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Production of L-Ornithine by Citrulline Auxotrophic Mutants of Glutamate-Producing Bacteria

CHOI, DAE KEON, WUK SANG RYU, BONG HYUN CHUNG, SOO WAN NAM AND YOUNG HOON PARK*

Genetic Engineering Research Institute, Korea Institute of Science and Technology, P.O. Box 17, Taedok Science Town, Taejon 305-606, Korea

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For the purpose of producing L-ornithine by microbial fermentation, mutant strains were developed from glutamate-producing bacteria by mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and UV irradiation. *Brevibacterium ketoglutamicum* BK1046, a L-citrulline auxotroph which is also resistant to arginine hydroxamate (Arghx), was isolated and selected as the best producer of L-ornithine. This strain was capable of producing L-ornithine at a concentration of 24 g/l after 69 hours of cultivation in the 2l jar fermentor. The optimum supplementary level of L-arginine, a substitute for L-citrulline, was found to be about 0.2 g/l in the shake-flask fermentation.

L-ornithine is an intermediate metabolite in arginine biosynthesis and belongs to the glutamic acid family (3). It is a basic amino acid and is also known to be effective for liver treatment. Its biosynthetic pathway and regulatory mechanisms have been well studied in *Escherichia coli* (13, 14) and also in glutamic acid-producing bacteria (4,10, 11, 16, 17). The formation of N-acetylglutamokinase, the second enzyme involved in these reactions, has been reported to be repressed by L-arginine in *Brevibacterium flavum* (15). Therefore the derivation of mutant strains, which are citrulline or arginine auxotrophs and also resistant to arginine analogs, is a prerequisite for the overproduction of L-ornithine. In addition, the activity of the second enzyme in the biosynthetic pathway from glutamate is inhibited by an excess amount of L-arginine and thus, the level of L-arginine should be carefully controlled in the course of the fermentative production of L-ornithine (9).

A few attempts have been made to obtain L-ornithine enzymatically from L-arginine, or by chemical systhesis (12). However, microbial fermentation has been employed as the most economically feasible process for large-scale production. The first large-scale fermentative production of L-ornithine was carried out with L-citrulline, or with L-arginine-requiring mutants derived from glutamate-producing strains. Kinoshita *et al.* (5) reported that a citrulline-requiring mutant of *Corynebacterium glutamicum* accumulated L-ornithine in a very high yield under appropriate fermentation conditions.

Recently, we developed a new high-yield strain for L-ornithine production. This strain, *Brevibacterium ketoglutamicum* BK1046, is resistant to arginine hydroxamate (Arghx) and also requires L-citrulline or L-arginine for growth. Batch fermentation was carried out with the strain to produce L-ornithine in a jar fermentor.

*Corresponding author

Key words: L-ornithine, glutamate-producing bacteria, analog resistant mutant, auxotrophic mutant

MATERIALS AND METHODS

Microorganisms

The microorganisms used in this study were *Corynebacterium glutamicum* ATCC 13032, a glutamate producing strain, *Arthrobacter citreus* ATCC 21040 and *Brevibacterium ketoglutamicum* ATCC 21092. *A. citreus* and *B. ketoglutamicum* were L-citrulline auxotrophic mutants. These parent strains produced only very small amounts of L-ornithine, as shown in Table 1. Mutant strains were derived from these strains by mutagenesis using NTG and UV irradiation.

Culture Media and Conditions

YPD medium was used for the seed culture and M9 medium as a minimal medium, with some modifications

Table 1. Characteristics of the strains used in this study.

Strains	Characteristics*	L-ornithine produced(g/l)
Corynebacterium glutamicum ATCC 13032	wild type	0
Arthrobacter citreus ATCC 21040	Cit^-	0.9
Brevibacterium ketoglutamicum ATCC 21092	Cit^-	1.8

* Cit^-; L-citrulline auxotroph.

(7). If needed, M9 medium was supplemented with 100 μg/ml of L-citrulline. The medium composition used in shake-flask fermentation was (in g/l of distilled water): glucose, 50; KH_2PO_4, 0.5; $(NH_4)_2SO_4$, 10; $MgSO_47H_2O$, 1; yeast extract, 1; $CaCO_3$, 2; and 0.1% (v/v) of stock solution containing 100 μM of various trace minerals. The pH of the initial medium was adjusted to 7.0 with 4N NaOH solution. Main fermentation was carried out in a 2l jar fermentor (Korea Fermentor Co., Ltd) with one liter culture volume containing 10% glucose, 1% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.1% $MgSO_47H_2O$, 0.5% yeast extract, and 0.1% (v/v) of mineral stock solution. Temperature and pH were maintained at 30℃ and 7.0 with 4N NH_4OH, respectively. Air was supplied at an aeration rate of 1 vvm and the agitation speed was kept at 600 rpm.

