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Enhancement of L-ornithine production by disruption of three genes encoding putative oxidoreductases in *Corynebacterium glutamicum*

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Abstract Recently, *Corynebacterium glutamicum* has been shown to exhibit gluconate bypass activity, with two key enzymes, glucose dehydrogenase (GDH) and gluconate kinase, that provides an alternate route to 6-phosphogluconate formation. In this study, gene disruption analysis was used to examine possible metabolic functions of three proteins encoded by open reading frames having significant sequence similarity to GDH of *Bacillus subtilis*. Chromosomal in-frame deletion of three genes (NCgl0281, NCgl2582, and NCgl2053) encoding putative $NADP^+$-dependent oxidoreductases led to the absence of GDH activity and correlated with increased specific glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities. This finding suggested that enhanced carbon flux from glucose was directed toward the oxidative pentose phosphate (PP) pathway, when the mutant was cultivated with 6 % glucose. Consequently, the mutant showed 72.4 % increased intracellular NADPH and 66.3 % increased extracellular L-ornithine production. The enhanced activities of the oxidative PP pathway in the mutant explain both the increased intracellular NADPH and the high extracellular concentration of L-ornithine. Thus, the observed metabolic changes in this work corroborate the importance of NADPH in L-ornithine production from *C. glutamicum*.

Keywords *Corynebacterium glutamicum* · Glucose dehydrogenase activity · L-Ornithine · NADPH

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Introduction

Corynebacterium glutamicum is an important organism, which is widely used in the industrial production of various amino acids. Recently, more efficient amino acid producing strains of *C. glutamicum* have been developed through the characterization of the genes involved in their biosynthesis as well as elucidation of the carbon flux distribution in the central metabolic network under particular conditions of growth [3, 15–17]. Specific genes involved in the central metabolic network can be experimentally deleted or overexpressed so that the metabolic network can direct more carbon toward the production of metabolites.

During growth on glucose, the oxidative part of the pentose phosphate (PP) pathway, including the glucose 6-phosphate dehydrogenase (G6PD) and the 6-phosphogluconate dehydrogenase (6PGD), becomes a major source of the anabolic redox cofactor NADPH for the biosynthetic reactions of amino acids, including L-lysine and branched chain amino acids such as L-isoleucine and L-valine in *C. glutamicum* [2, 13]. Thus, it is highly relevant to redirect the carbon flux toward the oxidative PP pathway for biosynthesis of the desired metabolites, for which NADPH supply is necessary. For example, attempts have been made to redirect the carbon flux from glycolysis to the oxidative PP pathway to increase the production of L-lysine in *C. glutamicum* (1) by disruption of the phosphoglucose isomerase (PGI) gene *pgi* [15], (2) by the introduction of a mutant allele encoding a feedback-resistant 6PGD [19], and (3) by homologous overexpression of the fructose 1,6-bisphosphatase gene *fbp* [4, 6] or the G6PD gene *zwf* [3]. Further, improvements in L-lysine production with additional sources of NADPH supply have been achieved by expression of the membrane-bound transhydrogenase genes from *Escherichia coli* in *C. glutamicum* [9] as well as by replacing the glycolytic NAD-dependent

glyceraldehyde 3-phosphate dehydrogenase gene of *C. glutamicum* with the NADP-dependent glyceraldehyde 3-phosphate dehydrogenase gene of *Streptococcus mutans* [23]. In our previous study of the cellular response to a PGI-deficient mutant, we found that a blockage of the Embden–Meyerhof–Parnas (EMP) pathway caused flux rerouting and triggered an operation of the gluconate bypass, consisting of two key enzymes, glucose dehydrogenase (GDH) and gluconate kinase (GntK), to provide an alternative route for the glucose to enter the PP pathway [8]. Subsequently, the specific activity of 6PGD, which can regulate the carbon flux of the oxidative PP pathway [16, 17] and is, in part, responsible for NADPH regeneration, was increased to elevate the intracellular level of NADPH as well as the extracellular L-ornithine production by genetically inactivating the gluconate bypass enzyme, GntK [8]. These results revealed that the blockage of the gluconate bypass by the absence of GntK activity significantly induced the increased carbon flux from glucose toward the oxidative PP pathway with concomitant increase in extracellular L-ornithine production. In contrast to *Pseudomonas* [10] and *Bacillus* [20, 25], there has been no genetic evidence for the direct oxidation of glucose to gluconate via GDH in *C. glutamicum*. In order to corroborate the physiological function of the gluconate bypass affecting the activity of the oxidative PP pathway, we constructed *C. glutamicum* mutants with defined in-frame deletion mutations in three homologues of the *B. subtilis* GDH protein. We then evaluated the effects of these mutations on NADPH regeneration and L-ornithine formation in a *C. glutamicum* strain.

