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ORIGINAL PAPER

Double deletion of *dtsR1* and *pyc* induce efficient L-glutamate overproduction in *Corynebacterium glutamicum*

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Abstract *Corynebacterium glutamicum* strains are used for the fermentative production of L-glutamate. Five *C. glutamicum* deletion mutants were isolated by two rounds of selection for homologous recombination and identified by Southern blot analysis. The growth, glucose consumption and glutamate production of the mutants were analyzed and compared with the wild-type ATCC 13032 strain. Double disruption of *dtsR1* (encoding a subunit of acetyl-CoA carboxylase complex) and *pyc* (encoding pyruvate carboxylase) caused efficient overproduction of L-glutamate in *C. glutamicum*; production was much higher than that of the wild-type strain and Δ*dtsR1* strain under glutamate-inducing conditions. In the absence of any inducing conditions, the amount of glutamate produced by the double-deletion strain Δ*dtsR1*Δ*pyc* was more than that of the mutant Δ*dtsR1*. The activity of phosphoenolpyruvate carboxylase (PEPC) was found to be higher in the Δ*dtsR1*Δ*pyc* strain than in the Δ*dtsR1* strain and the wild-type strain. Therefore, PEPC appears to be an important anaplerotic enzyme for glutamate synthesis in Δ*dtsR1* derivatives. Moreover, this conclusion was confirmed by overexpression of *ppc* and *pyc* in the two double-deletion strains (Δ*dtsR1*Δ*ppc* and Δ*dtsR1*Δ*pyc*), respectively. Based on the data generated in this investigation, we suggest a new method that will improve glutamate production strains and provide a better understanding of the interaction(s) between the anaplerotic pathway and fatty acid synthesis.

Keywords *Corynebacterium glutamicum* · Glutamate · Pyruvate carboxylase · PC · Phosphoenolpyruvate carboxylase · PEPC

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Introduction

Corynebacterium glutamicum is an aerobic, nonpathogenic, biotin-auxotrophic, Gram-positive soil bacterium that was isolated during a screening program for L-glutamate-producing bacteria [15]. Different strains of this species are used for the fermentative production of L-glutamate and several other amino acids [7, 9, 17]. Glutamate secretion can be induced by incubating the biotin-auxotrophic wild type in a biotin-limited medium [34], although the wild type does not produce glutamate under ordinary culture conditions. The addition of selected detergents, such as Tween 40 or Tween 60 [5] or penicillin [21] to the medium can also induce glutamate overproduction. Application of the cell-wall arabinogalactan synthesis inhibitor ethambutol causes glutamate efflux [28]. A recent study suggested that the *NCgl1221* gene encodes an L-glutamic acid exporter [19]. Despite the growing literature on the genetics, physiology and metabolism of *C. glutamicum*, the molecular mechanisms underlying glutamate overproduction have yet to be elucidated, particularly in terms of changes in metabolic flux.

Early biochemical analysis demonstrated that 2-oxoglutarate dehydrogenase (ODH) activity markedly decreased

under glutamate-producing conditions [12], leading to an increase in the metabolic flux toward glutamate synthesis at the ODH branch point [35]. A recent study reported that a *C. glutamicum* mutant with a deletion of the *odhA* gene encoding the E1 subunit of the 2-oxoglutarate dehydrogenase complex (ODHC) excreted high levels of glutamate in the absence of any inducing conditions [2], suggesting that the ODH branch point is the bottleneck of the glutamate biosynthesis pathway. A novel regulatory mechanism involving ODH inhibitor protein OdhI and serine/threonine protein kinase G was found to control the ODHC in *C. glutamicum* [20, 29]. Previous studies found that the amount of DtsR protein, a homolog of the β subunit of the biotin enzyme acetyl-CoA carboxylase complex, declined in response to biotin limitation or Tween 40 addition [14]. Therefore, the decreasing level of the DtsR protein may somehow lead to glutamate production. Disruption of the *dtsR1* gene, which encodes a component of a biotin-containing enzyme complex that is involved in fatty acid synthesis, caused a reduction in ODHC activity, which indirectly contributed to glutamate biosynthesis [13]. However, these changes would be insufficient because the ODH branch is merely the final branch in the glutamate biosynthetic pathway.

Efficient glutamate production also requires that the anaplerotic pathway provides a balanced supply of acetyl-CoA and oxaloacetate (OAA), which are believed to form a precursor of glutamate, namely, citrate. The importance of a precursor supply for amino acid synthesis came into focus when Menkel et al. [18] found that the supply of OAA or aspartate might be a bottleneck for optimal lysine production. *C. glutamicum* is known to possess a complex set of anaplerotic pathways for supplying OAA, including reactions from phosphoenolpyruvate to OAA catalyzed by PEPC and from pyruvate to OAA catalyzed by pyruvate carboxylase (PC) [6, 25]. The genes of PEPC (*ppc*) and PC (*pyc*) from *C. glutamicum* have been cloned and sequenced [16, 23]. Generally, the reaction catalyzed by PC, a biotin-requiring enzyme, is very unstable in *C. glutamicum*, whereas the flux of PEPC is relatively constant according to metabolic flux analysis [36]. Using recombinant strains that overexpressed or were deficient in PC activity, it was observed that PC activity plays an important role in glutamate production with *C. glutamicum* [25, 26]. Subsequent investigations also indicated that the PC reaction is a major bottleneck in glutamate production [22]. Accordingly, PC became a primary target for the optimization of amino acid production. PEPC is known to be present with high specific activity in *C. glutamicum* strains [6, 11]. Previous studies suggested a key role of PEPC in the carbon flow to amino acids derived from the TCA cycle, and so the enzyme was proposed to be an important potential target for breeding *C. glutamicum* amino acid-producing strains because of its relatively high activity and regulatory properties [6, 39]. Moreover, it was suggested that the PEPC-catalyzed anaplerotic reaction is necessary for glutamate production induced under biotin-limited conditions by disrupting and overexpressing the genes encoding PEPC (*ppc*) and PC (*pyc*) [37]. However, some studies revealed that PEPC is dispensable for growth and lysine production by constructing and analyzing PEPC-negative mutants of *C. glutamicum* [8, 27].

In this study, for the first time, the disruption of *pyc* stimulated efficient L-glutamate production when it occurred concurrently with the deletion of *dtsR1*, which was triggered by the increased activity of PEPC, whereas glutamate production in Δ*dtsR1*Δ*ppc* was drastically lowered by disrupting the *ppc* gene. The PEPC reaction plays an important role in the anaplerotic pathway during glutamate overproduction when *dtsR1* is deleted. We also discovered that both *dtsR1* and *pyc* deletion in the strain Δ*dtsR1*Δ*pyc* may somehow enhance the activity of PEPC. Data generated here suggest that the anaplerotic pathway interacts with fatty acid synthesis through some mechanism(s). Therefore, we propose a new way to improve glutamate production strains by optimizing metabolic pathways.