Isolation and Selection of L-Ornithine Overproducing Mutants

For the selection of Arghx resistant and L-citrulline-requiring mutants the cells were treated with NTG or UV irradiation, according to the methods of Miller (6) and Carlton (1), and cultivated on minimal agar plates containing 8 mg/ml of Arghx and 100 μg/ml of L-citrulline at 30℃ for 4 to 6 days. After colonies formed on the plates, they were replica plated to a minimal agar plates. Cells which grew on the former plates but not on the latter plates were selected as Arghx resistant and L-citrulline-requiring mutants and tested for the production of L-ornithine.

Analytical Methods

Cell growth was monitored by measuring optical density at 600 nm using a spectrophotometer (UVICON 930, USA). Glucose concentration was measured using a Glucose & Lactate Analyzer (YSI 2000, USA). L-ornithine concentration was determined colorimetrically (2) and amino acids were analyzed using a HPLC (Waters Associates, USA) with PICO.TAG column (15 cm×3.9 mm, 4 μm). Culture broth was filtered through a 0.45 μm filter. Fifty μl of sample was taken and dried under a vacuum. After drying, ten μl of redrying solution was added to the sample and shake gently. Redrying solution was consisted of a 2:2:1 mixture (by volume) of ethanol: water: triethylamine. It was dried again under a vacuum and derivatized by the addition of twenty μl of the phenylisothiocyanate (PITC) reagent solution. Derivatization reagent was consisted of a 7:1:1:1 solution (by volume) of ethanol: triethylamine: water: phenylisothiocyanate (PITC). The mixture was allowed to react at room temperature for twenty minutes and redried. To the redried sample, two hundred and fifty μl of sample diluent solution was added and mixed gently. For the preparation of sample diluent solution, seven hundred and ten mg of Na_2HPO_4 was dissolved in 1 l of distilled water and the pH was adjusted to 7.4 with 10% phosphoric acid. The resulting solution was mixed with acetonitrile so that acetonitrile equals 5% by volume. After mixing, the sample solution was used for HPLC analysis. Cell growth inhibition was determined by the comparison of the cell growth in minimal medium supplemented with increasing concentrations of Arghx with the cell growth in the minimal medium.

RESULTS AND DISCUSSION

Sensitivity of Glutamate-Producing Bacteria to Arghx

To isolate the mutants resistant to Arghx, an L-arginine analog, the sensitivity of glutamate-producing bacteria to Arghx was investigated. As observed in other analog resistant mutants the Arghx-resistant property is important since it results from the regulatory abnormality of the enzymes involved in the biosynthetic pathway of L-arginine, which consequently offers a potential for the overproduction of L-ornithine, especially when accompanied with the L-citrulline auxotrophic property. Sensitivity to Arghx was determined by cultivating a bacterial strain at 30℃ for 5 to 7 days on the minimal medium supplemented with varying concentrations of Arghx. *Arthrobacter citreus* ATCC 21040 and *Brevibacterium ketoglutamicum* ATCC 21092 were sensitive to Arghx at a concentration of 8 mg/ml, whereas the growth of *Corynebacterium glutamicum* ATCC 13032 was completely inhibited by Arghx at a concentration of 4 mg/ml. The sensitivity patterns of *B. ketoglutamicum* ATCC 21092 and BK1046 (an Arghx resistant mutant derived from ATCC 21092) to Arghx in a liquid minimal medium are shown in Fig. 1. The BK1046 mutant was found to be almost insensitive to Arghx at a concentration of 8 mg/ml, which suggested the possibility that this strain could be used for fermentative production of L-ornithine.

