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# Effect of Increased Glutamate Availability on L-Ornithine Production in *Corynebacterium glutamicum*

**Hwang, Joong-Hee, Gui-Hye Hwang, and Jae-Yong Cho***

*Division of Animal Science and Biotechnology, Sangji University, Wonju 220-702, Korea*

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**Glutamate availability in the *argF-argR-proBΔ* strain of *Corynebacterium glutamicum* was increased by addition of glutamate to the cell or inactivation of the phosphoenolpyruvate carboxykinase activity and simultaneous overexpression of the pyruvate carboxylase activity to assess its effect on L-ornithine production. When glutamate was increased in an L-ornithine-producing strain, the production of L-ornithine was not changed. This unexpected result indicated that the intracellular concentration and supply of glutamate is not a rate-limiting step for the L-ornithine production in an L-ornithine-producing strain of *C. glutamicum*. In contrast, overexpression of the L-ornithine biosynthesis genes (*argCJBD*) resulted in approximately 30% increase of L-ornithine production, from 12.73 to 16.49 mg/g (dry cell weight). These results implied that downstream reactions converting glutamate to L-ornithine, but not the availability of glutamate, is the rate-limiting step for elevating L-ornithine production in the *argF-argR-proBΔ* strain of *C. glutamicum*.**

**Keywords:** *argCJBD*, *Corynebacterium glutamicum*, glutamate availability, L-ornithine production, rate-limiting

*Corynebacterium glutamicum* is widely used for the fermentative production of various amino acids. L-Ornithine, an intermediate in the arginine biosynthetic pathway, is produced by the so-called cyclic pathway in *C. glutamicum* [22], in which four enzymes encoded by the *argCJBD* genes are involved and the acetyl group from acetylornithine is recycled with generation of acetylglutamate (Fig. 1). The transacetylation between acetylornithine and glutamate is mediated by the *argJ* gene product, monofunctional ornithine acetyltransferase (ArgJ), whose activity is feedback-inhibited by L-ornithine [22]. It has also been shown that *N*-acetylglutamate kinase (ArgB) activity is feedback-inhibited by arginine [22, 26].

