Original Article

Increased L-arginine Production by Site-directed Mutagenesis of N-acetyl-L-glutamate Kinase and *proB* Gene Deletion in *Corynebacterium crenatum*[*]

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Abstract

Objective In *Corynebacterium crenatum*, the adjacent D311 and D312 of *N*-acetyl-L-glutamate kinase (NAGK), as a key rate-limiting enzyme of L-arginine biosynthesis under substrate regulatory control by arginine, were initially replaced with two arginine residues to investigate the L-arginine feedback inhibition for NAGK.

Methods NAGK enzyme expression was evaluated using a plasmid-based method. Homologous recombination was employed to eliminate the *proB*.

Results The IC_{50} and enzyme activity of NAGK M4, in which the D311R and D312R amino acid substitutions were combined with the previously reported E19R and H26E substitutions, were 3.7-fold and 14.6% higher, respectively, than those of the wild-type NAGK. NAGK M4 was successfully introduced into the *C. crenatum* MT genome without any genetic markers; the L-arginine yield of *C. crenatum* MT-M4 was 26.2% higher than that of *C. crenatum* MT. To further improve upon the L-arginine yield, we constructed the mutant *C. crenatum* MT-M4 △*proB*. The optimum concentration of L-proline was also investigated in order to determine its contribution to L-arginine yield. After L-proline was added to the medium at 10 mmol/L, the L-arginine yield reached 16.5 g/L after 108 h of shake-flask fermentation, approximately 70.1% higher than the yield attained using *C. crenatum* MT.

Conclusion Feedback inhibition of L-arginine on NAGK in *C. crenatum* is clearly alleviated by the M4 mutation of NAGK, and deletion of the *proB* in *C. crenatum* from MT to M4 results in a significant increase in arginine production.

Key words: *Corynebacterium crenatum*; *N*-acetyl-L-glutamate kinase; Site-directed mutagenesis; L-arginine; *proB*

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INTRODUCTION

L-Arginine is a semi-essential amino acid discovered and named in 1886; this amino acid has been studied for its role in various biological processes, such as urine flow stimulation[1], ammonia detoxification[2], and immunomodulation[3]. L-arginine is also a precursor of nitric oxide, which is a key moiety of endothelium-derived relaxing factor[4]. The critical functions of L-arginine provide a powerful impetus to further improve upon bioconversion capacity and reduce production costs. One of the most effective solutions is the use of high L-arginine-producing strains with increased growth rates. For instance, random mutagenesis is a traditional method used to screen strains and cause marginal augmentation of the L-arginine yield. However, random mutagenesis is a complicated molecular breeding method that compromises the native robustness of an organism at the expense of enhanced productivity. In contrast to random mutagenesis, site-directed genetic manipulations are feasible strategies for the isolation of mutant strains of genetically defined hyperproducers.

C. crenatum AS 1.542 (CICC 20662) is an aerobic, Gram-positive, non-sporulating coryneform bacterium isolated from the soil. In our previous work, a mutant *C. crenatum* MT strain yielding 9.7 g/L L-arginine after shake-flask fermentation was performed by inactivating ArgR obtained via nitrosoguanidine mutagenesis[5]. The anabolic pathway of L-arginine in *C. crenatum* involves eight enzymatic reactions in which glutamate is used as a precursor (Figure 1). *N*-acetyl-L-glutamate kinase (NAGK) encoded by *argB* is a critical rate-limiting enzyme that catalyzes the second reaction in the pathway[6]. To construct a high L-arginine-yielding strain, researchers should relieve the L-arginine feedback inhibition of NAGK by site-directed mutagenesis. In analogous studies, L-lysine[7] and L-valine[8] have been produced in large quantities by careful engineering of *C. glutamicum*; furthermore, feedback inhibition-resistant key enzymes have been generated by site-directed mutagenesis. The presumptive arginine-binding sites of feedback inhibition undergo point mutations, thereby affecting *Corynebacterium* NAGK. Therefore, Xu et al.[9-10] reported that E19R, H26E, and H268D mutations in NAGK increased the IC_{50} and the highest level of L-arginine production attained as a result was 45.6 g/L, which is approximately 41.7% higher than that of the wild-type strain, due to the overexpression of the mutant NAGK carried by a multi-copy plasmid, pJCtac with kanamycin in the bioreactor medium, whereas the enzyme activities were decreased by 5.85%. Park et al.[11] reported that the production of arginine could reach 92.5 g/L in a 5 L bioreactor using a systems metabolic engineering strategy based on random mutagenesis involving a point mutation, M31V in NAGK. To further alleviate the inhibition of arginine, the A26V mutation in NAGK was introduced to the genome by the authors. Contrary to their expectations, this resulted in slower consumption of carbon sources and a decrease in L-arginine production. Thus, the mentioned positions of point mutations affecting the enzyme activity may touch on the reaction pocket of the enzyme and its substrate.