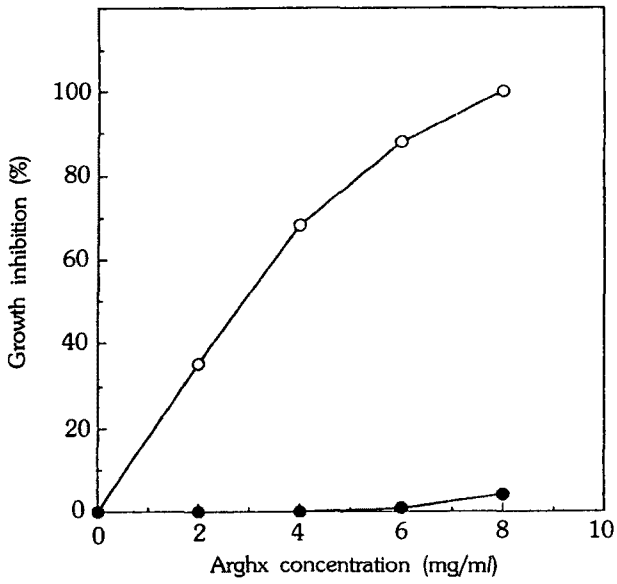

Fig. 1. Growth inhibition of ATCC 21092 (○) and BK 1046 (●) by arginine hydroxamate (Arghx).

Selection of L-Ornithine Overproducing Mutants

The pathway for L-ornithine biosynthesis from glutamate is known to consist of five enzyme reaction steps (10). While the structural genes for the enzymes are scattered among several regions of the DNA molecule, the groups of these structural genes individually constitute single regulatory units and are susceptible to repression by L-arginine simultaneously (12). The formation of N-acetylglutamokinase, the second enzyme in the L-ornithine biosynthetic pathway from glutamate, is also known to be repressed by the presence of L-arginine. Therefore, the derivation of mutants requiring L-citrulline or L-arginine for growth, and which are also resistant to L-arginine analogs, might be a promising way to find high-yield strains which are capable of overproducing L-ornithine.

A large number of L-citrulline auxotrophic and Arghx resistant mutant strains were derived from the glutamate-producing bacteria, *Corynebacterium glutamicum* ATCC 13032, *Arthrobacter citreus* ATCC 21040 and *Brevibacterium ketoglutamicum* ATCC 21092 (Table 2). The strains derived from *B. ketoglutamicum* ATCC 21092 were found to accumulate higher levels of L-ornithine than those derived from the other two strains. After extensive selection from thousands of mutant strains derived, seven L-ornithine overproducers were isolated, and designated as BK strains (Table 3). Among these BK strains BK1046 was found to produce the highest amount of L-ornithine, and was finally selected as the strain for further fermentation study.

Media Optimization

L-Ornithine is synthesized from glutamic acid with a supplement of energy (in the form of ATP), hydrogen donors and amino groups. The fermentative production of L-ornithine, therefore, requires a large amount of nitrogen and aerobic culture conditions. Although a large amount of ammonium ion is necessary, a high concentration is inhibitory not only to the cell growth, but also to the production of L-ornithine. On the contary, a shortage of ammonium ion promotes the formation of α-ketoglutaric acid, which results in a decrease in L-ornithine yield. The optimization of inorganic nitrogen concentration is therefore essential for the overproduction of L-ornithine.

BK1046 was cultivated in a fermentation medium containing various inorganic nitrogen compounds (Fig. 2). Among the compounds tested $(NH_4)_2SO_4$ and NH_4Cl

Table 3. Production of L-ornithine in shake flask fermentation using L-citrulline auxotrophic and Arghx resistant mutants derived from *Brevibacterium ketoglutamicum* ATCC 21092.

Strains	Dry cell wt. after 72 h cultivation (g/l)	Amount of L-ornithine accumulated (g/l)
BK102	2.6	7.2
BK104	2.8	7.8
BK603	2.9	7.4
BK610	2.4	7.5
BK703	2.8	7.6
BK1041	2.9	7.7
BK1046	2.7	8.5

Table 2. Derivation of L-ornithine producing auxotrophic mutants resistant to arginine-hydroxamate (Arghx) from their glutamate-producing parent strains.

Microorganisms	Concentration of Arghx (mg/ml)	No. of colonies tested	No. of producers isolated	Max. amount of L-orn. (g/l)
Corynebacterium glutamicum ATCC 13032	4	149	3	1.6
Arthrobacter citreus ATCC 21040	8	250	18	5.1
Brevibacterium ketoglutamicum ATCC 21092	8	1071	45	8.5

