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ORIGINAL ARTICLE

Site-directed mutagenesis and feedback-resistant N-acetyl-L-glutamate kinase (NAGK) increase *Corynebacterium crenatum* L-arginine production

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Abstract *N*-Acetyl-L-glutamate kinase (EC 2.7.2.8) is first committed in the specific L-arginine pathway of *Corynebacterium* sp. A limited increase of L-arginine production for the *arg*B overexpression in the engineering *C. creantum* SYPA-CCB strain indicated that L-arginine feedback inhibition plays an influence on the L-arginine production. In this study, we have performed site-directed mutagenesis of the key enzyme (NAGK) and the three mutations (E19R, H26E and H268D) exhibited the increase of $I^{R}_{0.5}$ efficiently. Thereby, the multi-mutated $NAGK_{M3}$ (including E19R/H26E/H268D) was generated and its $I^{R}_{0.5}$ of L-arginine of the mutant was increased remarkably, whereas the NAGK enzyme activities did not declined. To get a feedback-resistant and robust L-arginine producer, the engineered strains SYPA-CCB_{M3} were constructed. Introducing the *arg*B_{M3} gene enabled the NAGK enzyme activity insensitive to the intracellular arginine concentrations resulted in an enhanced arginine biosynthesis flux and decreased formation of by-products. The L-arginine synthesis was largely enhanced due to the overexpression of the *arg*B_{M3}, which is resistant to feedback resistant by L-arginine. Thus L-arginine production could reach 45.6 g/l, about 41.7% higher compared with the initial strain. This is an example of up-modulation of the flux through the L-arginine metabolic pathway by deregulating the key enzyme of the pathway.

Keywords *N*-Acetyl-L-glutamate kinase (NAGK) · Feedback inhibition · Site-directed mutagenesis · *Corynebacterium crenatum* · L-Arginine production

Abbreviations

NAGK	*N*-Acetyl-L-glutamate kinase
Ccre_NAGK	*N*-Acetyl-L-glutamate kinase of *Corynebacterium crenatum*
WT	Wild-type
NAG	*N*-Acetyl-L-glutamate
$I^{R}_{0.5}$	Inhibition constant (the L-arginine concentration yields 50% inhibition)
EMS	Ethylmethane sulfonate
DCW	Dry cell weight
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

L-Arginine has numerous applications in food flavor and pharmaceuticals. In humans, L-arginine is classified as a conditionally essential amino acid for protein synthesis, and its metabolism also gives rise to nitric oxide, which is a key component of endothelium-derived relaxing factor, metabolites of the urea cycle, creatine, proline, and polyamines (Grillo and Colombatto 2004; Ikeda 2003). The microbial production of L-arginine by *Corynebacterium* sp. has been studied and now most L-arginine has been

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produced by the direct fermentation method (Utagawa 2004; Glansdorff and Xu 2007; Ikeda et al. 2009). During the nearly 30 years, most amino acid producing bacterial strains have been constructed by random mutagenesis. Though the high production abilities and delicate constitutions of classical industrial producers have the merits and demerits of the classical approach, this approach based on random mutation and selection has sacrificed the native robustness of an organism in exchange for enhancing the production abilities to the limits. In efforts to improve the industrial productivity of strains, significant progress in the development of molecular techniques has been made (Wendisch 2006; Kalinowski et al. 2003; Hermann 2003) and the rational metabolic engineering by specific targeted modifications has been raised to overcome the limits and disadvantage of the random and classical mutagenesis (Holatko et al. 2009; Park and Lee 2008). The typical studies have described the high-production of L-valine and L-lysine by rationally constructed *C. glutamicum*, in which the feedback inhibition-resistant key enzymes were both generated by site-directed mutagenesis (Blombach et al. 2008, 2009; Elisakova et al. 2005).