Niersbach et al.[12] reported that D128 and D129 at the *Escherichia coli* ArgR C-terminus, which acts as a negative regulator of arginine biosynthesis, play an important role in linking ArgR with L-arginine. We found that two consecutive aspartic acid residues are located at positions 311 and 312 of *the C. crenatum* NAGK C-terminus. Thus, the two adjacent aspartic acids were speculated to also be an L-arginine pocket, although the crystal structure of *C. crenatum* NAGK has never been analyzed thus far. Combining the D311R and D312R amino acid substitutions with the previously reported E19R and H26E substitutions, we performed site-directed mutagenesis to construct three different mutant *argB* fragments: M2A with substitutions E19R and H26E; M2B with substitutions D311R and D312R; and M4 with substitutions E19R, H26E, D311R, and D312R. We then investigated the L-arginine feedback inhibition and enzyme activity characteristics of the three mutant forms. Our *in vitro* assay results showed that mutant *argB* M4 contained the optimal combination of amino acid substitutions, which not only improved enzyme activity by 14.6% but also enhanced IC_{50} by 3.7-fold. Mutant *argB* M4 was then introduced into the *C. crenatum* MT genome. L-arginine yield increased by 26.2% compared with that of *C. crenatum* MT at 108 h. The competitive branch of the L-proline biosynthetic pathway was also disrupted in our work, and the highest yield of L-arginine was 16.5 g/L, obtained with 10 mmol/L L-proline supplement. Meanwhile, we analyzed the reason for the decrease in the enzyme activity of NAGK M2A by means of a molecular docking approach.

MATERIALS AND METHODS

Strains and Cultivation Conditions

C. crenatum MT [derived from *C. crenatum* AS 1.542 (CICC 20662)] *argB* was replaced with *E. coli argB* to generate *C. crenatum* MT E-*argB*. *C. crenatum* MT E-*argB* was constructed in our previous work and used as a parent strain in the present study to re-engineer the bacterial strain. *E.coli* DH5α and BL21 strains were used as hosts in gene cloning and expression. All of the strains and plasmids used in this work are listed in Table 1. The shuttle vectors pXMJ19 and pk18 m*obsacB* int *sacB* were used for gene expression and deletion, respectively. *E. coli* and *C. crenatum* were grown in Luria-Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, and NaCl 10 g/L, pH 7.2) at 37 °C or 30 °C. Kanamycin (25 μg/mL) was added to the culture medium for the cultivation of *E. coli* and *C. crenatum* whenever necessary.

In shake-flask fermentation, *C. crenatum* strains were cultivated on LB agar plates for 24 h. A single colony was transferred into a 250 mL triangular flask containing 30 mL of the seed medium and cultivated with constant rotation on a rotary shaker (250 rpm) at 30 °C for another 24 h. The seed medium consisted of 30 g/L glucose, 20 g/L corn steep liquor, 20 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, and 1.5 g/L urea. Approximately 2 mL of seed culture was transferred into a 250 mL triangular flask containing 20 mL of fermentation medium and cultivated with constant rotation on a rotary shaker (250 rpm) at 30 °C for 108 h. The fermentation medium contained 120 g/L glucose, 25 g/L corn steep liquor, 45 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, and 30 g/L $CaCO_3$ (pH 7.0)[13]. Negligible amounts of L-proline were found in the fermentation medium.

DNA Isolation, Manipulation, and Analysis

The genomic DNA of *C. crenatum*was isolated as described previously[14]. The Plasmid Mini Kit I (Omega, USA) was used to isolate plasmid DNA. DNA was modified

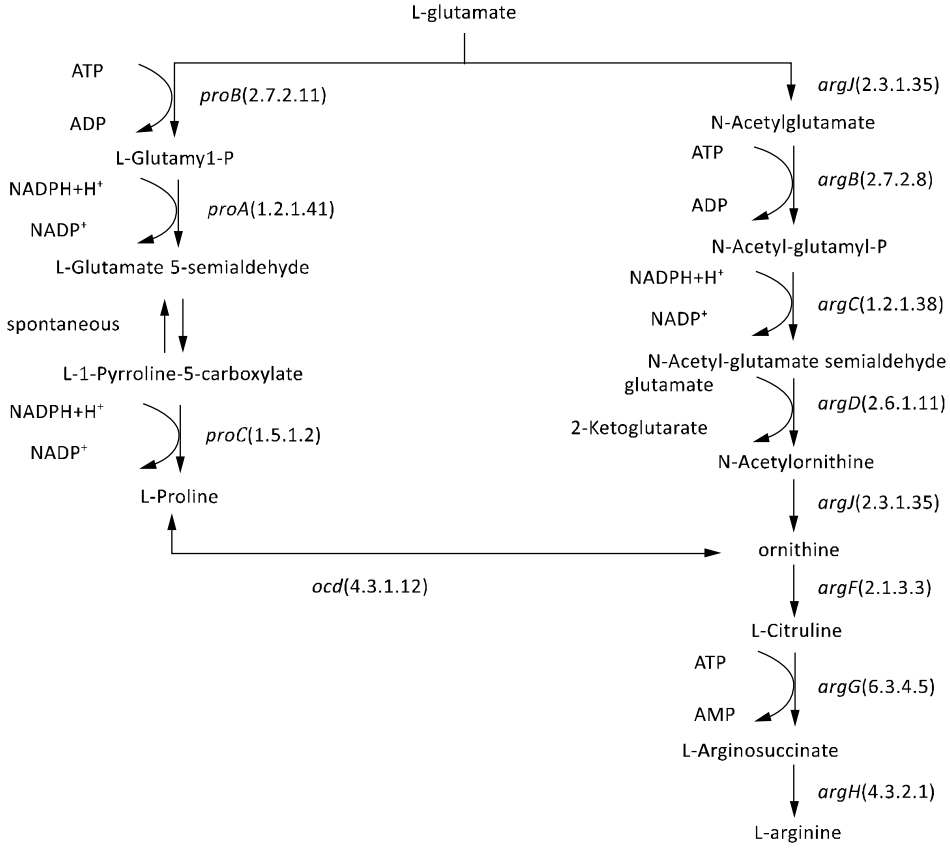

Figure 1. Biosynthetic pathways of L-Arginine and L-Proline in *C. crenatum.* Italic alphabet represented the gene involved in the reaction. The digit in the bracket reflected the number of relevant enzyme in KEGG database.

