars*), *iolT2* (*black bars*), *ppgk* (*hatched bars*), and *glk* (*dotted bars*) were standardized to the constitutive expression level of 16S rRNA. The transcript levels in *C. glutamicum* NC-3b were set to 1.0. Data represent mean values from three independent cultures, and the standard deviation from the mean is indicated as *error bars*

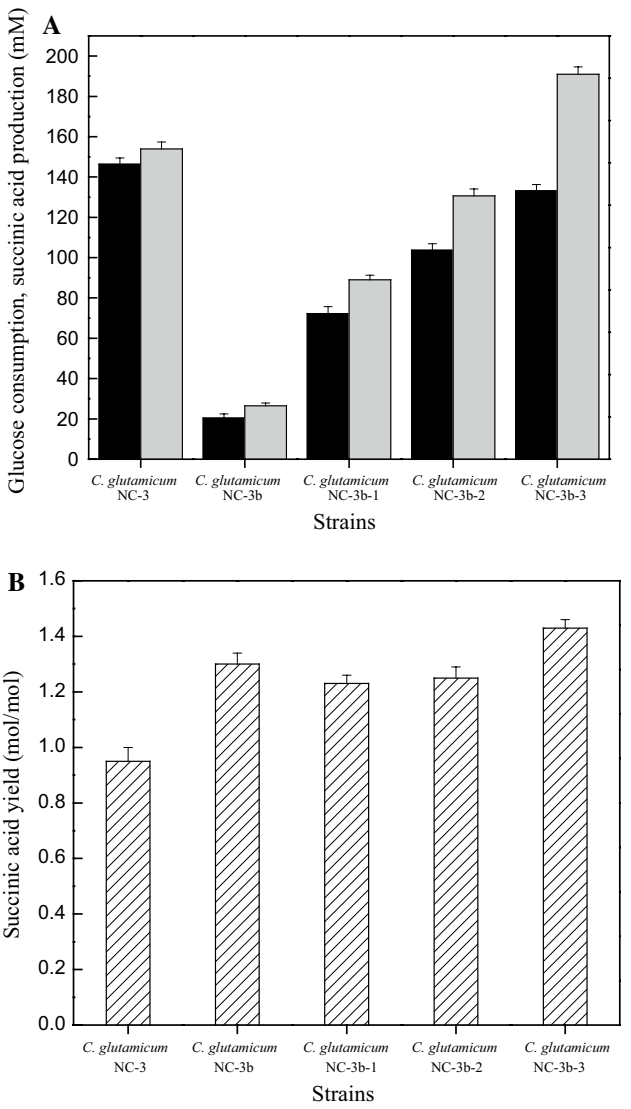

Fig. 4 Succinic acid production, glucose consumption (**a**) and succinic acid yield (**b**) of *C. glutamicum* NC-3, *C. glutamicum* NC-3b, *C. glutamicum* NC-3b-1, *C. glutamicum* NC-3b-2 and *C. glutamicum* NC-3b-3 in sealed bottles using anaerobic medium supplemented with 183 mM glucose. *Black bars* glucose consumption, *light gray bars* succinic acid production, *sparse line bars* succinic acid yield. Arithmetic means and absolute *errors* from at least three independent cultivation are given

Expression of *iolT1* and *ppgk* completely restores glucose utilization in *C. glutamicum* NC-3b-1

To test if the combined deletion of *iolR* and overexpression of the PTS-independent glucose transport genes could increase succinic acid production in the absence of PTS, *C. glutamicum* NC-3b-3 (Δ*iolR-iolT1-ppgk*) was constructed and its succinic acid production was analyzed. Notably, the growth of *C. glutamicum* strains NC-3b-2 (Δ*iolR-iolT1*) and NC-3b-3 (Δ*iolR-iolT1-ppgk*) was much faster than that of *C. glutamicum* NC-3b, but still slightly slower than that of *C. glutamicum* NC-3 (Fig. 2a). The glucose consumption rate of *C. glutamicum* strains NC-3b-2 and *C. glutamicum* NC-3b-3 was also much faster than that of *C. glutamicum*

NC-3b, and was equal to that of wild-type strain *C. glutamicum* NC-3 (Fig. 2b). Succinic acid accumulation in media containing 183 mM glucose was enhanced by 25 % for these strains, with yields of 191 ± 3.65 mM for *C. glutamicum* NC-3b-3 and 153 ± 3.43 mM for *C. glutamicum* NC-3 (Fig. 4a). The succinic acid yield of *C. glutamicum* NC-3b-3 (1.43 mol/mol) was 50.5 % higher than that of *C. glutamicum* NC-3 (0.95 mol/mol) (Fig. 4b), while the formation of by-products by strain NC-3b-3 was dramatically lower than that of *C. glutamicum*, the wild-type strain (pyruvic acid production, 5.82 and 20.3 mM; acetic acid production, 30.2 and 45.5 mM, respectively).

