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Enhancement of Ornithine Production in Proline-Supplemented *Corynebacterium glutamicum* by Ornithine Cyclodeaminase

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In this study, *Corynebacterium glutamicum* and its derived mutants were used to demonstrate the relationship between proline, glutamate, and ornithine. The maximum ornithine production was shown in the culture medium (3,295.0 mg/l) when the cells were cultured with 20 mM proline, and was 15.5 times higher than in the presence of 1 mM proline. However, glutamate, which is known as an intermediate in the process of converting proline to ornithine, did not have any positive effect on ornithine production. This suggests that the conversion of proline to ornithine through glutamate, is not possible in *C. glutamicum*. Comparative analysis between the wild-type strain, SJC 8043 (*argF*$^-$, *argR*$^-$), and SJC 8064 (*argF*$^-$, *argR*$^-$, and *ocd*$^-$), showed that *C. glutamicum* could regulate ornithine production by ornithine cyclodeaminase (Ocd) under proline-supplemented conditions. Therefore, proline directly caused an increase in the endogenous level of ornithine by Ocd, which would be a primary metabolite in the ornithine biosynthesis pathway.

Keywords: *Corynebacterium glutamicum*, ornithine, proline, ornithine cyclodeaminase

Corynebacterium glutamicum is a Gram-positive soil bacterium that is widely used for the production of a variety of amino acids [9, 20]. The ability of *C. glutamicum* strains to produce certain amino acids results from several improvements made through repeated random mutations and selection [9, 10].

The intermediate metabolite in arginine biosynthesis, ornithine, is effective in the treatment of liver diseases and helps strengthen the heart [21]. In bacteria, ornithine is synthesized by converting glutamate to ornithine in five steps through a series of acetylated intermediates [16]. Subsequently, ornithine is converted to citrulline by carbamoyltransferase (*argF*), and its biosynthesis is controlled by the repressor (*argR*) on the arginine operon [5, 17]. In addition, glutamate, the primary metabolite of ornithine and arginine biosynthesis, is converted to glutamyl phosphate by γ-glutamyl kinase [4, 22, 23].

Several studies have reported an increase in ornithine production by fermentation using auxotrophic mutants, which are citrulline- and arginine-required mutants of *Acinetobacter lwoffi* and *Brevibacterium ketoglutamicum*, respectively [2, 14]. However, these methods have several problems, such as the production of by-products or the induction of undesirable metabolic pathways caused by the overexpression of specific genes or a reversion of the auxotrophic mutants [13, 15]. On the other hand, an ornithine production-controlling reaction such as feeding of feedback effectors and culture condition control using supplements is able to simplify and minimize the process of amino acid production desired [8, 10].

Therefore, this study examined the production of ornithine through proline and glutamate supplementation to increase the primary metabolite in the ornithine biosynthesis pathway in *C. glutamicum*. This is the first report of ornithine production *via* ornithine cyclodeaminase (Ocd) in only proline-supplemented *C. glutamicum*.

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Materials and Methods

Bacterial Strains and Growth Conditions

C. glutamicum ATCC 13032 was used in this study. The deletion mutants of *C. glutamicum*, SJC 8043 (*argF*$^-$, *argR*$^-$) and SJC 8064 (*argF*$^-$, *argR*$^-$, ocd$^-$), were kindly provided by Sangji University, Korea [11]. For the ornithine production experiments, a seed culture was prepared by inoculating the LB medium, and growing the inoculated cells to an optical density (OD) of 18.0–20.0, with further growth for 20 h. The cells were inoculated in 100 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, 20 mg $MnSO_4 \cdot H_2O$, 20 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/l] for the main culture. All cultures were grown at 30°C and 150 rpm on a rotary shaker, and the samples were withdrawn at regular intervals so that the ornithine and biomass concentrations could be determined.