Materials and methods

Bacterial strains and growth conditions

The wild-type *C. glutamicum* SJC 8039 (*C. glutamicum* ATCC 13032 *argF*Δ *argR*Δ) [7] strain was used in this study as the parent strain for constructing the mutant strains. 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 in recovery glucose medium (80 g brain heart infusion, 20 g glucose, and 60 g sorbitol per liter) and growing them 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 per liter] in a 100-ml baffled flask at an OD_{600} of 0.4–0.5. They were grown until the stationary phase was reached. All cultures were grown at 30 °C on a rotary shaker at 200 rpm, 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 given in Table 1. Chromosomal DNA isolated from *C. glutamicum* ATCC 13032 was used as the template in PCR, in which *Pfu* DNA polymerase was used to amplify DNA fragments of the genes of interest.

Site-specific gene disruption was conducted using the non-replicable integration vector pK18mobsacB [21], which allows for the marker-free deletion of the target gene. pK18mobSacB integration vectors, harboring the internal in-frame deletion of the target gene, 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 by a method described previously by Yoon and Cho [24]. The locus tag numbers of the DNA sequences reported in this study are NCgl0281, NCgl2582, and NCgl2053.

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. Activities of G6PD, 6PGD, GDH, and isocitrate dehydrogenase (ICD) in crude cell extracts were measured by spectrophotometric determination of NADPH formation at 340 nm, as described previously [1, 5, 18].

Analytical methods

Cell growth in the CGI broth was estimated at OD_{600} using spectrophotometry, and amino acid concentrations (grams per liter of culture medium) were determined using an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) and a Zorbax Eclipse C_{18} column. NADPH concentrations were determined by the enzymatic cycling reaction using the EnzyChrom $NADP^+$/NADPH assay kit (BioAssay Systems, Hayward, CA, USA).

Results and discussion

Identification of the putative genes with GDH activity in *C. glutamicum*

To expand the carbon flux into the oxidative PP pathway by completely inactivating the gluconate bypass without

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		
SJC 8039	ATCC 13032 *argF*Δ *argR*Δ	[8]
SJC 8260	SJC 8039 NCgl2053Δ NCgl2582Δ NCgl0281Δ	This study
Plasmids		
pK18mobsacB	Mobilizable vector, *oriT sacB* Km^R	[21]
pSJ3513	pK18mobsacB derivative with 2,940-bp *Hin*dIII fragment of the NCgl2053 ORF containing internal in-frame deletion of *Sma*I fragment generated by crossover PCR with primer pairs 2053F1–2053R1 and 2053F2–2053R2 from *C. glutamicum* ATCC 13032 genomic DNA	This study
pSJ3515	pK18mobsacB derivative with 1,943-bp *Hin*dIII fragment of the NCgl2582 ORF containing internal in-frame deletion of *Kpn*I fragment generated by crossover PCR with primer pairs 2582F1–2582R1 and 2582F2–2582R2 from *C. glutamicum* ATCC 13032 genomic DNA	This study
pSJ3517	pK18mobsacB derivative with 2,330-bp *Hin*dIII fragment of the NCgl0281 ORF containing internal in-frame deletion of *Kpn*I fragment generated by crossover PCR with primer pairs 0281F1–0281R1 and 0281F2–0281R2 from *C. glutamicum* ATCC 13032 genomic DNA	This study
Primers		
2053F1	cccaagcttAAGACACCGGTCATGGTG (*Hin*dIII)	NCgl2053 (2256797–2256814)
2053R1	tccccgggTGCTACGGCAGCTCCAAT (*Sma*I)	NCgl2053 (2255368–2255385)
2053F2	tccccgggGACGAAGCCAGCTATGTG (*Sma*I)	NCgl2053 (2254726–2254743)
2053R2	cccaagcttACTCCTGTGCTTCCTTCC (*Hin*dIII)	NCgl2053 (2253251–2253268)
2582F1	cccaagcttCCTCCGCCCCAATTATTC (*Hin*dIII)	NCgl2582 (2840227–2840244)
2582R1	cgggtaccGGTCTCTGCAGCTTGTTC (*Kpn*I)	NCgl2582 (2841190–2841207)
2582F2	cgggtaccGGTCTGGTTTCGTTCCTG (*Kpn*I)	NCgl2582 (2841766–2841783)
2582R2	cccaagcttCCATCCTGCACTTCTCAG (*Hin*dIII)	NCgl2582 (2842710–2842727)
0281F1	cccaagcttGTCCAAGCCGATTGACTC (*Hin*dIII)	NCgl0281 (301129–301146)
0281R1	cgggtaccGATTGCTGCGACAGTCTC (*Kpn*I)	NCgl0281 (302296–302313)
0281F2	cgggtaccCAGGAACGTGGCAAGAAC (*Kpn*I)	NCgl0281 (302998–303015)
0281R2	cccaagcttCGGCTTCGGTGAGAACTT (*Hin*dIII)	NCgl0281 (304125–304142)