**Corresponding author*
Phone: 82-33-730-0555; Fax: 82-33-730-0503;
E-mail: jycho@sangji.ac.kr

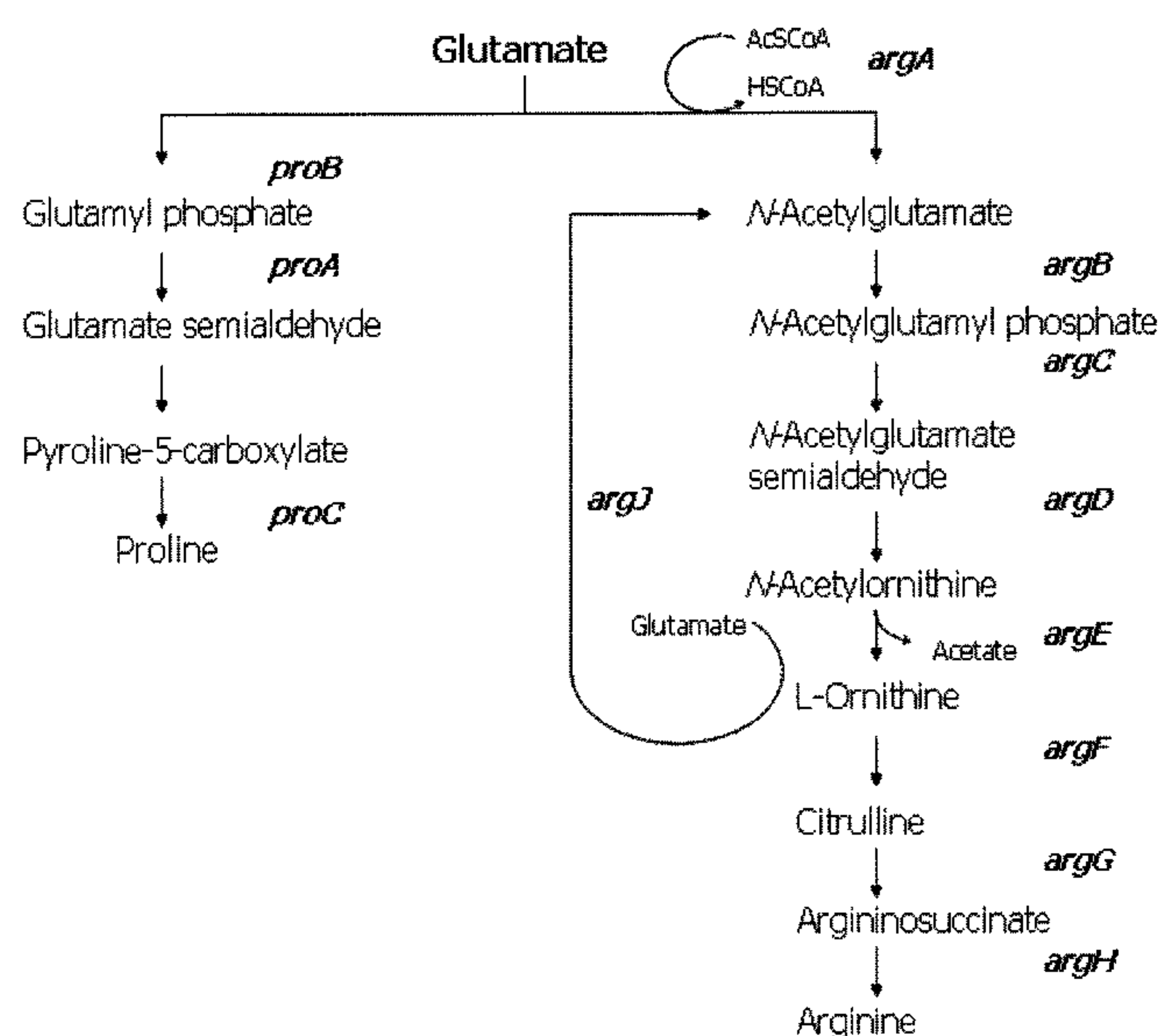


**Fig. 1.** Pathway of L-ornithine biosynthesis in *C. glutamicum*.
The genes that specify the enzymes are shown. It has been shown that *C. glutamicum* ATCC13032 displays *N*-acetylglutamate synthase activity [22], but the corresponding *argA* gene has not been found on the *C. glutamicum* ATCC13032 genome. The acetyl group of *N*-acetylornithine is recycled to *N*-acetylglutamate by the *argJ*-encoded enzyme as an alternative to deacetylation by the *argE* gene product. AcSCoA, acetyl coenzyme A; HSCoA, coenzyme A.

L-Ornithine has been produced commercially from a citrulline-requiring mutant of a coryneform bacterium [6], which was obtained by classical mutagenesis [15]. Although this auxotrophic mutant can produce a high level of L-ornithine, its growth culture may become unstable during the fermentation process owing to reversion of the auxotrophic mutant, and, consequently, the production of L-ornithine may become markedly reduced. Metabolic engineering has great potential to improve the production of many biological products by employing recombinant DNA techniques for overcoming existing limiting steps in biosynthetic pathways. Single or combined overexpression or disruption of genes encoding enzymes involved in the biosynthetic pathways for primary metabolites enables to redirect the carbon flux

towards a given metabolite [2, 14]. However, such approaches often raise the question of how to select genes to be manipulated for optimizing the biosynthetic pathway as means of improving industrial strain productivity. The importance of precursor supply for product formation has been brought to light, as the supply of oxaloacetate or aspartate was found to be rate-limiting for optimal lysine production in *C. glutamicum* [12, 18].