Material and methods

Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 1. *C. glutamicum* strains were cultivated aerobically at 30°C with Luria–Bertani (LB) medium. *Escherichia coli* DH5α was used in the recombinant DNA procedures and routinely grown in LB medium at 37°C. When necessary, kanamycin was added at a final concentration of 50 mg l^{-1} (*E. coli*) or 25 mg l^{-1} (*C. glutamicum*).

Construction of deletion vectors

For the purpose of deleting homologous genes, vectors for integration into the *C. glutamicum* chromosome were constructed. The oligonucleotides used as polymerase chain reaction (PCR) primers in this study are listed in Table 2. *Pfu* DNA polymerase was from Fermentas (Burlington, ON, Canada). The primer pairs A1/A2, B1/B2 and C1/C2 were used to amplify the upstream regions of the *dtsR1* gene, *pyc* gene and *ppc* gene, respectively. The amplification conditions were as follows: 26 cycles, denaturation at 95°C for 30 s, annealing at 54°C for 40 s, and an extension at 72°C for 25 s. The primer pairs A3/A4, B3/B4 and C3/C4 were used to amplify the downstream regions of these genes, respectively. The amplification conditions were as

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Source or reference
E. coli strain		
DH5α	supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Invitrogen
C. glutamicum strains		
ATCC13032	Wild-type, biotin-auxotrophic	[15]
Δ*dtsR1*	Wild type derivative with in-frame deletion of *dtsR1*(cg0812)	This work
Δ*dtsR1*Δ*pyc*	ΔdtsR1 derivative with in-frame deletion of *pyc*(cg0791)	This work
Δ*dtsR1*Δ*ppc*	ΔdtsR1 derivative with in-frame deletion of *ppc*(cg1787)	This work
Δ*pyc*	Wild-type derivative with in-frame deletion of *pyc*	This work
Δ*ppc*	Wild-type derivative with in-frame deletion of *ppc*	This work
Plasmids		
pK19mobsacB	Kan^R, *E. coli* vector for generating *C. glutamicum* deletion mutants	[31]
pK19ms-ΔdtsR1	pK19mobsacB derivative containing an overlap-extension PCR product that covers the flanking regions of the *dtsR1* gene	This work
pK19ms-Δpyc	pK19mobsacB derivative containing an overlap-extension PCR product that covers the flanking regions of the *pyc* gene	This work
pK19ms-Δppc	pK19mobsacB derivative containing an overlap-extension PCR product that covers the flanking regions of the *ppc* gene	This work
pVWEx1-pyc	Overexpression of the *pyc* gene encoding the pyruvate carboxylase	[26]
pHPppc	Overexpression of the *ppc* gene encoding the phosphoenolpyruvate carboxylase	[37]

Table 2 Oligonucleotides used in this work

Name	Sequence(5′–3′)	Restriction enzyme
A1	AAGCTTGCGGCTCTCTGGATCGTG	*Hin*dIII
A2	*CGCAGTACGCTCCACCGAATACGG*TGCCGTCC	
A3	*CCGTATTCGGTGGAGCGTACTGCG*TGATGGGTTC	
A4	AAGCTTCAGTGGCATGTGGCCGTGC	*Hin*dIII
B1	AAGCTTTCTGCAGGTGGAAGCG	*Hin*dIII
B2	*CTTATCGTCTGCGAGACTTA*TCACCGGTGAG	
B3	*TAAGTCTCGCAGACGATAAG*GGTATGCGCAATG	
B4	AAGCTTCCCTTCGTGCGGC	*Hin*dIII
C1	CTGATCGTGAAGCTTGCAG	*Hin*dIII
C2	*CAGCTCGGCACATTGAGTTT*CAGCCAGGTG	
C3	*AAACTCAATGTGCCGAGCTG*CAAACACCTC	
C4	GAAAGCTTGGACTGCACAC	*Hin*dIII

Restriction sites are underlined, and complementary sequences of the primer pairs used for overlap-extension PCR are shown in *italics*

follows: 26 cycles, denaturation at 95°C for 30 s, annealing at 56°C for 40 s, and an extension at 72°C for 25 s. After the first round of PCR using the chromosomal DNA of ATCC 13032 as a template, gel purification was used to purify the PCR products by QIAquick PCR purification Kit (Qiagen, Düsseldorf, Germany). The DNA sequences of 400 bp (the upstream or downstream region of the *dtsR1* gene), 650 bp (the upstream or downstream region of the *pyc* gene) and 640 bp (the upstream or downstream region of the *ppc* gene) were obtained. In the second round of overlap-extension PCR (overlap PCR), two resulting PCR products of the upstream and the downstream regions of the genes were performed as templates in a 1:1 molar ratio, and A1/A4, B1/B4 or C1/C4 were used as primers. The amplification conditions were as follows: 30 cycles, denaturation at 95°C for 30 s, annealing at 51°C for 40 s, and an extension at 72°C for 40 s. The DNA sequences of 800 bp (the flanking regions of the *dtsR1* gene), 1,300 bp (the flanking regions of the *pyc* gene) and 1,280 bp (the flanking regions of the *ppc* gene) were obtained. The fragments of overlap PCR were then inserted into the pMD19-T vector (TaKaRa, Otsu, Japan) and sequenced to verify an absence of the corresponding genes. The fused PCR fragments were excised by the restriction enzyme *Hin*dIII (TaKaRa) from the

pMD19-T vector and ligated with *Hin*dIII-digested pK19mobsacB. The newly constructed plasmids were identified by restriction endonuclease digestion and agarose gel electrophoresis.

Isolation of deletion mutants

Corynebacterium glutamicum ATCC 13032 was prepared in order to be transformed. Glycine (1.5%) [3] and 0.1% Tween 80 were used to inhibit the crosslinking of the peptidoglycan layer [1]. The cell pellets were washed four times with 40 ml of ice-cold 10% glycerol which was used as the electroporation solution [4]. One microgram of plasmid DNA was added to the electrocompetent cells and mixed, followed by the transfer of the mixture to an electroporation cuvette (interelectrode distance: 0.2 cm) on ice. Electroporation was performed by using the Gene Pulser System™ (Bio-Rad, Hercules, CA, USA) with parameters set to 25 μF, 200 Ω and 2.5 kV. A heat shock was applied after the electroporation [40].

