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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Implication of gluconate kinase activity in L-ornithine biosynthesis in *Corynebacterium glutamicum*

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Abstract With the purpose of generating a microbial strain for L-ornithine production in *Corynebacterium glutamicum*, genes involved in the central carbon metabolism were inactivated so as to modulate the intracellular level of NADPH, and to evaluate their effects on L-ornithine production in *C. glutamicum*. Upon inactivation of the 6-phosphoglucoisomerase gene (*pgi*) in a *C. glutamicum* strain, the concomitant increase in intracellular NADPH concentrations from 2.55 to 5.75 mmol g^{-1} (dry cell weight) was accompanied by reduced growth rate and L-ornithine production, suggesting that L-ornithine production is not solely limited by NADPH availability. In contrast, inactivation of the gluconate kinase gene (*gntK*) led to a 51.8 % increase in intracellular NADPH concentration, which resulted in a 49.9 % increase in L-ornithine production. These results indicate that excess NADPH is not necessarily rate-limiting, but is required for increased L-ornithine production in *C. glutamicum*.

Keywords *Corynebacterium glutamicum* · Gluconate kinase · L-ornithine production · NADPH · Pentose phosphate pathway

Introduction

Corynebacterium glutamicum has long since been a subject of interest for the industrial production of amino acids, mainly L-glutamate and L-lysine. Various genetic manipulations have been applied to *C. glutamicum* to successfully develop amino acid producing strains and the metabolic consequences of genetic manipulations have been studied.

In particular, the close connection between L-lysine production and carbon flux through pentose phosphate pathway (PPP) is crucial for improving L-lysine production in *C. glutamicum* [2, 9, 14, 15, 18]. The relationship between a metabolite production and NADPH regeneration has been well established for L-lysine biosynthesis in *C. glutamicum* [2, 15, 18, 23]. NADPH is generated predominantly by the oxidative part of the PPP enzymes, glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGD), during L-lysine production in this organism [13, 23]. Diverse metabolic flux studies have revealed a correlation between L-lysine production and the NADPH supplied by carbon flux through the PPP [2, 23]. There is much evidence to indicate that redirection of the carbon flux towards PPP is a general target for biosynthesis of the desired metabolites for which NADPH supply is necessary [2, 15, 16, 18, 20].

We developed an understanding of the metabolism, and identified rational targets to optimize L-ornithine production in *C. glutamicum* [10, 11]. L-ornithine is biosynthesized from the precursor L-glutamate by the so-called cyclic pathway in *C. glutamicum* [6], in which the third step is catalyzed by NADPH-dependent reductase encoded by the *argC* gene (Fig. 1). Previous work in our laboratory demonstrated that the homologous expression of NCgl1469 ORF exhibiting N-acetyl glutamate synthase activity in a mutant strain, which carries gene-disruptions in citrulline and proline biosynthesis and is blocked in the feedback repression by the arginine repressor (ArgR), resulted in a marginal increase of L-ornithine production [10]. However, a sufficient supply of NADPH may be required to maintain

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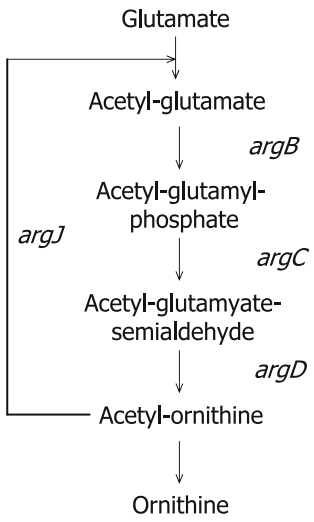

Fig. 1 Schematic representation of L-ornithine biosynthesis in *C. glutamicum*. Genes encode enzymes as follows: *argB* acetylglutamate kinase, *argC* *N*-acetylglutamate 5-semialdehyde dehydrogenase, *argD* acetylornithine aminotransferase, *argJ* ornithine acetyltransferase

a maximal carbon flux toward the L-ornithine biosynthetic pathway, in addition to overexpressing the NCgl1469 ORF. In the present study, the carbon flux of the oxidative PPP, which is regulated by changes in specific G6PDH and 6PGD activities [22] and is responsible for NADPH regeneration, was genetically engineered so as to investigate the possibility that intracellular availability of NADPH might be necessary for L-ornithine biosynthesis.