and analyzed by agarose gel electrophoresis. Ligation was performed according to a standard method[15]. PCR was conducted using a T Gradient thermocycler (Hangzhou Bio-Gener Technology Co., Ltd, China), Ex*Taq* DNA polymerase (Takara Bio, Inc., Japan), and chromosomal DNA as template. The PCR products were purified using a SiMax II™ Plasmid DNA Miniprep kit (SBS Genetech Co. Ltd., China). All of the primers (Table 2) used for PCR were purchased from Sangon Biotech (Shanghai, China).

Cloning and Expression of Site-directed Mutant NAGKs in E. coli

To construct recombinant expression vectors of pXMJ19-*argB* M2A (E19R and H26E), pXMJ19- *argB* M2B (D311R and D312R), pXMJ19-*argB* M4 (E19R, H26E, D311R, and D312R), and pXMJ19-*argB*, we amplified a fragment containing the intact *argB* by conducting PCR in which *C. crenatum* genomic DNA was used as a template. The three site-directed *argB* mutants were generated using an overlapping PCR-based method with the amplified intact *argB* as a template. All of the purified PCR products were digested and inserted into multiple cloning sites of pXMJ19. The four recombinant plasmids were transferred to *E. coli* BL21 by heat shock, and positive transformants were confirmed by sequencing.

Table 1. Strains and Plasmids in This Study

Strain/Plasmid	Characteristic	Source
Strain		
E. coli DH5α	Clone host strain	Invitrogen
E. coli BL21(DE3)	*OmpT hsdS*$_B$ (r_B^-, m_B^-) *gal dcm* (DE3) and expression host	Novagen
C. crenatum MT	Mutation strain and producing L-arginine	Our labs
E. coli BL21- pXMJ19	A derivative of *E. coli* BL21(DE3), harboring pXMJ19 expression plasmid	This study
E. coli BL21-pXMJ19- *argB*	A derivative of *E. coli* BL21(DE3), harboring pXMJ19-*argB* expression plasmid	This study
E. coli BL21-pXMJ19-*argB* M2A	A derivative of *E. coli* BL21(DE3), harboring pXMJ19-*argB* M2A expression plasmid	This study
E. coli BL21-pXMJ19- *argB* M2B	A derivative of *E. coli* BL21(DE3), harboring pXMJ19-*argB* M2B expression plasmid	This study
E. coli BL21-pXMJ19-*argB* M4	A derivative of *E. coli* BL21(DE3), harboring pXMJ19-*argB* M4 expression plasmid	This study
C. crenatum MT-M4	*C. crenatum* E-*argB* gene replaced by *C. crenatum argB* M4 gene	This study
C. crenatum MT E-*argB*	*C. crenatum* MT *argB* replaced by *E. coli argB*	Our labs
C. crenatum MT-M4 △*proB*	*C. crenatum* MT-M4 with *proB* deletion	This study
Plasmid		
pMD18-T	Clone vector , 2.7 kb, Amp^R, *lacZ*	TaKaRa
pK18*mobsacB*	Mobilizable vector, allows for selection of double crossover in *C. crenatum*, Km^R, *sacB*	(22)
pXMJ19	A shuttle expression vector, Cm^R	(23)
pXMJ19-*argB*	A derivative of pXMJ19, harboring *C. crenatum argB* gene	This study
pXMJ19-*argB* M2A	A derivative of pXMJ19, harboring *C. Crenatum* mutated *argB* (E19R H26E)	This study
pXMJ19-*argB* M2B	A derivative of pXMJ19, harboring *C. crenatum* mutated *argB* (D311R D312R)	This study
pXMJ19-*argB* M4	A derivative of pXMJ19, harboring *C. crenatum* mutated *argB* (E19R H26E D311R D312R)	This study
pK18*mobsacB*-CJ-*argB* M4-CD	A derivative of pK18*mobsacB*, harboring up arm, *C. crenatum argB* M4 and down arm fragments	This study
pK18*mobsacB*-△*proB*	A derivative of pK18*mobsacB*, harboring △*proB* fragment	This study

Note. Superscript 'R' indicates resistance to the following antibiotics: Amp ampicillin, Km kanamycin, CM chloramphenicol.

NAGKs were overexpressed by adding 1.0 mmol/L isopropyl-*b*-D-thiogalactopyranoside (IPTG) once the culture reached an OD_{600} of 0.5 to 0.6. Following induction for 8 h, cells were harvested by centrifugation at 5000 rpm. The cells were washed and resuspended in 200 mmol/L Tris-HCl (pH 8.0). The cells were then lysed by sonication; the lysate was clarified by centrifugation at 8000 rpm for 10 min. Insoluble solids and supernatants were evaluated forprotein expression using SDS-PAGE and subsequent staining with Coomassie R-250.