Two rounds of positive selection for homologous recombination were performed. Kanamycin resistance was first used to select for integration of the plasmid into the chromosome. The resistant clones were selected and tested by PCR analysis with the primer pairs of A1/A4, B1/B4 or C1/C4 using the chromosomal DNA as a template. DNA fragments of 800 and 2,432 bp were obtained from the deletion of the *dtsR1* gene. Deletion of the *pyc* gene yielded fragments of 1,300 and 3,850 bp. Upon deleting the *ppc* gene, 1,280 and 3,240 bp fragments were amplified (data not shown). Subsequently, clones that survived and grew in the presence of sucrose were selected, since it was assumed they had lost the pK19mobsacB vector [7]. The second recombination resulted either in the desired deletion or in the restoration of the wild-type characteristics. Clones selected by sucrose were tested by PCR analysis using A1/A4, B1/B4 or C1/C4 as primers to identify clones carrying the desired deletion or the allelic exchange. In the case of *dtsR1* gene deletion, only the 800 bp PCR fragment was obtained. The 1,300 and 1,280 bp DNA fragments were amplified in the case of *pyc* gene deletion and *ppc* gene deletion, respectively (data not shown).

Southern blot analysis

Southern blot analysis was used to further confirm the *C. glutamicum* deletion mutants (Fig. 1). The genome DNA of *C. glutamicum* deletion mutants was extracted using a genomic DNA Extraction Kit (Promega, Madison, WI, USA). For Southern blot analysis of *dtsR1* deletion, approximately 10 μg of genome DNA were digested with *Bam*HI and separated by electrophoresis in a 0.8% agarose gel. For the analysis of *pyc* deletion and *ppc* deletion, the genome DNA was digested with *Hin*dIII and *Nde*I, respectively. The DNA was transferred onto a nylon membrane (Hybond N+; Roche Molecular Biochemicals, Indianapolis, IN, USA) by a capillary transfer set-up and hybridized with digoxigenin-labeled DNA probes using DIG High Prime DNA Labeling and Detection Starter Kit 1 (Roche Molecular Biochemicals). DNA probes, which were amplified from chromosomal DNA of the wild type by PCR with the primers A1/A2, B1/B2 or C1/C2 [38], were generated by the random primed labeling technique. For the deletion of the *dtsR1* gene, hybridization signals were obtained at 3.6 kb with DNA from three strains (the wild-type strain, the Δ*pyc* strain and the Δ*ppc* strain) and 1.9 kb with DNA from the strains Δ*dtsR1*, Δ*dtsR1*Δ*pyc* and Δ*dtsR1*Δ*ppc* (Fig. 1a). For the deletion of the *pyc* gene, hybridization signals at 4.2 kb were obtained with DNA from four strains (the wild-type strain, the Δ*dtsR1* strain, the Δ*ppc* strain and the Δ*dtsR1*Δ*ppc* strain) and at 1.7 kb with DNA from two strains (strain Δ*pyc* and strain Δ*dtsR1*Δ*pyc*), respectively (Fig. 1b). For the disruption of the *ppc* gene, hybridization signals at 3.9 kb were obtained with DNA from the wild-type strain, the Δ*dtsR1* strain, the Δ*pyc* strain and the Δ*dtsR1*Δ*pyc* strain, and at 1.8 kb strain Δ*ppc* and strain Δ*dtsR1*Δ*ppc*, as expected (Fig. 1c).

Construction of the *ppc* and *pyc* overexpressing strains

The plasmid pHPppc, a derivative of expression vector pECt containing the *ppc* gene, was transformed into *dtsR1 ppc* double-disruptant cells by electroporation (see above), and the kanamycin-resistant transformants were selected. To induce the expression of the *ppc* gene cloned under the *trc* promoter on the pECt vector, 0.1 mM isopropyl- *β*-D-galactoside (IPTG) was added to the culture medium [37]. The pVWEx1-pyc plasmids constructed by ligating the *pyc* gene into the *C. glutamicum* expression plasmid pVWEx1 was introduced into *dtsR1 pyc* double-disruptant cells. To induce the expression of the gene cloned under *tac* promoter, 1 mM IPTG was used [26].

Culture conditions for L-glutamate production

For L-glutamate production by *C. glutamicum* strains, the cells scraped from the fresh LB plate were inoculated into LB-glucose medium (with 0.8 ml of 50% glucose stock solution in 20 ml LB) and cultured overnight in a 500-ml Erlenmeyer flask on a rotary shaker at 120 rpm [7]. For fermentation without induction, the overnight precultures were added to 20 ml of GH1 medium [per liter, 30 g glucose, 15 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.4 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.01 g $MnSO_4{\cdot}4H_2O$, 200 μg vitamin B_1 HCl, 300 μg biotin, 13.8 ml soybean protein hydrolysate