We have previously shown that the biosynthesis of L-ornithine could be rate-limited by the availability of glutamate in *Escherichia coli* [16], having many similarities with the L-ornithine biosynthesis in *C. glutamicum*. Therefore, to obtain information on the limiting step(s) in carbon flux to L-ornithine and to construct a well-defined recombinant L-ornithine-producing strain of *C. glutamicum*, we investigated directly the effects of glutamate supply and the combined amplification of the L-ornithine biosynthesis genes, *argCJBD*, on L-ornithine formation in an L-ornithine-producing strain of *C. glutamicum*.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

*Escherichia coli* DH5α (Gibco BRL) ($F^-$*recA1 endA1 hsdR17*[$r_k^-$ $m_k^+$] *supE44 thi-1 gyrA relA1*) was used for all recombinant DNA experiments that required a bacterial host. Cells of *E. coli* DH5α were grown in Luria-Bertani (LB) broth or on LB-agar plates prepared as described previously [23]. The wild-type strain of *C. glutamicum* ATCC13032 was employed for the construction of the mutant strains used in this study. The shake-flask cultures were prepared for testing the effects of mutagenesis on the L-ornithine production. For L-ornithine production experiments, a seed culture was made by inoculating cells into Recovery Glucose (RG) medium (80 g BHI, 20 g glucose, and 60 g sorbitol per liter) and growing the cells overnight. Cells were harvested, washed, and resuspended in 10 ml of MMY 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 $FeSO_4 \cdot 7H_2O$, 10 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 to an $OD_{600}$ of 0.4–0.5, and grown for 20 h. Kanamycin was added to a final concentration of 50 μg/ml when appropriate. All cultures were grown at 30°C and 200 rpm on a rotary shaker, and samples were withdrawn at regular intervals for measurement of L-ornithine and biomass concentrations.

### Plasmid and Strain Construction

The bacterial strains and plasmids constructed for this study are listed in Table 1. Oligonucleotide sequences used in this study are given in Table 1. Routine DNA manipulations, including DNA restriction with restriction enzymes, the Klenow fragment of DNA polymerase I or T4 DNA polymerase treatment, and ligation, were performed as described by Sambrook *et al.* [23]. Chromosomal DNA was isolated from *C. glutamicum* ATCC13032 as described previously [10] and used as a template in PCR using *Taq* or *Pfu* DNA polymerase to amplify DNA fragments of genes to clone. To construct gene-disrupted mutant strains, the target genes were cloned into the pBluescript II SK+ (Stratagene) and internally deleted. The resulting plasmid was then linearized with an appropriate restriction enzyme, and the fragment of target gene containing an internal deletion was inserted into the corresponding site of pK18mobsacB integration vector [24] to generate a gene disruption cassette. Site-specific gene disruption was performed by using these nonreplicable integration vectors, pSJ906, pSJ224, pSJ546, and pSJ711, which enable marker-free deletion of the target gene. Transformation of *C. glutamicum* was performed by electroporation using the methods of van der Rest *et al.* [27]. After integration of the plasmid, introduced into the chromosome by a single crossover, plasmid excision from the chromosome *via* a second recombination in the presence of 10% sucrose led to either construction of the gene-disrupted mutant or reconstitution of the wild-type genotype. Correct gene disruptions of the chromosomal genes in *C. glutamicum* were confirmed by a diagnostic PCR [13] using gene-specific primers outside the regions of the targeted gene (data not shown).