NAGK Activity Assay

To determine NAGK activity, we performed acolorimetric assay containing hydroxylamine[16]. The standard reaction mixture consisted of 40 mmol/L Tris-HCl (pH 8.0), 200 mmol/L $MgCl_2$, 20 mmol/L ATP (sodium salt), 40 mmol/L *N*-acetyl-L-glutamate (NAG), 400 mmol/L $NH_2OH{\cdot}HCl$, and the enzyme preparation in a total volume of 1.5 mL of aqueous solution. Enzymatic reactions were initiated by adding ATP (sodium salt); incubations were conducted at 37 °C for 1 h. Next, 0.5 mL of *N*-hydrochloric acid (containing 5% $FeCl_3{\cdot}6H_2O$ and 4% trichloroacetic acid) was added to each mixture to terminate the reactions. Finally, the mixtures were clarified by centrifugation at 13,000 rpm for 10 min, and the optical densities of the soluble fractions were determined at A_{540}. One unit of NAGK is defined as the amount of enzyme required to generate 1 μmol NAG oxygen oxime per minute or the amount of enzyme required to consume 1 μmol NAG.

L-Arginine Feedback Inhibition Experiments

To investigate the feedback inhibition concentration of L-arginine on NAGK activity, we added 0 to 6 mmol/L of L-arginine to the enzymatic reaction mixture; NAGK activity was then determined as described above. A feedback inhibition curve was constructed with NAGK activity on the *Y* axis and L-arginine concentration on the *X* axis. The IC_{50} is defined as the concentration of arginine that causes 50% inhibition.

Introduction of Specific Mutations to the Genome

The site-directed mutant *argB* M4 was amplified by PCR using pXMJ19-*argB* M4 as a template. *C. crenatum* MT E-*argB* was used as the original strain to replace the site-directed mutant *argB* M4. The upstream and downstream fragments of *argB* and *argB* M4 were purified and mixed as templates in an overlapping PCR-based experiment. A single, large fragment, which included an upstream fragment, *argB* M4, and a downstream fragment, was constructed to include *Sal* I and *Sph* I sites. The recombinant fragment was purified and inserted into pK18 m*obsacB*; the constructs were then transferred into *C. crenatum* MT E-*argB* by electroporation[17].

Table 2. Primers and their sequences in this study

Primers	Sequence (5'-3')	Restriction Enzyme
C.*argB*-F	CGC***AAGCTT***ATGAATGACTTGATCAAAG	*Hind* III
C.*argB*-his6R	CGC***TCTAGA***CAGTTCCCCATCCTT	*Xbal* I
CB-F	ATGAACTCAACGATGCGGTGT	
CB1926R	*t*Tc<u>CTGCAACCATGGCAACGCC</u>*cg*AGCGAGGACATTTGCGCGCACCTCAG	
CB1926F	*cg*<u>GGCGTTGCCATGGTTGCAG</u>*gAa*TTCCGTGACAAGATTGTTGTCGTG	
CB311312R	TTACAGTTCCCCATCCTTG*cg*G*cg*TTTTCTAAAAACGGTGCCTTC	
C. *argJ*-up-F1	CGGCCG***GTCGAC***TTGACACCCTGGATATTGATGG	*Sal* I
C. *argJ*-up-R1	<u>AGCCTAAATCTTTGATCAAGTCATTCATGGC</u>TGTGCCCTTTTCCCTG	
C. *argB*-F1	<u>GCCATGAATGACTTGATCAAAGATTTAGGCT</u>CTGAGGTG	
C. *argB*-R1	<u>CTGTGGCCAAGTTTCCAGCGTGCTCAT</u>TTACAGTTCCCCATCCTT	
C. *argD*-down-F1	<u>ATGAGCACGCTGGAAACTTGGCCACAG</u>GTCATTATTAATACGTACGGCAC	
C. *argD*-down-R1	CATCGG***GCATGC***TCAAGATGCCGTACTCATCGC	*Sph* I
proB-up-F	CGGCCG***GAATTC***TTGAACAGACCTCTCGTATC	*EcoR* I
proB-up-R	<u>GGACGAACCAATTTTCACCACCACT</u>CGCTTAGCGTTGGAGATGCG	
proB-down-F	<u>GTGGTGGTGAAAATTGGTTCGTCC</u>GTAAATCTTTGCTGGCTGTGGGC	
proB-down-R	CATCGG***TCTAGA***TTTAATATCCTCAACCCGCTC	*Xba* I

Note. Restriction enzyme sites were represented as italic and bold characters, overlapping region were marked by underline. Mutant sites were labeled by lowercase.

Thereafter, the clones exhibiting double recombination were detected on a 10% sucrose plate. The clones that were determined to be free of vector sequences and to contain the mutant *argB* M4 sequence were confirmed by PCR.

Chromosomal Deletion of the proB

Targeted gene deletion was performed by homologous recombination. 5′-upstream and 3′-downstream fragments of the *proB* were amplified by PCR using the primers listed in Table 2. The fusion arm containing 5′-upstream and 3′-downstream fragments was constructed using overlapping PCR. This fusion arm was then inserted into pK18 m*obsacB* using standard methods[18]. The *proB* was subjected to chromosomal deletion using a recombinant plasmid via two recombination events as described previously[19].