Analytical Methods

The level of cell growth was determined by measuring the OD at 600 nm. Ornithine analysis was performed according to the method reported by Chinard [3]. The reaction mixture contained 3 N HCl and 1% ninhydrin in a total volume of 1.0 ml. After incubation for between 40 min and 1 h at 100°C, 4 ml of 85% phosphoric acid was added and the mixture was measured at 526 nm. The residual glucose in the MMY broth was measured using a glucose (GO) assay kit (Sigma-Aldrich, Inc., U.S.A.).

Statistical Analysis

All the results were obtained from three independent samples from triplicate cultures carried out simultaneously for error analysis, and are shown along with the standard deviation represented by the error bars. The standard deviations, as well as the correlation between the concentration of proline and ornithine production, were otained using SigmaPlot software (SPSS, Chicago, IL. U.S.A.). The statistical significance was determined using a Student's *t*-test for two points. *P* values <0.05 were considered significant.

Results

Growth Rate and Ornithine Production in *C. glutamicum*

The wild-type strain *C. glutamicum* ATCC 13032 was cultivated in a MMY medium containing 6% (w/v) glucose, 1 mM arginine, and 1 mM proline. The final cell density of *C. glutamicum* was 24.9±1.7 at 48 h (Fig. 1), and the concentration of residual glucose was observed to be approximately 4% (w/v) at the stationary phase. The level of ornithine production by *C. glutamicum* in the stationary phase (at 24 h) was 290.1 mg/l. In this study, we found that the ornithine was produced by the consumption of glucose at high yield in MMY, and it consistent with the effect of organic acid production through glucose consumption [12, 19]. In addition, the concentrations of consumed glucose were similar in two other strains used in this study (data not shown), but the amounts of ornithine produced by the two mutant strains were remarkably higher than the wild-type strain (Table 1).

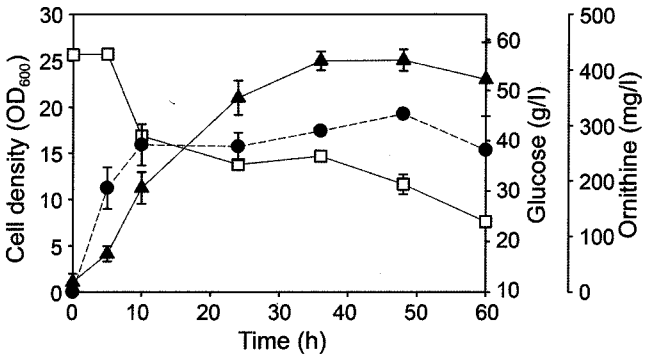

Fig. 1. The time profiles of ornithine, cell growth, and residual glucose concentration of the *Corynebacterium glutamicum* wild-type strain.
Cell density [▲]; residual glucose concentration [□]; ornithine concentration [●].

Ornithine Production Depending on Proline and Glutamate Concentrations

It has been reported that proline is converted to glutamate, which is the primary metabolite in the biosynthesis pathway of ornithine by pyrroline-5-carboxylate reductase (ProC) and proline dehydrogenase (PutA) in the metabolic pathway of proline [6, 18, 23]. Therefore, an attempt was made to regulate the concentration of glutamate, known as an

Table 1. Ornithine production by *C. glutamicum* wild type, SJC 8043 (*argF*$^-$, *argR*$^-$), and SJC 8064(*argF*$^-$, *argR*$^-$, *ocd*$^-$) during growth in proline-supplemented MMY medium in the presence of 1 mM arginine.

Strain	Ornithine concentration				
	MMY[a]		MMY+5 mM Proline[b]		
	mg/l	mg/g (dcw)[c]	mg/l	mg/g (dcw)[c]	Relative
C. glutamicum wild type	101.1±30.2	76.6±22.9	514.0±65.4	389.4±49.5	5.1
C. glutamicum SJC 8043	130.4±58.7	111.4±50.2	598.3±15.44	511.4±13.2	4.6
C. glutamicum SJC 8064	249.1±107	220.4±37.0	415.9±20.9	368.1±18.5	1.7

[a]The cells were grown on MMY medium with 1 mM arginine for 15 h.
[b]The cells were grown on MMY medium with 1 mM arginine and 5 mM proline for 15 h.
[c]Ornithine concentration was calculated from the free amount of ornithine production in 15 h by a known quantity of cells (dry cell weight). The data represent the mean of three independent cultures±standard deviation.