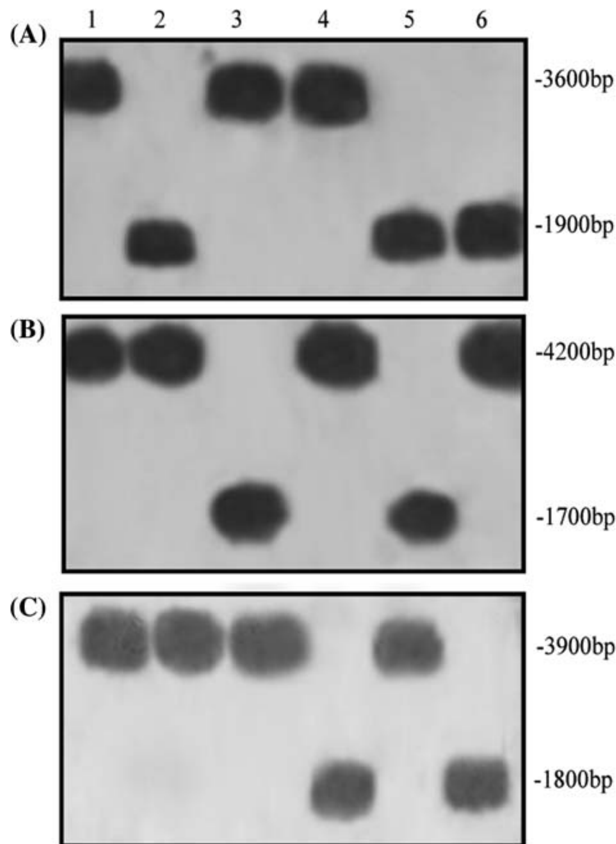

Fig. 1a–c Southern blot analysis of different *C. glutamicum* mutant strains using specific fragments amplified from chromosomal DNA of the wild type by PCR with the primers A1/A2, B1/B2 or C1/C2 used as probes. *Lane 1* shows the detection of the wild-type strain. *Lanes 2–6* show the analysis of Δ*dtsR1*, Δ*pyc*, Δ*ppc*, Δ*dtsR1*Δ*pyc* and Δ*dtsR1*Δ*ppc*, respectively. **a**, **b** and **c** indicate the genomes of different *C. glutamicum* strains detected by the *dtsR1*-specific probe (A1/A2) (wild-type, Δ*pyc* and Δ*ppc* gave hybridization signals at 3.6 kb; Δ*dtsR1*, Δ*dtsR1*Δ*pyc* and Δ*dtsR1*Δ*ppc* gave hybridization signals at 1.9 kb), *pyc*-specific probe (B1/B2) (wild-type, Δ*dtsR1*, Δ*ppc* and Δ*dtsR1*Δ*ppc* gave hybridization signals at 4.2 kb; Δ*pyc* and Δ*dtsR1*Δ*pyc* gave hybridization signals at 1.7 kb) and *ppc*-specific probe (C1/C2) (wild-type, Δ*dtsR1*, Δ*pyc* and Δ*dtsR1*Δ*pyc* gave hybridization signals at 3.9 kb; Δ*ppc* and Δ*dtsR1*Δ*ppc* gave hybridization signals at 1.8 kb), respectively

(total nitrogen, 35 g/l) and 50 g $CaCO_3$ (separately sterilized), adjusted to pH 8.0 with KOH] in a 500-ml flask with shaking. For Tween 40 (Sunshine, Nanjing, China) or penicillin (Sunshine) treatments, cells were cultured in GH2 medium [GH1 medium modified with 50 g glucose, 30 g $(NH_4)_2SO_4$ and 60 µg biotin]. The final concentration of Tween 40 or penicillin was 5 mg/ml or 0.4 U/ml, respectively. For biotin-limiting conditions, 2 ml aliquots of cell culture were inoculated into 20 ml GH3 medium (GH2 medium without biotin). During fermentation, samples were taken in order to measure the optical density at 600 nm (OD_{600}), the L-glutamate and the glucose.

Enzyme assays

All enzymes in the crude cell-free extracts obtained by sonication were measured. The crude extracts were prepared as described previously [10]. The crude extracts were further ultracentrifuged at 100,000×*g* for 30 min to assay the PEPC activity. The reaction mixture contained 100 mM TES-NaOH (pH 7.6), 2 mM PEP, 3.3 mM $MnSO_4$, 10 mM $NaHCO_3$, 0.15 mM NADH, 0.1 mM acetyl-CoA and 5 mM MDH, and the reaction was performed at 30°C. The rate of NADH consumption was measured spectrophotometrically at 340 nm. PC was assayed by a method based on Peters-Wendisch et al. [24]. The reaction mixture contained 100 mM TES-NaOH (pH 7.6), 25 mM $NaHCO_3$, 20 mM pyruvate, 4 mM ATP, 2 mM glutamate, 20 µM pyridoxal phosphate, and 2 units of pig heart GOT in a final volume of 1 ml. The reaction was performed at 30°C for 3 min. The aspartate that was formed was derivatized with phenylisothiocyanate (PITC) and analyzed by reverse-phase high-performance liquid chromatography (HPLC) using a PICO-Tag column (150 × 3.9 mm, L × I.D.; Waters Milford, MA, USA).

Analysis of growth, glucose consumption and L-glutamate production

Growth was monitored by measuring OD_{600} with the DU series 600 spectrophotometer (Beckman, Fullerton, CA, USA). The supernatant of the culture broth obtained by centrifugation was used for the determination of L-glutamate, as measured by HPLC (LC-10AT VP; Shimadzu, Japan) after derivation with phthalaldehyde (OPA) [33] using a Shim-Pack CLC-ODS column (250 mm × 4 mm, 5 µm) and detected at an absorbance of 210 nm. Mobile phase A was 0.1 M $KC_2H_3O_2$ (pH 5.89), and mobile phase B was methanol pumped at a flow rate of 1 ml min^{-1}. The column was maintained at 40°C during analysis. Glucose was analyzed using an anion exclusion column (Aminex® HPX-87H, 7.8 mm × 300 mm, Bio-Rad) by HPLC. The mobile phase was 0.005 M H_2SO_4 pumped at a flow rate of 0.5 ml min^{-1} [32]. The column was maintained at 55°C. The peak elution profile was monitored by a refractive index detector.

Results

Efficient glutamate overproduction induced by double disruption of the *dtsR1* gene and the *pyc* gene

Previous studies found that the decrease in the level of DtsR or a complex containing DtsR triggers increased synthesis of glutamate from 2-oxoglutarate by lowering ODHC activity [14]. We analyzed the growth and glutamate formation