Cell Concentration, Glucose, and L-arginine Assays

L-arginine concentration was determined using an L-8800 automated amino acid analyzer (Hitachi High-Technologies, Tokyo, Japan). Glucose concentration in the culture was measured by 3,5-dinitrosalicylic acid colorimetry[20]. The precipitation containing bacteria and $CaCO_3$ was diluted in 0.125 mol/L HCl. Subsequently, the optical density was measured at 562 nm and the cell concentration determined using a previously established relationship (1 OD=0.375 g/L). To verify the final results, we determined the concentrations of cells, arginine, and glucose in triplicate.

Statistical Analysis

All of the data were statistically analyzed using ANOVA, and significant differences were identified by Turkey's test (P<0.05). These analyses were conducted using GraphPad Prism 5.0 software (GraphPad software Inc., California, USA).

RESULTS

Cloning, Expression, and Enzyme Activity of NAGKs in E. coli

The *argB* fragment was obtained by PCR amplification and the mutant sites were introduced by overlapping PCR. The intact *argB* and the site-directed mutant DNA fragments were cloned into pXMJ19. The recombinant *E. coli* BL21 cells containing the each of the four plasmids were induced with 1 mmol/L IPTG at 30 °C. The results of SDS-PAGE showed a predominant band corresponding to the expected size (approximately 36 kD) in the supernatant of lysed whole cells (Figure 2). The specific enzyme activity of NAGK with the E19R and H26E mutations was remarkably decreased to 24.2% of that of the wildtype (Table 3). In contrast, the variants D311R and D312R showed a significant increase in specific enzyme activity. Furthermore, the specific enzyme activities of the assembly variant (E19R, H26E, D311R, and D312R) maintained an almost constant level, similar to those of D311R and D312R.

L-arginine Feedback Inhibition Experiment

To investigate the characteristics of NAGK M2B and NAGK M4, we added 0 to 6 mmol/L L-arginine to the enzyme reaction system and generated a feedback inhibition curve. After 1 mmol/L L-arginine was added to the enzyme reaction mixture in the presence of the wild-type NAGK, enzyme activity decreased by 50% (Figure 3). At 1.3 mmol/L L-arginine, half of the NAGK M2B enzyme activity was inhibited. Nevertheless, NAGK M4 showed a higher IC_{50} of 3.7 mmol/L. These results indicated that the IC_{50} of NAGK M4 was 3.7-fold higher than that of the wild-type NAGK (Table 4). Thus, the feedback inhibition of NAGK by L-arginine was successfully diminished by site-directed mutagenesis.

C. crenatum MT Transformation and L-arginine Production

We have shown that the mutant M4 is very effective in relieving L-arginine feedback inhibition *in vitro*. To improve L-arginine production and genetic stability of the recombinant *C. crenatum* strain, we successfully introduced the M4 point mutations into the *C. crenatum* MT genome. After *C. crenatum* MT and *C. crenatum* MT-M4 strains were cultivated for 108 h in fermentation medium, their corresponding L-arginine yields were compared. The fermentation curves showing cell concentration, glucose consumption, L-arginine production, and L-arginine yield are presented in Figure 4. *C. crenatum* MT-M4 showed no significant difference in growth rate (Figure 4A, P>0.05) and a slight increase in glucose uptake at 108 h of fermentation compared with *C. crenatum* MT (Figure 4B, P<0.05). *C. crenatum* MT-M4 produced 12.3 g/L L-arginine at 108 h and showed a 26.2% improvement, whereas the yield of L-arginine per gram of glucose showed no significant

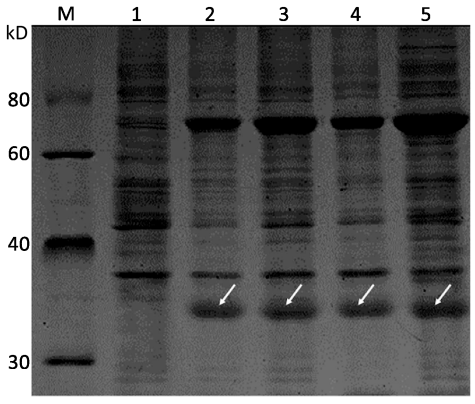

Figure 2. SDS-PAGE analyses of the overexpression of NAGKs in recombinant *E. coli* BL21. M: Protein RulerI maker (Low weight DR101 TRAN); line 1: *E. coli BL21-pXMJ19*; line 2: *E. coli BL21-pXMJ19-argB*; line 3: *E. coli BL21-pXMJ19-argB M2A*; line 4: *E. coli BL21-pXMJ19-argB M2B*; line 5 *E. coli BL21-pXMJ19-argB M4*.

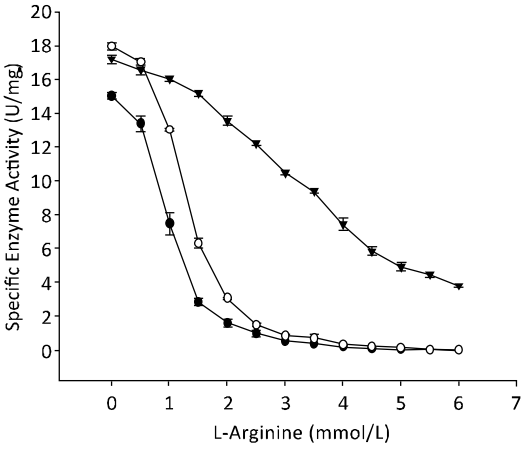

Figure 3. The feedback inhibition curves of NAGKs by L-arginine. The wild-type NAGK was indicated by filled circles; the NAGK M2B and the NAGK M4 were indicated by opened circles and filled inverted triangles, respectively. Data are means and standard deviations of at least three cultivations.