To construct strains overexpressing the L-ornithine biosynthesis genes, a 4,839-bp fragment of the *argCJBD* genes with their natural promoter(s) was amplified by PCR using the genomic DNA as the template and primer pair argCJBDF-argCJBDR. The PCR fragment was digested with XbaI and cloned into the corresponding site of pBluescript II SK+. The resulting plasmid was digested with SmaI in the sequence downstream of the *argCJBD* genes and ligated with the 407-bp blunt-ended EcoRI-ClaI fragment of the *rrnB* terminator amplified by a PCR using pTrc99A [1] as the template and primer pairs TrrnBF-TrrnBR. The resulting plasmid was linearized with NotI and KpnI, and the fragment containing the *argCJBD* genes and *rrnB* terminator was blunted with Klenow fragment and T4 DNA polymerase. The 5,330-bp blunt-ended DNA containing the *argCJBD* genes and *rrnB* terminator was subcloned into the blunt-ended KpnI site of pEK0 vector [9] to generate plasmid pEK-CJBD. To construct strains overexpressing the *pyc* gene, a 3,566-bp fragment of the *pyc* gene and 1,452-bp *araC*-$P_{araBAD}$ promoter region were first amplified by PCR using the *C. glutamicum* and *Escherichia coli* W3110 genomic DNA as the templates, respectively, and primer pairs pycF-pycR and ParaF-ParaR, respectively. The PCR fragments were digested with XbaI and NdeI, and cloned into the XbaI site of pBluescript II SK+. The resulting plasmid was digested with SmaI in the sequence downstream of the *pyc* gene, and ligated with the blunt-ended EcoRI-ClaI fragment of the *rrnB* terminator. The resulting plasmid was linearized with BanII, and the fragment containing the *pyc* gene flanked by the sequence of the *araC*-$P_{araBAD}$ promoter region and *rrnB* terminator was blunt-ended with T4 DNA polymerase I. Subsequently, this 5,521-bp blunt-ended DNA was subcloned into the blunt-ended KpnI site of pEK0 vector to generate plasmid pEK-PYC. The plasmids pEK-CJBD and pEK-PYC were transformed into *C. glutamicum* strains by electroporation, and the transformants were selected on RG plates containing kanamycin (50 μg/ml). Cells growing on the plates were tested for the presence of plasmids by plasmid rescue and used to test the effects of gene expression on L-ornithine production.

### Enzyme Assays

*C. glutamicum* cells were grown in MMY media, harvested by centrifugation during the exponential phase, and washed in 100 mM Tris/HCl buffer (pH 7.5). Cells were disrupted by using glass beads and the resulting homogenate was centrifuged to obtain crude extract [7]. All these treatments were performed at 4°C. *N*-Acetylglutamate 5-semialdehyde dehydrogenase (ArgC) activity was