Materials and methods

Bacterial strains and growth conditions

The wild-type *C. glutamicum* strain utilized in this study was *C. glutamicum* SJ8039 (*C. glutamicum* ATCC 13032, *argF*Δ, *argR*Δ) [10] and was employed as the parent strain for constructing the mutant strains used in this study. Shake flask cultures were prepared for testing the effects of mutagenesis on L-ornithine production. For the L-ornithine production experiments, a seed culture was prepared by inoculating cells into recovery glucose medium (80 g brain heart infusion, 20 g glucose, and 60 g sorbitol l^{-1}) followed by growing the cells overnight. Cells were harvested, washed, and resuspended in 10 ml of CGI medium [0.8 g KH_2PO_4, 10 g $(NH_4)_2SO_4$, 1 g $MgSO_4{\cdot}7H_2O$, 1.2 g Na_2HPO_4, 2 mg $MnSO_4{\cdot}H_2O$, 2 mg $FeSO_4{\cdot}7H_2O$, 1 mg $ZnSO_4{\cdot}7H_2O$, 10 g yeast extract, 20 g $CaCO_3$, and 60 g glucose l^{-1}] in a 100-ml baffled flask to an OD_{600} of 0.4–0.5, and grown for 20 h. All cultures were grown at 30 °C and 200 rpm on a rotary shaker, and samples were withdrawn at regular intervals to measure L-ornithine and biomass concentrations.

Site-specific gene disruption

The bacterial strains and plasmids constructed for this study are listed in Table 1. The oligonucleotide sequences utilized in this study are also provided in Table 1. Site-specific gene disruption was conducted using the nonreplicable integration vector, pK18mobsacB, which allows for the marker-free deletion of the target gene [21]. pK18mobSacB integration vectors, harboring the internally deleted *pgi* and *gntK* genes, were constructed to create the gene-disrupted mutant strains (Table 1). These recombinant plasmids were introduced into the wild-type *C. glutamicum* strain via electroporation, and the gene-disrupted mutant strains were created via a method described previously by Yoon and Cho [24]. The locus tag numbers of the DNA sequences reported in this study are NCgl0817, NCgl2399, and NCgl2905.

Enzyme assays

Corynebacterium glutamicum cells were grown in CGI media, harvested by centrifugation during the exponential phase, and washed in 100 mM Tris/HCl buffer (pH 7.5). The cells were disrupted using glass beads, and the resulting homogenate was centrifuged to obtain a crude extract. All treatments were performed at 4 °C, and the supernatant was used immediately for enzyme assay. Protein quantity was determined by the Bradford method [4]. Activities of G6PDH, 6PGD, and glucose dehydrogenase (GD) in crude cell extracts were measured by spectrophotometric determination of NADPH formation at 340 nm, as described previously [1, 8]. Gluconate kinase (GntK) activity was measured in crude cell extracts using the coupled enzymatic assay of 6PGD as described previously [8].

Analytical methods

Cell growth in the CGI broth was estimated at OD_{600} using spectrophotometry, and L-ornithine concentrations (g l^{-1} culture medium) were determined using an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA) and a Zorbax Eclipse C_{18} column. Dry cell weight (DCW) was estimated by correlating OD_{600} values with 0.3 g DCW l^{-1} [3]. NADPH concentrations were determined by the enzymatic cycling reaction using the EnzyChrom $NADP^+$/NADPH Assay kit (BioAssay Systems, Hayward, CA, USA).