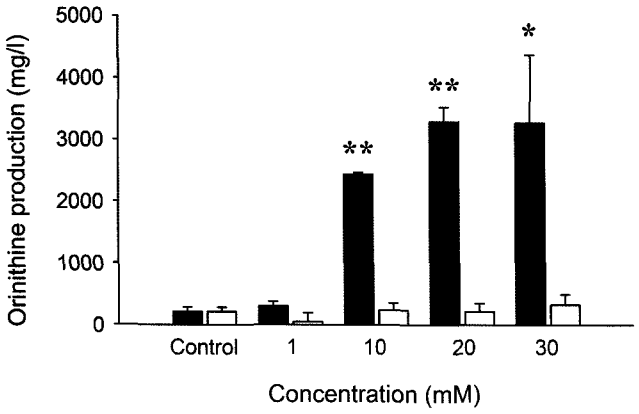

Fig. 2. Ornithine production in *C. glutamicum*.
The cultures were grown on MMY medium in the presence of 0 to 30 mM proline and glutamate, respectively. Black bars, proline supplement; White bars, glutamate supplement. The results are reported as the mean±SD and are statistically significant at $*p<0.05$ and $**p<0.01$ (n=3).

intermediate metabolite from proline to ornithine in MMY medium containing 6% glucose (w/v), 1 mM arginine, and 1 mM proline. As shown in Fig. 2, there was no increase in ornithine production by glutamate in *C. glutamicum* supplemented with each concentration of glutamate, and thus the conversion of glutamate to ornithine was not observed in the culture condition prepared in this study. However, proline had a positive effect on ornithine production depending on the proline concentration in *C. glutamicum* (Fig. 2). The maximum production of ornithine was observed in the presence of 20 mM proline in the culture medium (3,295.0 mg/l) and was 15.5-fold higher than with 1 mM proline (212.0 mg/l).

Effect of Ornithine Cyclodeaminase on Ornithine Production in Proline-Supplemented Condition

Based on a Kyoto Encyclopedia of Genes and Genomes (KEGG) database search (http://www.genome.jp/kegg), this study found Ocd (cg 1784, consisted of 1,149 nucleotides) as a key enzyme for ornithine biosynthesis from proline in *C. glutamicum* (Fig. 3). According to this pathway, proline can be used as a primary metabolite to produce ornithine directly, not *via* glutamate-5-semialdehyde as an intermediate. Moreover, in this pathway, proline is formed by the cyclodeamination of ornithine catalyzed by Ocd [1, 6, 8]. However, the direct conversion of proline to ornithine using Ocd has not been demonstrated.

Therefore, to determine the possible involvement of Ocd in the direct formation of ornithine from proline, *C. glutamicum* SJC 8064, in which the gene for Ocd is inactivated, was selected. Because SJC 8064 has two more inactivated genes for the ArgR repressor (ArgR) and ornithine carbamoyltransferase (ArgF), SJC 8043 (*argF*$^-$, *argR*$^-$) was also selected as a comparison with SJC 8064 (*argF*$^-$, *argR*$^-$, *ocd*$^-$). Three strains, consisted of the wild

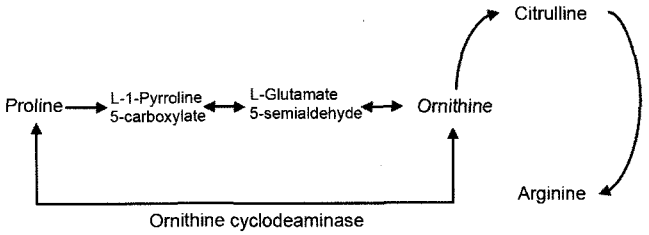

Fig. 3. Suggested pathway of ornithine biosynthesis by ornithine cyclodeaminase in *C. glutamicum*, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database search.