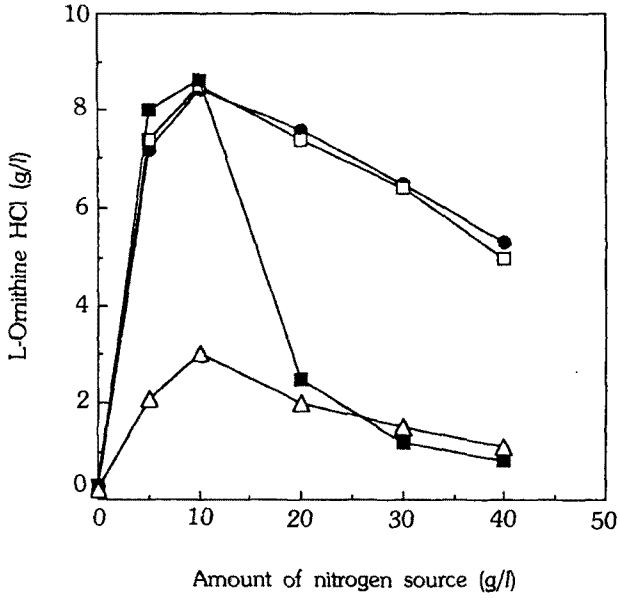

Fig. 2. Effects of inorganic nitrogen sources on L-ornithine prodution.
●; $(NH_4)_2SO_4$, □; NH_4Cl, ■; CH_3COONH_4, △; NH_4NO_3.

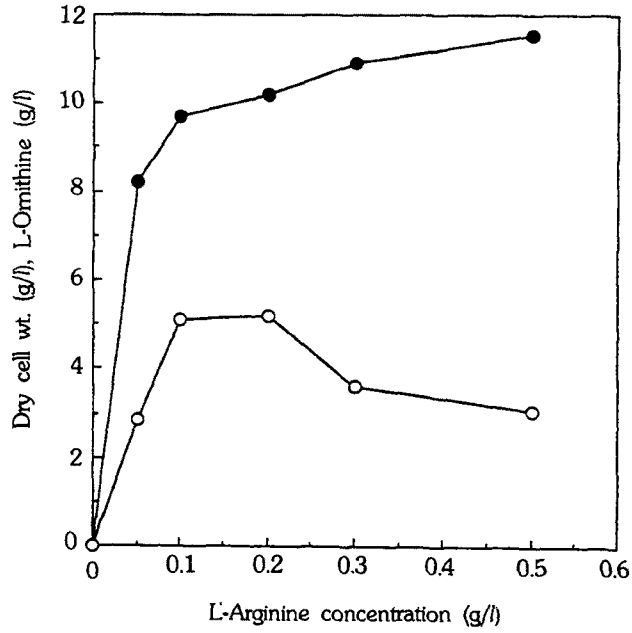

Fig. 3. Effect of L-arginine concentration on L-ornithine production (○) and cell growth (●).

were found to produce the highest L-ornithine yields. CH_3COONH_4 also produced a L-ornithine yield as high as $(NH_4)_2SO_4$ and NH_4Cl, up to the concentration of 10 g/l, but L-ornithine production was severely inhibited above this concentration. It has been reported that the culture broth becomes alkaline when CH_3COONH_4 is used as a nitrogen source, and major fermentation products are glutamic acid and alanine, rather than L-ornithine (8). L-ornithine production was inhibited by the addition of inorganic nitrogen sources above a concentration of 10 g/l. From these results $(NH_4)_2SO_4$, at a concentration of 10 g/l, was used as the inorganic nitrogen source for L-ornithine fermentation.

In addition to inorganic nitrogen sources the concentrations of carbon and inorganic salts were also optimized. Through a series of preliminary experiments glucose was found to be particularly suitable as a carbon source and cations such as K^+, Mg^{2+}, Fe^{2+} and Mn^{2+}, and anions such as PO_4^{-3}, Cl^- and SO_4^{-2} were found to be essential for L-ornithine production. The optimized levels of these inorganic ions were as follows; 0.015~0.15% of KH_2PO_4, 0.025~0.1% of $FeSO_47H_2O$, 0.0005~0.005% of $MnSO_44H_2O$, and 0.0001~0.001% of $ZnSO_47H_2O$. Although biotin was known to be one of the most important factors for glutamic acid fermentation it showed no significant effect on L-ornithine production. In addition, neither thiamine hydrochloride nor penicillin showed any significant effect on L-ornithine production.

Effect of L-Arginine Concentration on L-Ornithine Production

The effect of L-arginine concentration on L-ornithine production was examined with the BK1046 strain. Varying concentrations of L-arginine were tested in shake-flask experiments. The optimal concentration of L-arginine was found to be about 0.2 g/l as shown in Fig. 3. The BK1046 strain required L-arginine or L-citrulline for growth, but an excess amount of L-arginine resulted in a decreased L-ornithine yield. As already mentioned, the reduced L-ornithine production should be caused by the repression and/or inhibition of the enzymes involved in the biosynthetic pathway of L-ornithine due to the presence of an excess amount of L-arginine.