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

| Strain, plasmid, or primer | Description or sequence (5' to 3')[a] | Source, reference, or target[b] |
|---|---|---|
| Strains | | |
| *C. glutamicum* SJ8000 | *C. glutamicum* ATCC13032, *argF*Δ | This study |
| *C. glutamicum* SJ8039 | *C. glutamicum* ATCC 13032, *argF*Δ, *argR*Δ | This study |
| *C. glutamicum* SJ8074 | *C. glutamicum* ATCC 13032, *argF*Δ, *argR*Δ, *proB*Δ | This study |
| *C. glutamicum* SJ8145 | *C. glutamicum* ATCC 13032, *argF*Δ, *argR*Δ, *proB*Δ, *pck*Δ | This study |
| Plasmids | | |
| pEK0 | *E. coli-C. glutamicum* shuttle vector, $Km^R$ | [9] |
| pK18mobsacB | Mobilizable vector, *oriT sacB* $Km^R$ | [24] |
| pSJ906 | pK18mobsacB with 1,359-bp BamHI fragment of the *argF* gene containing internal deletion of 365-bp NcoI fragment | This study |
| pSJ224 | pK18mobsacB with 1,270-bp BamHI fragment of the *argR* gene containing internal deletion of 244-bp ClaI fragment | This study |
| pSJ546 | pK18mobsacB with 1,669-bp XbaI fragment of the *proB* gene containing internal deletion of 592-bp PstI fragment | This study |
| pSJ711 | pK18mobsacB with 1,482-bp EcoRI fragment of the *pck* gene containing internal deletion of 269-bp NruI fragment | This study |
| pEK-CJBD | pEK0 with 4,839-bp XbaI fragment of the *argCJBD* genes with their natural promoter(s) followed by 407-bp blunt-ended XbaI fragment of the $T_{rrnB}$ terminator | This study |
| pEK-PYC | pEK0 with 3,566-bp NdeI-XbaI fragment of the *pyc* gene encompassed by 1,452-bp XbaI-NdeI fragment of the *araC*-$P_{araBAD}$ promoter and 407-bp blunt-ended XbaI fragment of the $T_{rrnB}$ terminator | This study |
| Primers | | |
| argFF | cgcggatccTCTTGCCAAGGTTGACGG (BamHI) | *argF* (1469796-1469813) |
| argFR | cgcggatccCCTGGGTGATATCGATGC (BamHI) | *argF* (1471137-1471154) |
| argRF | cgcggatccGACGGCATCGATCGCACC (BamHI) | *argR* (1470761-1470778) |
| argRR | cgcggatccCAGCCGTGTGCAACGTGG (BamHI) | *argR* (1472013-1472030) |
| proBF | gctctagaCCTTCTCATTGACGACACC (XbaI) | *proB* (2468068-2468086) |
| proBR | gctctagaGAGTTGGATTATGGCGAGG (XbaI) | *proB* (2469718-2469736) |
| pckF | ccggaattcTGTGTCCAACAACGGTC (EcoRI) | *pck* (3025571-3025587) |
| pckR | ccggaattcTGGCGGAGGATCTTGTTG (EcoRI) | *pck* (3027035-3027052) |
| argCJBDF | ctagtctagaTCCAGTTCAGGAAGCACC (XbaI) | *argCJBD* (1465329-1465346) |
| argCJBDR | ctagtctagaACTTTGGTCCCTCGAGTG (XbaI) | *argCJBD* (1470150-1470167) |
| pycF | ggaattccatatgTCGACTCACACATCT (NdeI) | *pyc* (706684-706701) |
| pycR | ctagtctagaCCCTTTGAGCCTTGGTCT (XbaI) | *pyc* (710232-710249 |
| paraF | ctagtctagaCTGCCAATCAGCGCTCCC (XbaI) | *araC*-$P_{araBAD}$ (71485-71502) |
| paraR | gtcatgccatggTTTCACTCCATCC (NcoI) | *araC*-$P_{araBAD}$ (70051-70063) |
| TrrnBF | ccatcgatGCTGTTTTGGCGGATGAGAGAAG (ClaI) | $T_{rrnB}$ terminator |
| TrrnBR | cggaattcAAAAGGCCATCCGTCAGGATGGCC (EcoRI) | $T_{rrnB}$ terminator |

[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 the *C. glutamicum* ATCC13032 and *E. coli* K12 genomes (GenBank accession number BX927147 and U00096, respectively) is shown in parentheses.

measured in cell extracts by spectrophotometric determination of the formation of NADPH at 340 nm during the coupled enzymatic assay of *N*-acetylornithine aminotransferase and acetylglutamyl-phosphate reductase as described previously [8]. *N*-Acetylglutamate kinase (ArgB) activity was assayed by the ferric chloride method [26], and ornithine acetyltransferase (ArgJ) activity was measured by the ninhydrin procedure of Liu *et al.* [17]. *N*-Acetylornithine 5-aminotransferase (ArgD) activity was assayed according to Friedrich *et al.* [11], and pyruvate carboxylase activity was measured in cell extracts using the coupled enzymatic assay of citrate synthase and acetyl CoA with oxaloacetate generated by the action of pyruvate carboxylase [20]. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of product in 1 min at 30°C.

### Analytical Methods

During growth in MMY broth, cell growth was estimated by measuring the $OD_{600}$ using a spectrophotometer, and L-ornithine concentrations were determined by the colorimetric method with ninhydrin as described previously [5]. The levels of L-ornithine concentration are reported as milligrams of L-ornithine per liter culture medium. Dry cell weight was estimated based on the

correlation of 1 $OD_{600}$=0.28 g of dry cell weight per liter. The levels of L-ornithine production are represented as milligrams of L-ornithine per gram of dry cell weight.