Table 3. Protein Concentration and NAGK Initial Enzyme Activity of the Five Strains

NAGK Crude Enzyme	Expression Level	Total Protein Concen--tration (mg mL^{-1})	Total Enzyme Activity (U mL^{-1})	Specific Enzyme Activity (U mg^{-1})
E. coli BL21-pXMJ19	+++	1.27±0.02	ND	ND
E. coli BL21-pXMJ19-argB	+++	0.95±0.01	14.30±0.18	15.04±0.22
E. coli BL21-pXMJ19-argB M2A	+++	1.55±0.12	5.47±0.24	3.64±0.16***
E. coli BL21-pXMJ19-argB M2B	+++	0.94±0.01	16.92±0.19	17.97±0.35***
E. coli BL21-pXMJ19-argB M4	+++	2.12±0.08	36.51±0.51	17.23±0.43***

Note. Results are the means standard deviations from at least three independent experiments. Compared with the control group, $^{***}P<0.001$; ND: not detectable.

Table 4. Specific Enzyme Activity and IC_{50} Valuesof the NAGKs

NAGKs	Source	Mutation Sites	Specific Enzyme Activity (U mg^{-1})	IC50 (mmol/L)
NAGK	*E. coli BL21-pXMJ19-C. crenatum argB*	ND	15.04±0.22	1.00±0.01
NAGK M2B	*E. coli BL21-pXMJ19-C. crenatum argB* M2B	D311R D312R	17.97±0.35	1.3±0.01ns
NAGK M4	*E. coli BL21-pXMJ19-C. crenatum argB M4*	E19R H26E D311R D312R	17.23±0.43	3.7±0.03***

Note. *All values are means average values of three independent experiments; Compared with the control group, 'ns' designates $P>0.05$, $^{***}P<0.001$; ND (no mutation site).

difference as compared with those in *C. crenatum* MT (Figure 4C). These results indicated that L-arginine yield could be increased significantly by preventing L-arginine feedback inhibition of NAGK. Metabolic flux through the L-arginine production pathway could be increased by mutation of the rate-controlling enzyme. After inhibition of the key enzyme is lifted, sufficient precursor supply to the arginine biosynthetic pathway becomes an important factor to further improve arginine yield.

proB Gene Deletion and L-arginine Production

L-glutamate is a precursor of both L-arginine and L-proline. Gamma-glutamyl kinase, encoded by *proB*, is the first enzyme involved in L-proline biosynthesis. Thus, we constructed a *proB* deletion strain, *C. crenatum* MT-M4 △*proB,* to increase the precursor supply for arginine biosynthesis. The L-arginine produced by *C. crenatum* MT-M4 △*proB* in shake flask fermentation was then determined to be 14.6 g/L at 108 h (Figure 4C). This represented an approximately 52.1% (Figure 4A, $P<0.01$) and 196% (Figure 4D, $P<0.001$) increase in arginine production and the yield of L-arginine per gram glucose, respectively, relative to the same parameters in *C. crenatum* M4. However, the growth rate and glucose consumption of *C. crenatum* MT-M4 △*proB* were significantly decreased at 108 h of fermentation (Figure 5A, $P<0.05$, and 5B, $P<0.001$). Hence, L-proline deprivation in the fermentation medium may account for this remarkable decrease in growth rate and glucose uptake, indicating that gamma-glutamyl kinaseis an essential enzyme in the production of L-proline.

Proline Addition Experiment and Corresponding L-arginine Production

Proline is considered a growth-limiting factor of *C. crenatum* MT-M4 △*proB*. Thus, we explored the influence of various L-proline levels added to the fermentation medium on cell concentration and glucose consumption. The results showed that the growth rate and glucose consumption were improved by adding L-proline. After 20 mmol/L L-proline was added to the fermentation medium, the growth rate reached a level equal to that of *C. crenatum* MT-M4 (Figure 5A); glucose consumption also increased (data not shown). The growth rate and