Table 1 Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence (5′–3′)[a]	Source, reference, or target[b]
C. glutamicum strains		
SJC8039	ACTC13032, *argF*Δ, *argR*Δ	[10]
SJC8242	SJC8039, *pgi*Δ	This study
SJC8399	SJC8039, NCgl2399Δ, NCgl2905Δ	This study
Plasmids		
pK18mobsacB	Mobilizable vector, *oriT sacB* Km^R	[21]
pSJ1032	pK18mobsacB derivative with 2,270-bp *Xba*I fragment of *pgi* containing internal in-frame deletion of 570-bp *Nco*I fragment	This study
pSJ1034	pK18mobsacB derivative with 2,819-bp *Xba*I fragment of the NCgl2399 ORF containing internal in-frame deletion of *Kpn*I fragment generated by crossover PCR with primer pairs GcF1-GcR1 and GcF2-GcR2 from *C. glutamicum* SJC8039 genomic DNA	This study
pSJ1035	pK18mobsacB derivative with 2,267-bp *Xba*I fragment of the NCgl2905 ORF containing internal in-frame deletion of *Sma*I fragment generated by crossover PCR with primer pairs GcF3-GcR3 and GcF4-GcR4 from *C. glutamicum* SJC8039 genomic DNA	
Primers		
GcF1	gctctagaCCCCAGAACATGCTGACG (*Xba*I)	NCgl2399 (2629324–2629341)
GcR1	ggggtaccCGCTGCTAGGGCTTTACC (*Kpn*I)	NCgl2399 (2630703–2630720)
GcF2	ggggtaccGGAACCGTCTTCGTCCACC (*Kpn*I)	NCgl2399 (2630928–2630946)
GcR2	gctctagaGTCACCGCTGGTACATCC (*Xba*I)	NCgl2399 (2632332–2632349)
GcF3	gctctagaCATTGGAGCATCCGTAGC (*Xba*I)	NCgl2905 (3212005–3212022)
GcR3	tccccgggGCCTTCACCATCGACCAA (*Sma*I)	NCgl2905 (3210861–3210878)
GcF4	tccccgggGTTCCCGAACAGATCCCC (*Sma*I)	NCgl2905 (3210303–3210320)
GcR4	gctctagaCGCTCTGACCTGCCTAAC (*Xba*I)	NCgl2905 (3209216–3209233)
PF1	gctctagaTGTACACCGCCAAAGACC (*Xba*I)	*pgi* (910012–910029)
PR1	gctctagaGAAATCGCCGCAACCAAC (*Xba*I)	*pgi* (907190–907207)

[a] Underlined sequences indicate restriction sites for restriction enzymes, as shown in *parentheses*. *Uppercase letters* refer to the sequences of bacterial genes

[b] Numerical position on *C. glutamicum* ATCC13032 genome (GenBank accession number NC_003450) is shown in *parentheses*

Results and discussion

Evaluation of the *pgi* mutant strain

Redirecting carbon flux to the PPP by disrupting the *pgi* gene might be successful in increasing the formation of metabolites for which intracellular NADPH supply is of critical importance [15, 16]. Therefore, a simple metabolic engineering strategy to redirect all carbon flux through the oxidative PPP by blocking the entry of carbon into the Embden–Meyerhof–Parnas (EMP) pathway was tested by disrupting the *pgi* gene in *C. glutamicum* SJ8039. When glucose was used as the carbon source, the *pgi*-disrupted mutant strain showed an increased intracellular NADPH concentration in comparison with that in the parent strain (Table 2). It can be assumed that the *pgi*-disrupted mutant strain overproduced NADPH, as all carbon flux had to be channeled through the PPP. However, a significantly reduced growth rate was observed in the *pgi*-disrupted mutant strain, a phenomenon that has been reported previously for *pgi*-disrupted mutants of other microbial strains of *C. glutamicum* [15], *Escherichia coli* [5], and *Saccharomyces cerevisiae* [7].