For the analysis of glutamate accumulation in the culture, aliquots were withdrawn and the cells were removed by centrifugation (5 min at 13,000 ×*g*). Glutamate in the supernatants was analyzed by HPLC following the method described elsewhere [25].

**Genbank Accession Number**

The GenBank accession number of the DNA sequences, *argF*, *argI*, *argR*, *proB*, *pck*, *pyc*, and *argCJBD*, which are described in this work is BX927147, and the nucleotide sequences of the *araC*-$P_{araBAD}$ promoter region and *rrnB* terminator have been deposited in GenBank under the accession numbers of U00096 and U13872, respectively.

## RESULTS AND DISCUSSION

### Construction of a Recombinant Strain Overproducing L-Ornithine

To address the question of whether the glutamate supply is rate-limiting for the biosynthetic pathway that converts glutamate to L-ornithine in *C. glutamicum*, we first established an experimental system consisting of the well-defined *C. glutamicum* mutant strain, which accumulates an increased level of L-ornithine. Thus, we disrupted *argF*, the structural gene for ornithine carbamoyltransferase, on the chromosome of the wild-type strain, as described in Materials and Methods. The resulting mutant strain SJ8000 was tested for L-ornithine accumulation and growth in MMY medium containing 1 mM arginine. As shown in Table 2, the strain SJ8000 required L-arginine for growth and accumulated significantly more L-ornithine within 20 h than the wild-type strain [6.78 *vs*. 0.1 mg/g (dry cell weight)]. However, the accumulation of L-ornithine from the strain SJ8000 under the conditions tested could be limited by transcriptional repression of the arginine biosynthetic pathway by ArgR [4]. We, therefore, disrupted *argR*, the gene encoding the arginine repressor, on the chromosome of the SJ8000. This resulted in a mutant (SJ8039), which produced significantly more L-ornithine than the parental strain [9.65 *vs*. 6.78 mg/g (dry cell weight)] in the medium with 1 mM L-arginine, and it was confirmed to be due to increased enzyme activities associated with the L-ornithine biosynthesis genes *argCJBD* (data not shown). In addition, the presence of 10 mM proline or the *proB* gene disruption in SJ8039 could further increase the level of L-ornithine production [13.06 or 12.65 mg/g (dry cell weight), respectively]. These results appeared to be attributed to an increased level of the intracellular glutamate concentration due to either feedback inhibition by proline of the first enzyme in the proline pathway (γ-glutamyl kinase) or the *proB* gene disruption.

### L-Ornithine Synthesis is Not Limited by Increased Availability of Glutamate

To address the question of whether the intracellular level of L-glutamate in the strain SJ8074 was limited for L-ornithine production, we first tested the effect of the addition of external L-glutamate to the culture, expecting that some are taken up and directed to the reaction for L-ornithine biosynthesis. The result showed that the cultures produced similar levels of L-ornithine [from 13.29 to 14.75 mg/g (dry cell weight)] and the levels of L-ornithine in the cells were not affected by the presence of glutamate (up to 50 mM), suggesting that the biosynthesis of L-ornithine is not limited by the availability of L-glutamate in this situation. In a parallel study, we tested if the accumulation of L-ornithine is increased when the supply of endogenous L-glutamate is replenished by inactivation of phosphoenolpyruvate carboxykinase, which catalyzes the first step in gluconeogenesis [21], and by simultaneous overexpression of pyruvate carboxylase, which plays the major role as the anaplerotic enzyme [19], since the inactivation of phosphoenolpyruvate carboxykinase and overexpression of pyruvate carboxylase in strains related to *C. glutamicum* ATCC13032 led to an improvement in glutamate production [19, 21]. Thus, to investigate the *in vivo* effect of the inactivation of phosphoenolpyruvate carboxykinase and overexpression of pyruvate carboxylase on the L-ornithine production, the *pck* gene on the chromosome of strain SJ8074 was disrupted, and the *pyc* gene was overexpressed on the *E. coli-C. glutamicum* shuttle