of *C. glutamicum* wild-type ATCC 13032 and the deletion strain Δ*dtsR1* in batch cultures. As shown in Fig. 2b, the deletion of the *dtsR1* gene progressively triggered glutamate overproduction with an excess of biotin, though it inhibited the growth of *C. glutamicum*. The growth rates of wild-type and strain Δ*dtsR1* were 0.37 and 0.26 h^{-1}, respectively, under noninducing conditions; 0.32 and 0.21 h^{-1}, respectively, under biotin limitation; and 0.33 and 0.17 h^{-1}, respectively, in the presence of Tween 40. It was also observed that oleate or oleate ester supported the growth of the mutant (data not shown). The glutamate concentration in the medium reached 78.1 ± 3.9 mM after 28 h, compared with 0 mM in the wild type without any induction. For the Δ*dtsR1* strain, glutamate induction conditions (biotin limitation and Tween 40 addition) promoted glutamate production; the concentration of glutamate in the medium after 28 h reached 92.2 ± 4.6 mM with biotin limitation and 99.7 ± 3.8 mM with the addition of Tween 40, which were 18.5 and 21.7% higher, respectively, than that of the culture without inducing conditions, and were as much as that of the wild type under inducing conditions (Figs. 2, 3, 4). The glutamate yield of strain Δ*dtsR1* was 0.48 under noninducing conditions, 0.60 under biotin limitation, and 0.62 under Tween 40 addition. The glutamate production of strain Δ*dtsR1* did not change when cultured under oleate or oleate ester addition conditions, though its growth was enhanced by oleate or oleate ester. Due to the importance of the anaplerotic pathway for glutamate production (it supplies acetyl-CoA and OAA), attention should be paid to PEPC and PC. Therefore, we constructed the double-deletion strain Δ*dtsR1*Δ*pyc* and Δ*dtsR1*Δ*ppc* to observe the influence of enzymes involved in the anaplerotic pathway on glutamate biosynthesis. We analyzed the growth and glutamate formation of these two double-deletion strains under three culture conditions: (1) an excess of biotin, (2) biotin limitation and (3) addition of Tween 40 or penicillin (Figs. 2, 3, 4). The double-deletion strain, Δ*dtsR1*Δ*pyc* and Δ*dtsR1*Δ*ppc*, produced glutamate without any inducing treatment (Fig. 2c, d). Surprisingly, the Δ*dtsR1*Δ*pyc* strain produced more glutamate than either the Δ*dtsR1* strain or the Δ*dtsR1*Δ*ppc* strain without treatment (Fig. 2b–d). The glutamate yield of the Δ*dtsR1*Δ*pyc* strain was 0.63, while that of the Δ*dtsR1*Δ*ppc* strain was 0.42 without induction. The deletion of the *ppc* gene, together with the disruption of the *dtsR1* gene, lowered the amount of glutamate formed compared with single deletion of the *dtsR1* gene (Fig. 2b, d). The final concentration of glutamate reached 95.2 ± 5.3 mM in Δ*dtsR1*Δ*pyc*, which was approximately 17.8 and 33.7% higher than those for Δ*dtsR1* and Δ*dtsR1*Δ*ppc*, respectively, which produced 78.1 ± 4.6 and 63.3 ± 3.3 mM after being cultured for 28 h (Fig. 2b–d). Therefore, the deletion of the *pyc* gene enhanced glutamate synthesis on the basis of the *dtsR1* deletion. In contrast, a lack of the *ppc* gene did not promote metabolic flux in the Δ*dtsR1*Δ*ppc* strain. However, additional disruption of the *pyc* gene and the *ppc* gene inhibited the growth of *C. glutamicum* mutants (Δ*dtsR1*Δ*pyc* and Δ*dtsR1*Δ*ppc*) compared with the growth of the Δ*dtsR1* strain (Fig. 2c, d). The growth rates of strain Δ*dtsR1*Δ*pyc* and strain Δ*dtsR1*Δ*ppc* were 0.16 and 0.20 h^{-1}, respectively, without treatments. We further investigated whether the inducing conditions had additive effects on glutamate synthesis in the Δ*dtsR1*Δ*pyc* strain. As shown in Figs. 2c, 3c and 4c, glutamate synthesis of the Δ*dtsR1*Δ*pyc* strain was increased with inducing conditions compared with synthesis under noninducing conditions, and was substantially higher than that of the wild-type strain under inducing conditions. When biotin was limited, the concentration of glutamate in the Δ*dtsR1*Δ*pyc* strain reached 116.5 ± 4.3 mM after 28 h, which was 13.1% higher than the wild type (Fig. 3c). The synthesis of glutamate in the Δ*dtsR1*Δ*pyc* strain with Tween 40 addition reached

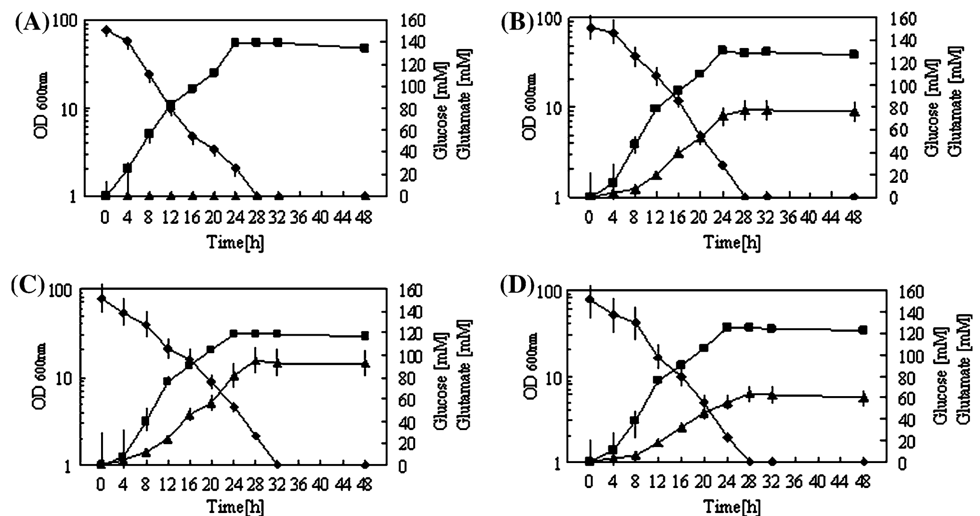

Fig. 2a–d Growth (OD_{600}, *squares*), glucose consumption (*diamonds*) and glutamate formation (*triangles*) of different *C. glutamicum* strains cultured at 30°C under noninducing conditions. **a** *C. glutamicum* wild-type ATCC 13032; **b** the Δ*dtsR1* mutant; **c** the Δ*dtsR1*Δ*pyc* mutant; **d** the Δ*dtsR1*Δ*ppc* mutant. Mean values and standard deviations of at least six independent cultures grown under these conditions are shown