Fed-batch succinic acid production using *C. glutamicum* strains NC-3 and NC-3b-3

To further investigate whether the production of succinic acid could be improved by increasing the available PEP, two independent fed-batch fermentations were carried out under identical conditions. The two experiments had comparable final succinic acid yields, succinic acid production rates, and formation of by-products. The fermentation with *C. glutamicum* NC-3 is shown in Fig. 5a, and described in detail below. Initially, the anaerobic fermentation medium contained 367 mM glucose and 300 mM $NaHCO_3$. At 8 and 44 h post-inoculation, $NaHCO_3$ was added to the medium (stock concentrations of 250 and 100 mM, respectively). At 14 and 44 h, glucose was added to a concentration of 90 mM. Fermentation using *C. glutamicum* NC-3b-3 is shown in Fig. 5b. Initially, the anaerobic fermentation medium contained 367 mM glucose and 300 mM $NaHCO_3$. At 8, 22, and 38 h post-inoculation, $NaHCO_3$ was added to 100 mM, and at 8 and 22 h post-inoculation, glucose was added to 90 mM.

As shown in Fig. 5, the final succinic acid concentration of the *C. glutamicum* NC-3b-3 culture at 48 h was 11.6 % greater than that of *C. glutamicum* NC-3 (769 and 689 mM, respectively). Over the course of the 48-h fermentation, 522.2 and 616.7 mM glucose had been consumed (Fig. 5). Additionally, a higher succinic acid yield was obtained from *C. glutamicum* NC-3b-3 (1.47 mol/mol glucose) than from wild-type *C. glutamicum* NC-3 (1.11 mol/mol glucose). By-product formation in the *C. glutamicum* NC-3b-3 fermentation was dramatically lower than that of *C. glutamicum* NC-3 (pyruvic acid production, 44.3 and 56.1 mM; acetic acid production, 108.3 and 250.9 mM, respectively). Thus, the deletion of *iolR* and combined overexpression of *iolT1* with *ppgk* in the PTS-deficient *C. glutamicum* NC-3b strain enabled 11.6 % greater succinic acid production and a 32.4 % higher succinic acid yield than that of *C. glutamicum* NC-3 in a 3-l fermentation bioreactor system.

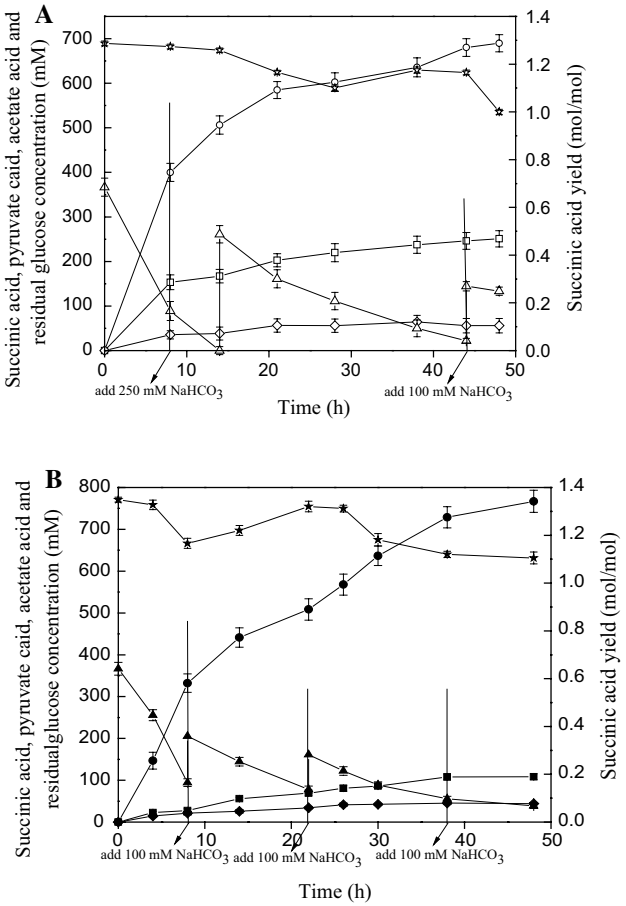

Fig. 5 Representative anaerobic fed-batch fermentation with *C. glutamicum* NC-3 (**a**) and *C. glutamicum* NC-3b-3 (**b**) showing succinic acid production during the utilization of glucose and formation of the by-products. **a** Succinic acid (*open circles*), glucose consumption (*open triangles*), acetate acid (*open squares*), pyruvate acid (*open diamonds*), succinic acid yield (*open stars*). **b** Succinic acid (*filled circles*), glucose consumption (*filled triangles*), acetate acid (*filled squares*), pyruvate acid (*filled diamonds*), succinic acid yield (*filled stars*). The experiment was performed in a 3-L fermentation bioreactor system, and the cells, pregrown aerobically, were resuspended in 1-L anaerobic fermentation medium. Two independent fermentations were performed, with both showing comparable results with respect to product yield and production