We next examined how this mutation influences G6PDH and 6PGD activity, as the reduced growth rate in the *pgi*-disrupted mutant strain may have been limited by the activities of these enzymes (Table 3). Interestingly, higher 6PGD activity was observed for the *pgi* mutant strain

Table 2 Effects of the *pgi* and *gntK* mutation in the *C. glutamicum* wild-type strain on growth, NADPH, and L-ornithine production

C. glutamicum strain	Growth (h^{-1})[a]	NADPH (mmol g^{-1} DCW)	L-Ornithine concentration (g l^{-1})	L-Ornithine yield (g g^{-1} DCW)
SJC8039	0.69	2.55	8.78	0.71
SJC8242	0.55	5.75	1.37	0.14
SJC8399	0.61	3.87	13.16	1.14

The values are averages based on the results obtained with at least three independent experiments and the standard deviations were consistently <10 %

[a] The rates observed during the exponential growth phase

grown on glucose, as compared with that of the parent strain, indicating that blocking the EMP pathway affected carbon metabolism at the level of enzyme expression. However, no discernible change in G6PDH activity was observed. This finding agreed with previous results showing that regulation of the oxidative part of the PPP of *C. glutamicum* mainly occurs by 6PGD [17]. *C. glutamicum* may have another route for biosynthesizing 6-phosphogluconate (Fig. 2), which is an intermediate of the oxidative PPP, i.e., the gluconate bypass that is typically found in *Pseudomonas* [12] and *Bacillus* [19, 25]. GD and GntK enzyme activities were determined in cell-free extracts of the parent and mutant strains to identify the physiological function of the enzymes in the gluconate bypass. The parent strain used in this study exhibited both GD and GntK activities (Table 3). More interestingly, a significant increase in GD and GntK activities was observed in the *pgi*-disrupted mutant strain, suggesting that glucose can also be catabolized, at least partly, through the gluconate bypass to produce a substantial excess of 6-phosphogluconate and NADPH. This pattern of induction is consistent with a key role of the 6-phosphogluconate active with $NADP^+$ to produce the reducing power required for biosynthetic reactions.

The growth rate of the *pgi* mutant strain on glucose was shown to be decreased significantly. The simplest interpretation might be that increased formation of gluconate from glucose, due to the increased GD activity in the *pgi* mutant strain, derepressed *gntK* expression and repressed glucose transport, as reported previously [8], resulting in

Table 3 Specific activities of the key central carbon metabolism enzymes in the *pgi* and *gntK* mutant strains

C. glutamicum strain	Specific activity (U mg $protein^{-1}$)[a]			
	G6PDH	6PGD	GD	GntK
SJC8039	0.12	0.25	1.82	0.51
SJC8242	0.10	0.59	2.48	1.04
SJC8399	0.12	0.69	2.87	0.11

[a] The values are averages based on the results obtained with at least three independent experiments and the standard deviations were consistently <10 %

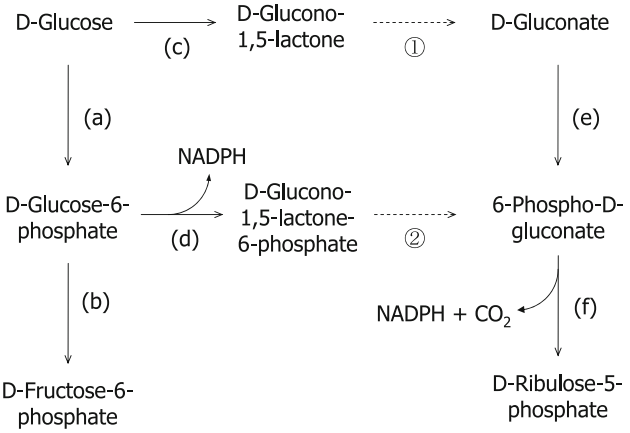

Fig. 2 Metabolic schematic of glucose and gluconate metabolism: **a** PEP:glucose phosphotransferase system; **b** phosphoglucose isomerase (*pgi*); **c** glucose dehydrogenase (*gdh*); **d** glucose 6-phosphophate dehydrogenase (*zwf*); **e** gluconate kinase (*gntK*); **f** 6-phosphogluconate dehydrogenase (*gndA*). *Dotted arrows* (① and ②) represent possible routes for gluconolactonase and 6-phosphogluconolactonase, respectively, but no genes are known to date

the reduced growth rate of the mutant strain on glucose. Consistent with this speculation, we found that the *pgi* mutant strain showed a reduced growth rate on glucose but not on sucrose/fructose (data not shown). The differences between the carbon sources are most likely a consequence of different cellular activities of central carbon metabolism. However, the production of excess NADPH in the *pgi* mutant strain did not result in an increase in L-ornithine production. These results indicate that redirecting carbon flux through the PPP and gluconate bypass led to a dramatic increase in intracellular NADPH supply, which was not necessarily rate-limiting for L-ornithine production.