type and two mutant strains, were used and the level of ornithine production from supplemented proline 5 mM after 15 h incubation was determined. All the strains tested showed different levels of ornithine production under normal conditions because of their mutant type. In SJC 8064, the basal level of ornithine was about 2-fold higher than the wild type and the SJC 8043. However under proline (5 mM)-supplemented conditions, there was no significant increase in ornithine production from SJC 8064, considering the basal level of ornithine production. Table 1 shows a decrease in ornithine production from SJC 8064 along with that from the wild type, under supplemented conditions. There was no decrease observed with SJC 8043 as a parental strain containing *ocd*, under proline-supplemented conditions. The increased level of ornithine production of the wild type, SJC 8043, and SJC 8064 under proline supplemented conditions was 5.1, 4.6, and 1.7, respectively.

Originally, it was reported that Ocd converts ornithine to proline and ammonia directly in several bacterial strains, including *Pseudomonas*, *Agrobacterium tumefaciens*, *Rhizobium*, *Clostridium*, and *Brucella abortus* [1]. Indeed, we found that there was a 40% increase in ornithine production from the wild-type strain on the MMY medium supplying 2% $(NH_4)_2SO_4$ as an ammonia source compared with 0% $(NH_4)_2SO_4$ in the presence of 5 mM proline (data not shown). In summary, our results show that proline and ammonia are converted to ornithine in *C. glutamicum*, catalyzed by Ocd, even though the direct conversion of proline to ornithine using Ocd has not been demonstrated.

DISCUSSION

The present study describes the role of Ocd for the ornithine production in *C. glutamicum*. Based on the current metabolic pathway for the production of ornithine from proline [3, 22, 23], the results for the enhanced production of ornithine under proline supplementation suggests that it probably was due to an increase in the endogenous level of glutamate, which is a precursor molecule for producing ornithine, a primary metabolite in the ornithine biosynthesis pathway. However, in this study, there was no increase

in ornithine production under glutamate-supplemented conditions in *C. glutamicum*. Hwang *et al.* [11] reported that biosynthesis of ornithine is not limited by increased availability of glutamate level in addition of external glutamate to the culture, expecting that some are taken up and directed to the reaction for ornithine biosynthesis. According to their report, this situation presumed that the presence of rate limitations in the ornithine biosynthesis pathway is downstream of glutamate in *C. glutamicum*. In addition, based on the report by Lee and Cho [15], ornithine must be limited to allow an increased production of other metabolites of interest or repressed by a transcriptional regulator (*e.g.*, arginine repressor, ArgR).

However, *C. glutamicum* SJC 8064 (*argF*$^-$, *argR*$^-$, *ocd*$^-$), which was selected to examine the formation of ornithine from proline, was unaffected by the increase in ornithine production under proline-supplemented conditions. This suggests that a significant level of ornithine was formed from proline in *C. glutamicum* through the activity of Ocd. Until now, there are no reports showing that proline directly converts ornithine based on the reverse reaction of Ocd, and the precise function is still unclear.

Originally, it was reported that Ocd converts ornithine to proline and ammonia directly in several bacterial strains, including *Pseudomonas*, *Agrobacterium tumefaciens*, *Thizobium*, *Clostridium*, and *Brucella abortus* [1]. Indeed, we found that there was a 40% increase in ornithine production from the wild-type strain on the MMY medium supplying an ammonia source, and in addition, indirect clue for the reversible activity of Ocd is the fact that in the absence of proline supplement, the SJC 8064 produced about 2-fold higher than the wild type and SJC 8043.

In total, these results show that proline does influence the production of ornithine and enhances the production of ornithine by Ocd in *C. glutamicum*. In addition, Ocd under proline-supplemented conditions is a key enzyme for enhancing the biosynthesis of ornithine from proline in *C. glutamicum*.

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