Discussion

Previous studies have shown that *C. glutamicum* strains with a PTS^- $glucose^+$ phenotype have characteristics that are useful for biotechnological applications [4]. Mutation of *ptsG* increased production of succinic acid from the fermentation of glucose by *C. glutamicum*, and decreased conversion of PEP to pyruvate reduced the formation of by-products derived from pyruvate [3]. In *C. glutamicum*, each mole of glucose taken up by the PTS requires the consumption of 1 mol of PEP, and PTS inactivation has a strong

effect on the metabolic flux distribution throughout the central metabolic network [37]. In a PTS mutant, the pentose phosphate pathway and tricarboxylic acid cycle fluxes were enhanced, and there was an increase in the exchange rate between PEP and oxaloacetic acid, involving Ppc and Pck, compared with the wild-type strain [37]. This suggests that available PEP relative to pyruvate in the PTS mutant strain contributes to a better balance in the supply of carbon from central metabolism into the succinic acid-biosynthetic pathway through the two anaplerotic reactions involving pyruvate carboxylase and PEP carboxylase.

Although the PTS is the major route of glucose uptake, a lower glucose utilization rate and slower growth were observed in the *ptsG*-deficient *C. glutamicum* strain (Fig. 2). This study has developed a strategy for engineering *C. glutamicum* to use a PTS-independent glucose uptake route instead of the original PTS pathway. This strategy contains just two steps: (1) disruption of *iolR*, and (2) overexpression of the PTS-independent glucose utilization genes. RT-PCR was applied to detect the effects of *iolR* on the expression of *iolT1*, *iolT2*, *ppgk*, and *glk*. Deletion of *iolR* partly restored the glucose utilization and growth rates of *C. glutamicum* NC-3b (Fig. 2), and increased the transcript levels of *iolT1*, *iolT2*, *ppgk*, and *glk*. The inactivation of *iolR* also relieved repression of *iolT*, most likely acting on the promoter of this gene. Introduction of a single-base deletion (320delA) into the transcriptional regulator gene *iolR* also allowed a *ptsG*-deficient *C. glutamicum* strain to utilize glucose through the PTS-independent glucose transport system [11]. The current study is the first to demonstrate that transcription of *ppgk* and *glk* is repressed by the transcriptional regulator *iolR*. It is probable that *iolR* has negative effects on the promoter operator region of glucokinase genes.

To more precisely analyze the effects of deleting *iolR* and independently overexpressing *iolT1* and *ppgk*, *iolT1* was overexpressed in *C. glutamicum* NC-3b to rule out the effect of *iolR*. *iolT1* and *ppgk* were chosen because their overexpression in a Δ*hpr* strain had a greater positive effect on biomass formation from a medium containing 10 mM inositol and 200 mM glucose than the overexpression of *iolT2* and *glk*, as well as affecting the growth of the GSM strains [19]. The results showed that overexpression of *iolT1* and *ppgk* was necessary to restore glucose transport; however, growth and glucose uptake were partly restored by overexpression of *iolT1* and *ppgk* on their own. Although Lindner et al. improved the yield of L-lysine by overexpressing *ppgK* with either *iolT1* or *iolT2* in a PTS-deficient strain, growth needed to be prolonged, which would decrease the productivity of the reaction [19]. In the current study, the engineered strain *C. glutamicum* NC-3b-3 showed glucose uptake and growth rates comparable to those of the wild-type strain *C. glutamicum* NC-3. Batch fermentation of *C. glutamicum* NC-3 and *C. glutamicum* NC-3b-3 revealed that strains engineered for PTS-independent glucose uptake showed improved production of succinic acid because of reduced pyruvate and increased PEP availability in the cells.

By decoupling glucose transport from PEP consumption, the metabolic availability of this intermediate molecule was significantly higher than with a PTS^+ strain. In *C. glutamicum*, PEP also participates as a substrate in the reaction catalyzed by pyruvate kinase (*pyk*). Future work will include the deletion of *pyk* to further increase PEP availability. It can also be expected that the production of other chemicals, such as phenylalanine, shikimate, and dehydroshikimate, which are synthesized using PEP as a precursor, will be improved in a PTS^- $glucose^+$ strain.

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Conflict of interest The authors declare that they have no conflict of interest.

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