**Table 2.** Growth and accumulation of free L-ornithine in *C. glutamicum* ATCC13032 derivatives.

| Strain[a] | Genotype | Dry cell weight (g/l) | L-Ornithine concentration (mg/l) | L-Ornithine production[b] (mg/g [dry cell weight]) |
|---|---|---|---|---|
| *C. glutamicum* ATCC13032 | Wild type | 13.75±0.03 | 1.44±0.23 | 0.10±0.01 |
| *C. glutamicum* SJ8000 | *argF*Δ | 14.49±0.18 | 98.30±5.29 | 6.78±0.36 |
| *C. glutamicum* SJ8039 | *argF*Δ, *argR*Δ | 12.76±0.33 | 123.08±2.25 | 9.65±0.23 |
| *C. glutamicum* SJ8074 | *argF*Δ, *argR*Δ, *proB*Δ | 13.10±0.47 | 165.60±6.29 | 12.65±0.20 |

[a]The cells were grown on MMY medium with 1 mM arginine for 20 h, except for SJ8074 grown on MMY medium with 1 mM arginine plus 1 mM proline for 20 h.

[b]L-Ornithine production was calculated from the free amount of L-ornithine accumulated in 20 h by a known amount of cells. Data represent the mean of triplicate cultures±standard deviation.

**Table 3.** Specific activities of the L-ornithine biosynthesis enzymes in cell-free extracts.

| Strain | Genotype | Vector | Specific activity (units/mg protein)[a] | | | |
|---|---|---|---|---|---|---|
| | | | ArgC | ArgJ | ArgB | ArgD |
| *C. glutamicum* SJ8074 | *argF*Δ, *argR*Δ, *proB*Δ | pEK0 | 0.035 | 0.028 | 0.018 | 0.072 |
| | | pEK-CJBD | 0.146 | 0.035 | 0.103 | 0.151 |

[a]The values are means of triplicate cultures and the standard deviations were, in all cases, below 10%.

vector pEK0 under the control of the arabinose-inducible *araC*-$P_{araBAD}$ promoter [3] in the resulting Δ*pck* strain SJ8145. Strain SJ8145 grew as well as the parental strain. However, in contrast to the parental strain, it did not grow on minimal medium containing either acetate or lactate as the sole carbon source (data not shown), indicating that phosphoenolpyruvate carboxykinase in this strain was abolished. As a result, the strain SJ8145 accumulated approximately 57% more glutamate than that of parental strain (SJ8074) within 16 h after the addition of Tween 60 (14.3 *vs.* 9.1 mM; the mean values obtained from three independent cultivations and the values for replicate assays differed from the mean by <10%). To ascertain the expression of the plasmid-borne *pyc* gene, pyruvate carboxylase activity was determined in the crude cell-free extracts of the pEK-PYC-carrying strain, and the activity was compared with those of cells from the same strain carrying the vector pEK0 without insert. The specific pyruvate carboxylase activity of SJ8145 carrying the plasmid pEK-PYC in cells grown in the presence of 10 mM arabinose was approximately 57% higher than that in the same strain carrying The pEK0 plasmid (0.023 *vs.* 0.036 units/mg protein; the mean values obtained from three independent cultivations and the values for replicate assays differed from the mean by <10%). At the same time, this strain SJ8145 carrying the plasmid pEK0-PYC, when grown in the presence of 10 mM arabinose, accumulated approximately 97% more glutamate than the control strain SJ8145 carrying the vector pEK0 without insert, within 16 h after the addition of Tween 60 (17.94 *vs.* 9.10 mM; the mean values obtained from three independent cultivations and the values for replicate assays differed from the mean by <10%). However, the effect of lack of phosphoenolpyruvate carboxykinase activity and of elevated pyruvate carboxylase activity on L-ornithine production was negligible [12.17 *vs.* 12.24 mg/g (dry cell weight), respectively]. These results indicate that the increased availability of glutamate might be effective only to a certain genetic background (*e.g.*, SJ8039), and that an increased glutamate pressure cannot eventually be translated into more L-ornithine production in the strain SJ8074, because of the presence of rate limitation(s) in the L-ornithine biosynthesis pathway downstream of glutamate under the conditions we tested.