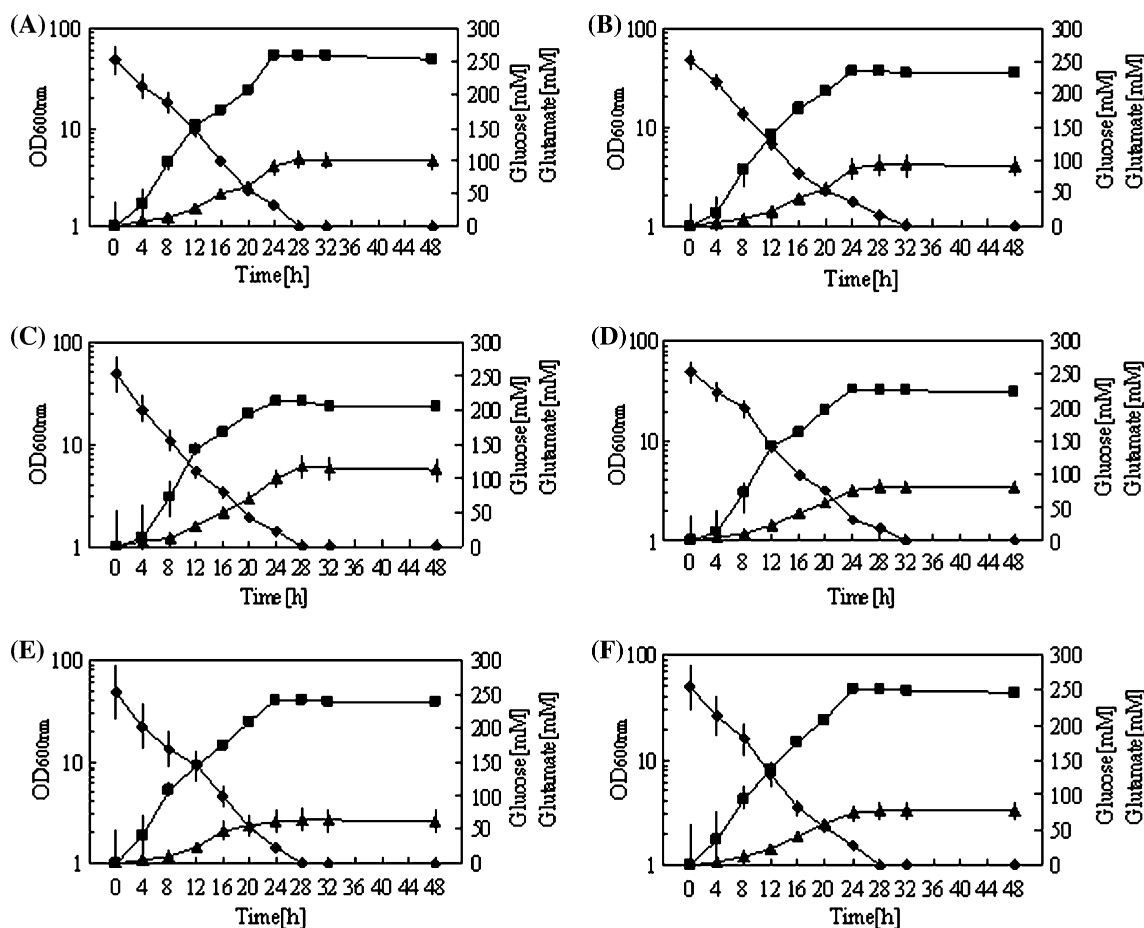

Fig. 3a–f Growth (OD_{600}, *squares*), glucose consumption (*diamonds*) and glutamate formation (*triangles*) of different *C. glutamicum* strains cultured at 30°C with biotin limitation. **a** *C. glutamicum* wild-type ATCC 13032; **b** the Δ*dtsR1* mutant; **c** the Δ*dtsR1*Δ*pyc* mutant; **d** the Δ*dtsR1*Δ*ppc* mutant; **e** the Δ*pyc* mutant; **f** the Δ*ppc* mutant. Mean values and standard deviations of at least six independent cultures grown under these conditions are shown

120.2 ± 3.7 mM after 28 h, which was 9.8% higher than the wild type (Fig. 4c). The glutamate yields of strain Δ*dtsR1*Δ*pyc* under biotin limitation and Tween 40 addition were, respectively, 0.67 and 0.68, as compared to 0.63 under noninducing conditions. Glutamate analysis of the strain Δ*dtsR1*Δ*ppc* revealed that its glutamate production was still lower than those of the Δ*dtsR1* strain and the Δ*dtsR1*Δ*pyc* strain, though its production increased under inducing conditions. These results indicate that double deletion of the *dtsR1* gene and the *pyc* gene induced a drastic metabolic flux from 2-oxoglutarate to glutamate synthesis, thereby increasing glutamate synthesis. However, the absence of the *ppc* gene did not promote glutamate production, based on *dtsR1* deletion.

PEPC plays a key role in Δ*dtsR1* derivatives for glutamate overproduction

To elucidate the reason for efficient glutamate overproduction in the double-disruption mutant strain (Δ*dtsR1*Δ*pyc*) during formation, we assayed the specific activity of PEPC and PC in the Δ*dtsR1* derivatives. We found that PEPC activity in the Δ*dtsR1*Δ*pyc* strain was 2.5 times that in the wild type when cultured without induction (Table 3). Concurrent deletion of the *dtsR1* gene and the *pyc* gene caused a distinctly increased change in PEPC activity. To find out which deletion changes the PEPC activity, we determined the PEPC activities in the Δ*dtsR1* strain and the Δ*pyc* strain, respectively. We found that the PEPC activity upon the deletion of the *dtsR1* gene was 75.6% higher than for the wild type, and the activity upon the deletion of the *pyc* gene was 24.3% higher than for the wild type (Table 3). The disruptions of the *dtsR1* gene and the *pyc* gene both play a role in increasing the PEPC activity. We also observed the influence of PC on glutamate production in Δ*dtsR1* derivatives. The PC activity in the Δ*dtsR1* derivatives (Δ*dtsR1* and Δ*dtsR1*Δ*ppc*) barely changed when they were cultured under noninducing conditions. It seems that PC is dispensable in Δ*dtsR1* derivatives for glutamate production. Therefore, PEPC is the major anaplerotic enzyme