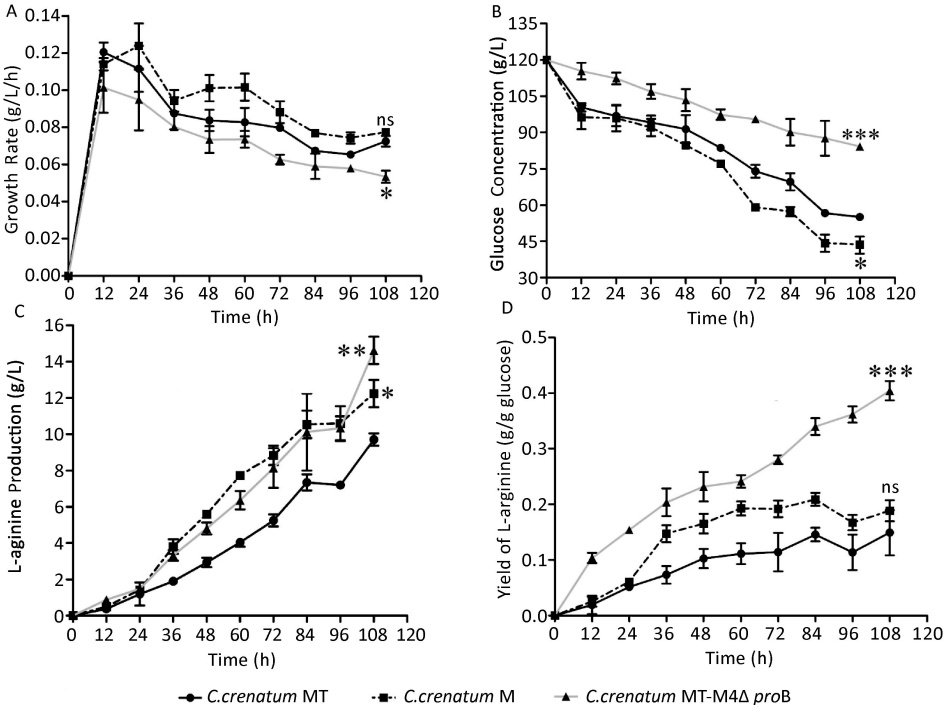

Figure 4. Comparison of L-arginine production between the *C. crenatum* MT and *C. crenatum* MT-M4, and *C. crenatum* MT-M4 *proB*, respectively. All values are means and standard deviations of at least three independent experiments. (A) Cell growth rate. (B) Glucose uptake rate. (C) L-arginine production. (D) Yield of L-arginine (g/g glucose). Compared with the control group (*C. crenatum MT*), 'ns' designates $P>0.05$; * designates $P<0.05$; ** designates $P<0.01$ and *** designates $P<0.001$, respectively.

glucose consumption revealed a similar relationship. L-arginine yield was also increased due to L-proline supplementation. After 10 mmol/L L-proline was added to the culture solution, L-arginine concentration reached 16.5 g/L at 108 h, approximately 70.1% higher than that of *C. crenatum* MT (Figure 5B, $P<0.001$).

DISCUSSION

In *Corynebacterium*, NAGK (encodedby *argB*) enzyme activity is inhibited by L-arginine. In order to carefully engineer a high-producing L-arginine strain, releasing the feedback inhibition of NAGK by L-arginine was the most urgent issue. In this study, site-direct mutagenesis was employed to reengineer *C. crenatum* NAGK using mutant sites that helped to reduce the affinity of L-arginine for NAGK that were derived from the published data and our speculation based on the L-arginine pocket of the *E. coli* ArgR C-terminus. Because the results of our kinetic studies showed that D311R and D312R mutations alleviated the feedback inhibition of L-arginine, we speculated that these two residues could participate in arginine binding. NAGK enzyme activity was increased 19.5% and the IC_{50} was increased 1.3-fold in the D311R/D312R mutant compared with the wild type. Indeed, the mutant NAGK M4 not only displayed a 14.6% increase in enzyme activity but also a 3.7-fold enhanced IC_{50}. This result further confirmed our speculation regarding the function of D311 and D312 as an arginine pocket. Therefore, the mutant NAGK M4 described here will provide a useful reference for engineering a *Corynebacteria* strain for further improvement of the industrial production of L-arginine.

To investigate the decrease in the enzyme activity of NAGK M2A, we submitted the *C. crenatum* NAGK sequence to the SWISS-MODEL Workspace and obtained a 289 amino acid NAGK model, based on *Mycobacterium tuberculosis* NAGK (PBD code: 2ap9_E), without amino acid residues 1 to 4 and 294 to 317, and then employed it to compare the docked conformation between NAGK M2A and NAG with that of the wild-type NAGK and NAG using AutoDock software. The results suggested that NAG was located in the pocket of the wild-type NAGK from amino acid residues 5 to 293 (Figure 6A); His26 at this moment was tightly closed to the NAG substrate. However, NAG was found closer to the gate with a portion of its chain outside the pocket of NAGK M2A (Figure 6B); the glutamate residue replacing His26 was also separated from NAG. Thus, the decrease in steric contact between NAGK and NAG may lead to a serious decrease in enzyme activity toward the NAG substrate. The H26E mutation causes a slight conformational change, possibly resulting in a pocket that is in a more open conformation than the original. Glu19 may not be directly involved in binding because this residue is located far from the pocket. Our results revealed that amino acid residues in active pockets play important roles in substrate binding. However, we cannot explain why the combination of NAGK M4 with D311R and D312R with H26E and E19R not only promoted recovery but also improved enzyme activity. Hence, further studies