[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* ATCC 13032 genome (GenBank accession number NC_003450) is shown in parentheses

gluconate accumulation, which may lead to growth defect on glucose [5], we first sought to identify the glucose dehydrogenase (GDH, EC 1.1.1.47) gene expressing the key enzyme activity of the gluconate bypass, which can provide an alternate route of 6-phosphogluconate formation in *C. glutamicum* [8]. The whole genome database of *C. glutamicum* was searched using the basic local alignment search tool (BLAST) based on homology to known GDH. Three open reading frames (ORFs) encoding putative oxidoreductases, NCgl0281, NCgl2582 and NCgl2053, were found, and the deduced amino acid sequences showed 36, 34, and 31 % identities, respectively, to *Bacillus subtilis* GDH (BSU02830). NCgl0281, NCgl2582, and NCgl2053 were partially disrupted in the chromosome of the L-ornithine-producing strain *C. glutamicum* SJC 8039 to test the physiological relevance of three putative oxidoreductases in the central carbon metabolism in *C. glutamicum*.

The specific GDH activities were determined in cell extracts of *C. glutamicum* SJC 8039 and the deletion mutants grown in CGI medium containing 6 % glucose to investigate whether these ORFs encode the GDH activities in *C. glutamicum*. As shown in Table 2, the combined deletion of NCgl0281, NCgl2582, and NCgl2053 in the *C. glutamicum* SJC 8039 strain showed almost no detectable GDH activity, whereas the SJC 8039 NCgl2053Δ, SJC 8039 NCgl2582Δ, and SJC 8039 NCgl0281Δ mutants showed residual GDH activity, corresponding to 84.3, 78.7, and 66.7 % of the parent strain, respectively. The corresponding gene products may represent a functionally diverse family of oxidoreductases that catalyze the oxidation of glucose to gluconate in the presence of oxidized cofactor $NAD(P)^+$, because the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) predicts that they have the characteristic $NAD(P)^+$-binding motifs and catalytic

Table 2 Specific GDH activities in the *C. glutamicum* deletion mutants

C. glutamicum strain	SJC 8039	SJC 8039 NCgl0281Δ	SJC 8039 NCgl2582Δ	SJC 8039 NCgl2053Δ	SJC 8039 NCgl0281Δ NCgl2582Δ NCgl2053Δ
Specific activity (U mg protein^{-1})[a]	1.08	0.72	0.85	0.91	0.04

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

sequence patterns for a wide range of oxidoreductase activities. These results suggest that all three proteins encoded by NCgl0281, NCgl2582, and NCgl2053 ORFs could be the isoenzymes of GDH for the direct oxidation of glucose to gluconate in *C. glutamicum*. Thus, the mutant strain SJC 8039 NCgl0281Δ NCgl2582Δ NCgl2053Δ was used for further studies.

Physiological consequences of GDH deficiency in *C. glutamicum*

For growth comparison of the *C. glutamicum* SJC 8039 and its GDH-deficient strain, shake flask cultivations were performed in CGI medium containing 6 % glucose. The maximum specific growth rate of the GDH-deficient strain as well as its biomass formation was comparable to that of the parent strain SJC 8039 on glucose (data not shown), indicating that the GDH activity is dispensable for glucose catabolism in *C. glutamicum*. A similar observation—that a small part of the glucose catabolism was catalyzed through the gluconate bypass—was made with some bacilli [25].