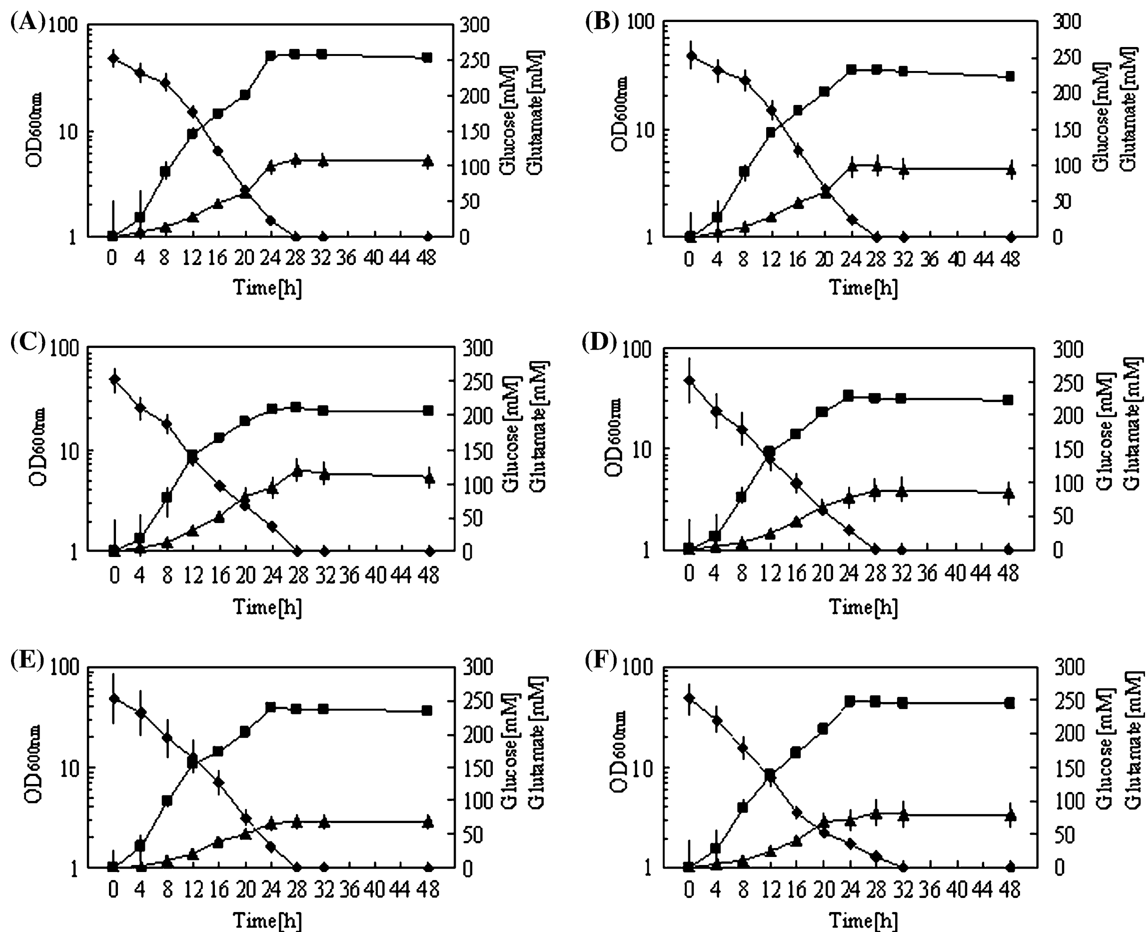

Fig. 4a–f Growth (OD_{600}, *squares*), glucose consumption (*diamonds*) and glutamate formation (*triangles*) of different *C. glutamicum* strains cultured at 30°C under the addition of Tween 40 (5 mg/ml). **a** *C. glutamicum* wild-type ATCC 13032; **b** the Δ*dtsR1* mutant; **c** the Δ*dtsR1*Δ*pyc* mutant; **d** the Δ*dtsR1*Δ*ppc* mutant; **e** the Δ*pyc* mutant; **f** the Δ*ppc* mutant. Mean values and standard deviations of at least six independent cultures grown under these conditions are shown

in Δ*dtsR1* derivatives for glutamate overproduction. This conclusion is consistent with previous studies which indicated that PEPC activity plays an important anaplerotic role, especially at the beginning of the production phase [3]. In order to further confirm our conclusions, we transformed *C. glutamicum* Δ*dtsR1*Δ*ppc* with the plasmid pHP-ppc and *C. glutamicum* Δ*dtsR1*Δ*pyc* with the plasmid pVWEx1-pyc under noninducing conditions. The PEPC and PC activities were significantly increased by the transformations of pHPppc and pVWEx1-pyc, respectively (data not shown). Results revealed that glutamate concentration was greatly increased after the *ppc* gene was overexpressed in *C. glutamicum* Δ*dtsR1*Δ*ppc*, and the yield reached 0.62, which was as high as that in the strain Δ*dtsR1*Δ*pyc*. On the other hand, overexpression of the *pyc* gene in *C. glutamicum* Δ*dtsR1*Δ*pyc* did not promote glutamate biosynthesis; it lowered it (Table 4). It is therefore clear that PEPC is much more important than PC in the anaplerotic pathway when the TCA cycle is interrupted by the decreased ODHC activity due to the disruption of the *dtsR1* gene.

However, deletion of the *pyc* gene in the wild-type strain has a greater influence on glutamate production than deletion of the *ppc* gene. Production of glutamate in the Δ*pyc* strain was approximately 61.5 and 64.4% of the wild type under biotin limitation and the addition of Tween 40, respectively (Figs. 3e, 4e). For the Δ*ppc* strain, the concentrations of glutamate were 77.3 and 76.1%, respectively, of that for the wild type under these same two inducing conditions (Figs. 3f, 4f). Therefore, it appears that the PC reaction played an important role in glutamate production with *C. glutamicum* when the *dtsR1* gene was not disrupted, which agrees with previous studies [25, 26].

Discussion

Our studies have shown that L-glutamate overproduction in the *C. glutamicum* mutant strain Δ*dtsR1*Δ*pyc* was greater than that of the deletion strain Δ*dtsR1* in the absence of glutamate-inducing conditions. Moreover, L-glutamate

Table 3 Specific activities of pyruvate carboxylase (PC) and phosphoenolpyruvate carboxylase (PEPC) in different *C. glutamicum* strains with three culture conditions for glutamate production after 18 h of cultivation

Strain	PC activity (U/mg DCW ± SD)[a]			PEPC activity (U/mg DCW ± SD)[a]		
	Without induction	Tween 40 addition	Biotin limitation	Without induction	Tween 40 addition	Biotin limitation
Wild type	32 ± 7	58 ± 10	12 ± 3	37 ± 17	36 ± 3	39 ± 5
Δ*dtsR1*	31 ± 13	47 ± 2	8 ± 7	65 ± 19	66 ± 8	65 ± 14
Δ*dtsR1*Δ*pyc*	<1	<1	<1	92 ± 7	94 ± 12	91 ± 16
Δ*dtsR1*Δ*ppc*	36 ± 5	48 ± 8	11 ± 8	<1	<1	<1
Δ*pyc*	<1	<1	<1	46 ± 4	48 ± 11	45 ± 9
Δ*ppc*	38 ± 3	59 ± 15	15 ± 6	<1	<1	<1

[a] Mean values ± standard deviations were obtained from at least three independent cultivations with at least two determinations per experiment

Table 4 Growth (OD_{600}), glutamate formation (mM) and yield (g glutamate/g glucose) for different *C. glutamicum* strains under noninducing conditions after 28 h of cultivation

C. glutamicum strain	Plasmid-bound overproduction enzyme	OD_{600}	Glutamate (mM)	Yield (g glutamate/g glucose)
Δ*dtsR1*Δ*ppc*		33.6 ± 0.4	62.7 ± 3.1	0.42
Δ*dtsR1*Δ*ppc* (pHPppc)	PEPC	37.0 ± 5.7	92.4 ± 11.3	0.62
Δ*dtsR1*Δ*pyc*		35.8 ± 1.2	95.3 ± 4.0	0.63
Δ*dtsR1*Δ*pyc* (pVWEx1-pyc)	PC	36.4 ± 2.1	77.4 ± 2.4	0.51