Effects of GntK mutation on L-ornithine production

An alternative attempt to maximize carbon flux through the oxidative PPP was targeted to increase intracellular NADPH supply with balanced growth by disrupting the putative GntK genes (NCgl2399 and Ncgl2905 ORFs) in the parent strain, resulting in the creation of an in-frame double deletion mutant strain of the NCgl2399 and NCgl2905 ORFs. GntK enzyme activities in cell-free

extracts of the parent and double deletion mutant strains were determined. However, the specific enzyme activity assay showed residual GntK activity of 22 % by the parent strain in the double deletion mutant strain. The low residual activity in the specific GntK enzyme activity assay with the double deletion mutant strain might be due to a sugar kinase activity, which retains the ability to catalyze identical biochemical reactions on numerous substrates in vitro.

A significant increase in intracellular NADPH concentration and L-ornithine production was observed for the double-deletion mutant strain, with a slower growth rate when the double-deletion mutant and parent strains were compared. We determined the specific activities of G6PDH and 6PGD in cell-free extracts of the double-deletion mutant and parent strain to elucidate the underlying mechanism for the increased NADPH and L-ornithine production by the double-deletion mutant strain. As shown in Table 3, the specific activity of 6PGD increased significantly in the double-deletion mutant strain, as compared with that in the parent strain. The double-deletion mutant strain appeared to compensate for loss of operation of the gluconate bypass, by expanding the PPP to provide the 6-phosphogluconate required for formation of the reducing power. The observation that the parent strain, but not the mutant strain, was able to grow normally on gluconate (data not shown), confirmed that the GntK activity encoded by NCgl2399 and NCgl2905 ORFs represented the active route for 6-phosphogluconate formation. The apparent increase in L-ornithine production, resulting from blocking GntK activity, can be interpreted by an effect of increased NADPH regeneration by the stimulated 6-phosphogluconate formation via the oxidative PPP with a concomitant reduction of 6-phosphogluconate formation through GntK activity. The reduced growth rate of the double deletion mutant strain could be due to increased gluconate accumulation in the cell that repressed glucose transport, as described previously [8].

Based on these results, the mechanisms of increased L-ornithine production from glucose in the double-deletion mutant strain can be proposed. The increased capacity to regenerate NADPH with balanced growth by the increased level of 6PGD was responsible for the increase in L-ornithine production from *C. glutamicum*. The finding that L-ornithine production was improved by redirecting the carbon flux through the PPP only when the NADPH supply was adequate in the double-deletion mutant strain, but not in the *pgi* mutant strain, suggests that the whole metabolome is influenced in such a way that the pools of redox cofactors are more favorable for those biosynthetic pathways that depend on NADPH as a cofactor. The importance of reducing power in L-lysine production from *C. glutamicum* was also observed through the overexpression of the *zwf* gene [2]. G6PDH, along with 6PGD, are allosterically regulated in *C. glutamicum* [17]. Thus, it is likely that deregulation of this enzyme in the GntK-deficient mutant strain might lead to a larger effect on L-ornithine production. The present results have clearly demonstrated the potential of targeted inactivation of the gluconate bypass to significantly extend the performance of existing production strains, including L-arginine, L-lysine and L-threonine, as redirecting the carbon flux towards the PPP is desirable for a sufficient NADPH supply to produce those metabolites. In future studies, metabolic flux analysis methods will be used to quantitatively investigate the complex responses of the metabolic network to disruption of the *pgi* or *gntK* genes in *C. glutamicum*.

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