To elucidate the metabolic alterations associated with GDH deficiency, specific activities of enzymes involved in the oxidative PP pathway for generation of the anabolic redox cofactor NADPH were measured in the *C. glutamicum* SJC 8039 and its GDH-deficient strain grown on 6 % glucose. Table 3 shows the specific activities of G6PD and 6PGD in cell extracts of the parent and its GDH-deficient strain grown to the mid-exponential growth phase. The specific activities of G6PD and 6PGD of the GDH-deficient strain were significantly higher than those of the parent strain. Consistent with our earlier study of mutant deficiency in gluconate kinase (GntK), we found in the present study that a loss of operation of the gluconate bypass system, by inactivating GDH activity, significantly induces the oxidative PP pathway enzymes, G6PD and 6PGD. However, it appears that the consequence of GDH inactivation has a different effect in activating the oxidative activity of the PP pathway from that observed previously in GntK inactivation. In *C. glutamicum*, at least some of the glucose can be taken up by the non-phosphotransferase system (non-PTS) on high glucose concentration, as shown by Lindner et al. [11, 12] and unphosphorylated glucose, if any, can be linked to gluconate bypass, instead of being converted into glucose 6-phosphate to be channeled into the EMP and the oxidative PP pathways. The increased flux from glucose 6-phosphate toward the oxidative PP pathway by completely eliminating the competing pathway forming 6-phosphogluconate in the GDH-deficient strain could be due to an increase of the key enzyme activities of the oxidative PP pathway, G6PD and 6PGD, along with an increase in the concentration of glucose 6-phosphate by PTS-independent phosphorylation of glucose. However, in the GntK-deficient strain, the accumulated gluconate inside the cell, due to the inactivation of GntK, might interfere with the strong binding of the GntR-type transcriptional repressors GntR1 and GntR2 to their target promoters, including the 6PGD gene [5], which can lead to activation of the oxidative PP pathway.

Table 3 Effects of GDH inactivation in the *C. glutamicum* strain on NADPH and specific activities of the key enzymes generating NADPH

C. glutamicum strain	NADPH (μM)	Specific activity (U mg protein^{-1})		
		G6PD	6PGD	ICD
SJC 8039	2.03	0.12	0.35	0.85
SJC 8039 NCgl0281Δ NCgl2582Δ NCgl2053Δ	3.50	0.21	0.47	0.91

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

To test the physiological relevance of the increased specific enzyme activities of G6PD and 6PGD by loss of GDH activity, the intracellular levels of NADPH in the parent and its GDH-deficient strains were compared during growth on high glucose concentration (Table 3). The GDH-deficient strain showed a 1.7-fold increase in NADPH production compared to the parent strain, which was in agreement with the result of the specific enzyme activity assays of G6PD and 6PGD and suggested that the oxidative PP pathway flux in the GDH-deficient strain was significantly increased.

Whereas the major pathway of NADPH formation for the biosynthetic demand in *C. glutamicum* on glucose is the oxidative PP pathway with the two NADPH-generating enzymes G6PD and 6PGD, *C. glutamicum* may employ $NADP^+$-dependent ICD in the TCA cycle to increase NADPH production. In order to confirm this possibility, the specific ICD activities of the parent and its GDH-deficient strains were compared during growth on 6 % glucose. As shown in Table 3, the parent strain showed a specific ICD activity of 0.85 U mg protein^{-1}. Similar specific activity of ICD was detected in the GDH-deficient strain, suggesting that an increased supply of NADPH in the GDH-deficient strain mostly comes from the increased activity of the two NADPH-generating enzymes G6PD and 6PGD. This result shows that the inactivation of GDH activity in fact led to an enhancement of NADPH availability by activating the oxidative reactions of the PP pathway enzymes, G6PD and 6PGD, in the *C. glutamicum* SJC 8039 strain.