The data represent the mean values from at least six cultivations and the standard deviations

synthesis in Δ*dtsR1*Δ*pyc* was significantly higher than in the wild type under biotin limitation or the addition of Tween 40. Enzyme assays showed that PEPC activity in the Δ*dtsR1*Δ*pyc* strain was higher than that in the Δ*dtsR1* strain under three different conditions (without induction, under biotin limitation and with Tween 40 addition). We further noted that the activity of PEPC in the Δ*dtsR1* strain was 1.8 times that of the wild-type strain. Moreover, we also found that lack of the *pyc* gene in the wild-type strain also increased the PEPC activity. The activity of PEPC is enhanced by disrupting either the *dtsR1* gene or the *pyc* gene, and is especially enhanced when the two genes are disrupted concurrently. The efficient glutamate overproduction induced by double deletion of *dtsR1* and *pyc* is triggered by enhanced PEPC activity. Glutamate production in Δ*dtsR1* without induction is caused by not only the lower level of ODHC [14] but also the increased activity of PEPC. Therefore, the PEPC reaction constitutes the principal anaplerotic route when the TCA cycle is interrupted by decreased ODHC activity, as caused by the disruption of *dtsR1*. We further overexpressed the *pyc* gene and the *ppc* gene in the strains Δ*dtsR1*Δ*pyc* and Δ*dtsR1*Δ*ppc*, respectively. Glutamate production in Δ*dtsR1*Δ*ppc* was greatly increased after the *ppc* gene was overexpressed, while overexpression of *pyc* in Δ*dtsR1*Δ*pyc* decreased the glutamate production. This result confirms our conclusion about the importance of the PEPC reaction in the anaplerotic pathway after the *dtsR1* gene is disrupted.

Efficient glutamate production requires a sufficient supply of acetyl-CoA and OAA to form the precursor upstream of the glutamate biosynthesis pathway [30, 36]. In the Δ*dtsR1* derivatives (Δ*dtsR1*, Δ*dtsR1*Δ*pyc* and Δ*dtsR1*Δ*ppc*), OAA could not be supplied in sufficient quantities by the TCA cycle due to the decrease in ODHC activity; the OAA supply would therefore depend on the anaplerotic pathway. There are two important anaplerotic enzymes that supply OAA in *C. glutamicum*: PEPC and PC [30]. Peters-Wendisch et al. [24] reported that the in vitro specific activity of PEPC was much higher than that of PC, though PC was formerly considered to be the principal anaplerotic enzyme. PEPC is sensitive to various metabolite effectors, such as fatty acids, acetyl-CoA and fructose-1,6-bisphosphate acting as activators, and aspartate and malate acting as allosteric inhibitors [30]. Moreover, glutamate excretion is a feedback inhibitor of PEPC activity. PC from *C. glutamicum* shows no (or only a slight) dependence on the presence of acetyl-CoA [30]. The accumulation of acetyl-CoA in Δ*dtsR1* derivatives caused by the deletion of *dtsR1* may stimulate the activity of PEPC, which leads to increased PEPC activity in the strains Δ*dtsR1* and Δ*dtsR1*Δ*pyc*, while

the activity of PC was not influenced by the deletion of *dtsR1*. Therefore, we believe that the PEPC reaction is predominant in Δ*dtsR1* derivatives. The link between DtsR1 (acetyl-CoA carboxylase complex) and enzymes involved in the anaplerotic pathway, such as PC and PEPC, may occur through some activators and inhibitors. The deletion of *pyc* also increased the activity of PEPC, which may occur because the inhibition of PC to PEPC is relieved. Therefore, we detected higher PEPC activity in Δ*dtsR1*Δ*pyc* than in Δ*dtsR1* and also higher glutamate production in Δ*dtsR1*Δ*pyc* than in *dtsR1*. However, disruption of the *ppc* gene in Δ*dtsR1*Δ*ppc* induced a decrease in glutamate synthesis during fermentation. We can thus conclude that a sufficient supply of OAA is mainly provided in Δ*dtsR1* derivatives by the PEPC reaction, and that PC is dispensable for glutamate production in Δ*dtsR1* derivatives, which has not been previously reported. Previous studies using recombinant strains that overexpressed or were deficient in PC activity suggested that PC activity plays an important role in glutamate production with *C. glutamicum* [25, 26]. In our experiment, deletion of the *pyc* gene alone inhibited glutamate production to a greater degree than lack of the *ppc* gene compared to the wild type under the two inducing conditions (Figs. 3, 4). Overexpression of *pyc* in Δ*pyc* led to distinctly increased glutamate production, while expression of *ppc* had little effect on glutamate synthesis (data not shown). Thus, when *dtsR1* is not disrupted, PC activity plays an important role in glutamate production in *C. glutamicum*, which agrees with previous studies.

In summary, we determined that the PEPC reaction constitutes the major anaplerotic pathway for glutamate production when *dtsR1* is disrupted. As previously described, the lack of *dtsR1* causes decreased ODHC activity, which leads to the constitutive overproduction of glutamate through the optimization of the glutamate biosynthetic pathway [14]. The additional disruption of *pyc* in the strain Δ*dtsR1* results in a sufficient supply of OAA due to the increased activity of PEPC, leading to increased glutamate production of Δ*dtsR1*Δ*pyc* under noninducing conditions which almost reaches that of the wild-type strain under inducing conditions. Thus, disruption of *dtsR1*, together with deletion of *pyc*, produces efficient glutamate formation in *C. glutamicum* due to the increased activity of PEPC. Disrupting either (or both) of *dtsR1* and *pyc* was found to enhance the activity of PEPC in our experiment. Although some activators and inhibitors have been identified, further studies are required to fully understand the mechanisms that regulate the interactions between fatty acid synthesis and the anaplerotic pathway. In addition, the interrelationship between the enzymes involved in the anaplerotic pathway must be analyzed to obtain a detailed molecular understanding of glutamate production. We also observed that inducing conditions had additive effects on glutamate synthesis in the Δ*dtsR1*Δ*pyc* strain without any increase in PEPC activity. We think that the inducing conditions may cause additional changes in metabolic flux or membrane tension, which enhanced glutamate excretion in the Δ*dtsR1*Δ*pyc* strain. Therefore, the Δ*dtsR1*Δ*pyc* strain produces a larger amount of glutamate under inducing conditions than it does when it is cultured without induction.

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