Batch Fermentation of L-Ornithine

Batch fermentation was conducted in a 2 l jar fermentor containing 1 l liquid medium. Yeast extract at a concentration of 5 g/l was used as a nitrogen source, along with 10 g/l of $(NH_4)_2SO_4$. The L-arginine required for cell growth was supplied at a concentration of 0.18 g/l from a yeast extract containing about 3.5% L-arginine. A typical time course of the fermentation is shown in Fig. 4. Twenty four g/l of L-ornithine was accumulated after 69 hours of cultivation. L-ornithine formation appears to be associated with cell growth. It is notable that cell growth was remarkably retarded after 20 hours. This is believed to be due to a shortage of L-arginine that develops as fermentation proceeds. During this phase some revertant cells were found. This was confirmed by a growth test on the minimal medium devoid of L-citrulline and L-arginine. The ratio of revertant cells to total cells was found to be about 0.2 after 48 hours.

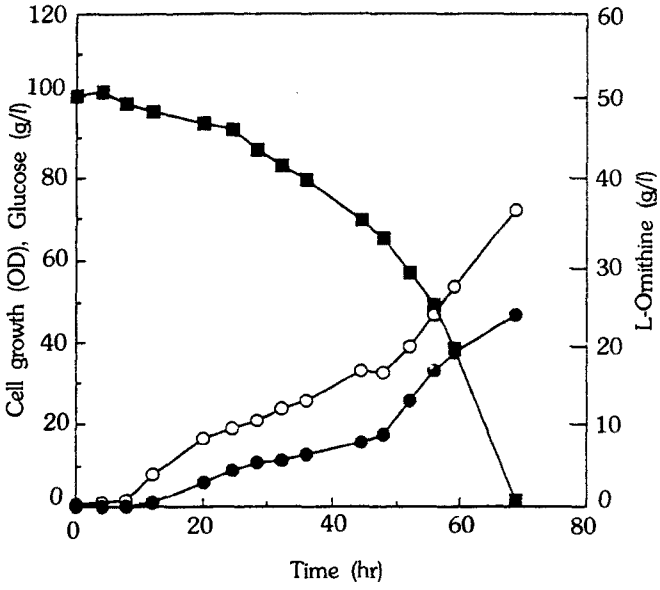

Fig. 4. Time courses of L-ornithine fermentation.
○; cell growth, ■; glucose, ●; L-ornithine.

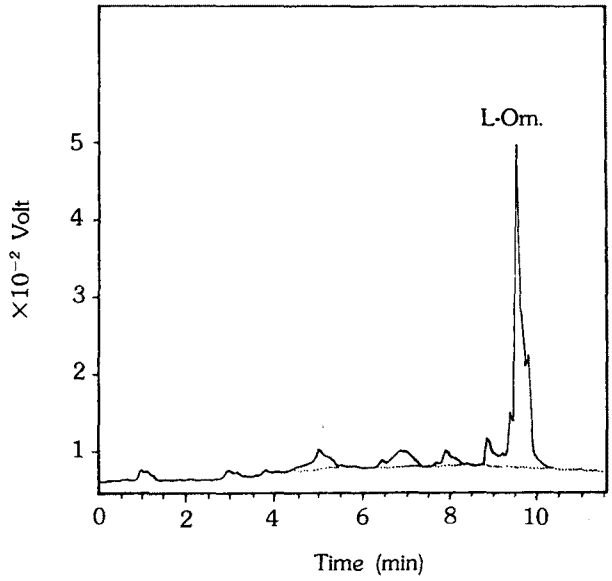

Fig. 5. HPLC chromatogram of amino acids in the fermentation broth after 69h cultivation.

Revertant cells which did not possess an auxotrophic property could produce L-arginine. The cells started to grow rapidly again using the L-arginine produced by the revertant cells, and reached a stationary phase after exhaustion of glucose at 69 hours. After the fermentor operation the fermentation broth was subjected to a HPLC for the analysis of amino acids. L-ornithine was accumulated at a concentration of 24 g/l, whereas other amino acids were rare (Fig. 5).

In conclusion, *Brevibacterium ketoglutamicum* BK 1046, a L-ornithine-overproducing strain, was successfully derived from its glutamate-producing parent strain by mutagenesis using NTG and UV. It produced 24 g/l of L-ornithine with a molar yield of 33% (one mole ornithine per mol glucose was defined as 100%) within a culture period of 69 hours. Further study is underway to enhance L-ornithine production through the selection of better L-ornithine-producing strains, and an improved fermentor operation including fed-batch culture.

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