Effect of GDH deficiency on L-ornithine production

The parent strain used in this study for constructing the GDH-deficient mutant, *C. glutamicum* SJC 8039, produced significant levels of L-ornithine when grown on glucose [8]. This is simply the consequence of disrupting the arginine-mediated feedback repressor gene *argR*, which in turn derepressed the expression of the L-ornithine biosynthetic genes *argCJBD*, and also disrupting the *argF* gene to prevent conversion of L-ornithine to citrulline. In order to test if the increased NADPH supply in the GDH-deficient strain can function as a driving force for increasing L-ornithine production, the parent and its GDH-deficient strains were compared for L-ornithine production on glucose. As shown in Fig. 1, the inactivation of GDH activity in *C. glutamicum* SJC 8039 was found to significantly improve L-ornithine production. The present study revealed that the inactivation of GDH activity leads to increased specific enzyme activities of the oxidative PP pathway, including those of G6PD and 6PGD, and to an increased level of intracellular NADPH as well as extracellular L-ornithine production. These findings also complement the previous results, which demonstrated that increased activity of 6PGD, a key enzyme of the oxidative PP pathway, due to the inactivation of GntK activity in the gluconate bypass in *C. glutamicum* SJC 8039, is linked to the elevated production of NADPH and L-ornithine on glucose.

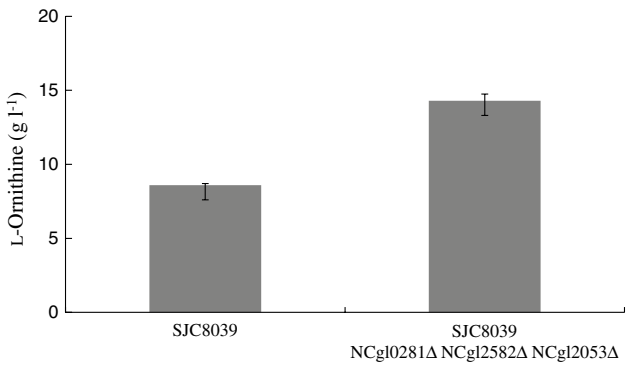

Fig. 1 L-Ornithine production of *C. glutamicum* SJC 8039 and its GDH-deficient strain. The values are averages based on the results obtained from at least three independent experiments, where the standard deviation was <5 %

An attempt was made in this work to increase the intracellular supply of NADPH for L-ornithine biosynthesis using *C. glutamicum* as the host strain, because 2 moles of NADPH are required for the synthesis of 1 mole of L-ornithine. Although G6PD and 6PGD are the main sites of central carbon metabolism generating NADPH in *C. glutamicum*, GDH activity catalyzing the oxidation of glucose to gluconate with $NADP^+$ as a coenzyme has been detected in the *C. glutamicum* SJC 8039 strain [8]. Therefore, the increased NADPH generation in the GDH-deficient strain can be attributed to an internal alteration of the NADPH balance, resulting from the flexibility of the oxidative part of PP pathway. The redistribution of the carbon flux into the oxidative part of the PP pathway is adjusted according to the NADPH demand and controlled by changing the activity of two key enzymes of the pathway generating NADPH: G6PD and 6PGD [14, 16]. Our strategy of blocking the entry of glucose into the gluconate bypass to expand the oxidative reactions of the PP pathway under growth conditions of high glucose concentration appears to be more relevant to the non-PTS glucose uptake system, which is not coupled to glucose phosphorylation for entry into the EMP and the oxidative PP pathways. Recently, increased NADPH-generating capacity in the oxidative part of the cyclized PP pathway was described for a glyceraldehyde 3-phosphate dehydrogenase (GapA) deletion mutant in *C. glutamicum* where no phosphoenolpyruvate was formed during glucose catabolism, and thus glucose uptake via the PTS was impossible [22]. Future optimization of L-ornithine production in the GDH-deficient strain could be achieved by constructing a PTS-negative mutant and ensuring overexpression of the inositol transporters IolT1 or IolT2, as well as the PolyP/ATP-dependent glucokinase gene *ppgK*, to allow for a sufficient growth rate of the PTS-negative mutant with an increased availability of glucose 6-phosphate by PTS-independent phosphorylation of glucose [12]. From a biotechnological point of view, it would be interesting to study if the metabolic engineering strategy of redirecting carbon flux toward the oxidative PP pathway by blocking the gluconate bypass can be generalized for the biosynthesis of products demanding NADPH as a cofactor.

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