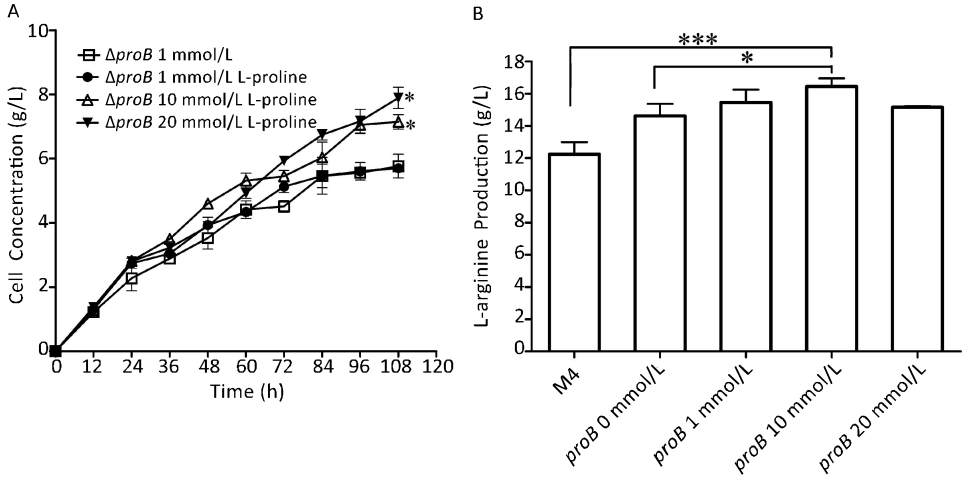

Figure 5. L-arginine production of *C. crenatum* MT-M4 *proB* supplemented with different concentration of proline. (A) Cell concentration, (B) L-arginine production at 108 h. Compared with the control group, *designates $P<0.05$ and ***designates $P<0.001$, respectively.

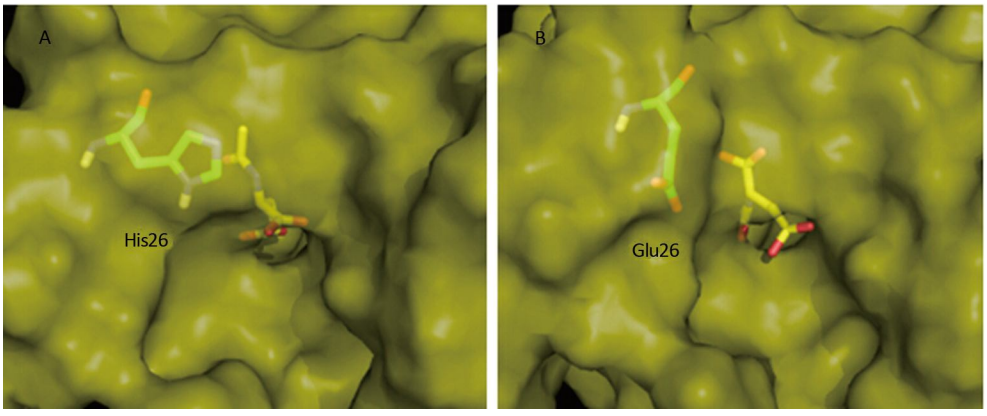

Figure 6. The model of docked conformation between NAGK and NAG. (A) The docked conformation between wild-type NAGK and NAG, (B) The docked conformation between NAGK M2 and NAG.

should be conducted to reveal the crystal structure of NAGK from *Corynebacterium* andto elucidate the mechanism of catalysis of NAGK towards this substrate as well as its inhibition by arginine.

Plasmid-mediated structural gene amplification is a common strategy in genetic engineering. However, several problems restrict the application of this technique in industrial production. Firstly, a plasmid-based expression system is prone to structural and segregation instability, as well as allele segregation, which result in decreased productivity of the desired compound. Tyo et al.[21] reported that plasmid-carrying strains lose poly-3-hydroxybutyrate productivity after 40 rounds of subculturing. Secondly, plasmids generally carry genetic markers, particularly antibiotic resistance markers, which consume a large amount of antibiotic; these markers affect the physiological function of *Corynebacterium* and raise fears of spreading antibiotic resistance genes[19]. Here, the optimal mutant gene obtained by *in vitro* experiments was introduced into the *C. crenatum* MT genome. The result showed that the L-arginine yield of *C. crenatum* MT-M4 increased by 26.2% through only one copy in the genome without any genetic markers.

Metabolism *in vivo* is a complex process requiring multifarious enzymatic and spontaneous reactions. As such, factors other than the removal of feedback inhibition of NAGK should be considered as ways to improve L-arginine yield. Glutamate derived from the tricarboxylic acid cycle is a common precursor of L-arginine and L-proline[22]. To further obtain a high-L-arginine-producing strain, gamma-glutamyl kinase of the L-proline biosynthetic pathway was successfully deleted using homologous recombination. The results showed that L-arginine concentration was increased significantly at 108 h. However, cell growth remarkably decreased because the cells were deprived of L-proline. Ooyen et al.[23] reported that proline can be exogenously added to improve glucose consumption; therefore, cell growth is promoted and lysine yield is further increased. Lee et al.[24] found that proline can be catalyzed directly by *ocd*-ornithine cyclodeaminase to produce ornithine, one of the intermediates in the arginine biosynthetic pathway (Figure 1). Thus, an L-proline supplementation experiment was performed to increase cell growth and improve L-arginine production. As expected, cell growth and glucose consumption were stimulated; L-arginine production was also increased after L-proline supplementation was performed. After 10 mmol/L L-proline was added to the medium, L-arginine yield reached the optimal level (Figure 5B, $P<0.05$).

In conclusion, feedback inhibition of NAGK M4 by L-arginine could be relieved by site-directed mutagenesis and the enzyme activity could be increased by mutation; NAGK M4 was successfully introduced into the *C. crenatum* genome; *proB* deletion and exogenous addition of proline could induce arginine accumulation. This work highlighted the importance of lifting the inhibition on biosynthetic pathways and eliminating competitive pathways for a target compound, providing a good strategy for metabolic engineering.

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