**Effect of Overexpression of L-Ornithine Biosynthesis Genes on the L-Ornithine Production in an L-Ornithine-Producing Strain**

In subsequent experiments, the pEK0 plasmid expressing the *C. glutamicum argCJBD* genes under the control of their natural promoter(s) was constructed to form pEK-CJBD. Thus, strain SJ8074 was transformed with pEK-CJBD to study the L-ornithine-producing ability of the resulting recombinant strain. In order to assess the expression of the L-ornithine biosynthesis genes cloned, the specific activities of *N*-acetylglutamate 5-semialdehyde dehydrogenase (ArgC), ornithine acetyltransferase (ArgJ), *N*-acetylglutamate kinase (ArgB), and *N*-acetylornithine 5-aminotransferase (ArgD) were determined in the crude cell-free extracts of the recombinant strain SJ8074, carrying pEK-CJBD. As shown in Table 3, the recombinant strain carrying pEK-CJBD showed approximately 1.25- to 5.7-fold higher specific enzyme activities than the host strain carrying the vector pEK0 without insert, showing that the increased enzyme activities are due to overexpression of the plasmid-borne L-ornithine biosynthesis genes. However, the recombinant strain carrying pEK-CJBD showed that the specific activity of ornithine acetyltransferase was increased the least, compared with the control strain, indicating that the enzyme activity of ornithine acetyltransferase is probably feedback-inhibited by L-ornithine overproduced in strain SJ8074, since a corynebacterial ornithine acetyltransferase has already been shown to be feedback-inhibited by L-ornithine [22]. To test the effect of the overexpressed L-ornithine biosynthesis genes on the carbon flux from glutamate to L-ornithine,

**Table 4.** Growth and accumulation of free L-ornithine in the recombinant strain of *C. glutamicum* SJ8074.

| Strain | Genotype | Plasmid | Dry cell weight (g/l) | L-Ornithine concentration (mg/l) | L-Ornithine production (mg/g[dry cell wt]) |
|---|---|---|---|---|---|
| SJ8074 | *argF*Δ, *argR*Δ, *proB*Δ | pEK0 | 12.35±0.06 | 157.27±0.50 | 12.73±0.03 |
| | | pEK-CJBD | 10.87±0.18 | 179.14±0.44 | 16.49±0.26 |

Values are same as in Table 2.

accumulation of L-ornithine by the recombinant strain carrying pEK-CJBD was determined (Table 4). Overexpression of the L-ornithine biosynthesis genes, *argCJBD*, resulted in approximately 30% increase in the L-ornithine production [12.73 *vs.* 16.49 mg/g (dry cell weight)], suggesting that L-ornithine production by strain SJ8074 is not limited by the availability of glutamate, but the overall reaction converting glutamate to L-ornithine could mainly be limited for elevating L-ornithine production.

However, the fact that the increase of L-ornithine production in the pEK-CJBD-carrying strain SJ8074 was not higher can be due to regulatory characteristics of *argJ* expression, ultimately limiting the supply of *N*-acetylglutamate for the L-ornithine production, or to the presence of other potential limiting steps in the L-ornithine biosynthesis pathway, possibly including the conversion of L-ornithine into other metabolites and the export of it out of the cells. Optimization of the degree of overexpression of the L-ornithine biosynthesis genes, *argCJBD*, and an improvement of the *N*-acetylglutamate supply for the L-ornithine biosynthesis may further enhance the L-ornithine production in a well-defined recombinant strain of *C. glutamicum*. The strain *C. glutamicum* SJ8074 would be the basis for our further metabolic engineering studies of L-ornithine overproduction.

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