The biosynthesis of L-arginine in bacteria was a focus of research interest for the past decades on metabolic regulation, from which yielded several findings of general interest. The historical perspective for genetic and molecular studies of the "arginine regulon" and the summary of L-arginine biosynthetic enzymes were reviewed. There are three routes for the biosynthesis of L-arginine proceeding from L-glutamate. They are, respectively, the linear way, the cycle way and a new pathway which employs a novel family of transcarbamylases for arginine biosynthesis (Cunin et al. 1986; Wendisch et al. 2007). In the cycle pathway, the metabolic pathway for the arginine formation consists in seven successive steps (Fig. 1). The genes involved in the arginine biosynthesis pathway in *Corynebacteria* are organized as the cycle pathway, in which the feedback inhibition control occurs by a single enzyme (NAGK encoded by *arg*B) (Sakanyan et al. 1996). Ramón-Maiques et al. (2006) had shed light on L-arginine inhibition by determining the crystal structures of arginine-complexed *T. maritime* and *P. aeruginosa* NAGKs. Both the NAGKs are highly similar ring-like hexamer having a central orifice of ~30 Å diameter. Then Fernandez-Murga and Rubio (2008) had revealed that arginine-insensitive *Escherichia coli* NAGK (EcNAGK) is homo-dimeric, whereas arginine-inhibitable NAGKs, including Paer_NAGK and Tmar_NAGK, are hexamers in which an extra N-terminal kinked helix (N-helix) interlinks three dimers. The influence of point mutations affecting residues of the putative arginine sites on L-arginine inhibition of Paer_NAGK had showed that mutations H271N and E284D increased the concentration of arginine needed for 50% inhibition ($I_{0.5}$) > tenfold, however, mutations Y21A, K213A, G290A could increase the $I_{0.5}$ > 50-fold. Knowledge of the regulatory mechanisms controlling the function or synthesis of arginine biosynthetic enzymes in

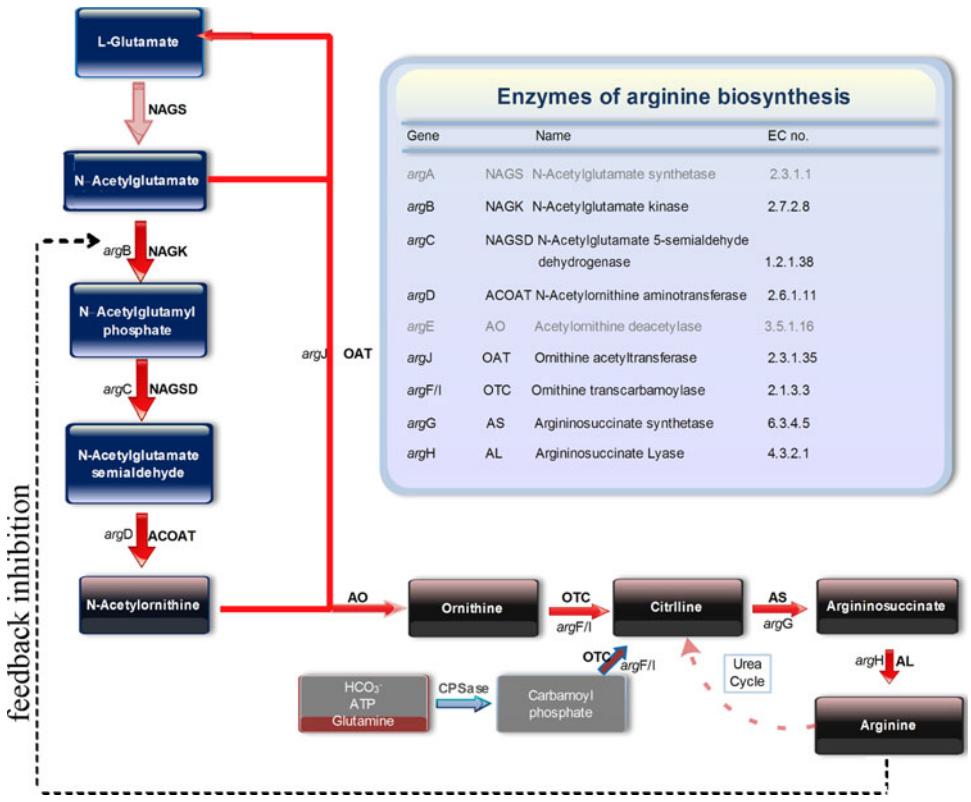


Fig. 1 Outline of pathways for L-arginine biosynthesis from L-glutamate. *Dotted lines* indicate feedback inhibition

prokaryotes has been used to engineer L-arginine over-producing strains amenable to industrial exploitation (Lu 2006; Utagawa 2004; Glansdorff and Xu 2007; Ikeda et al. 2009).

In our previous work, a new L-arginine hyper-producing strain, *Corynebacterium crenatum* SYA5 was isolated from soil sample and the L-arginine concentration in batch fermentation mode could reach 30.6 g/l under optimal culture conditions by its mutant strain *C. crenatum* SYPA5-5 (Xu et al. 2009). In this study, *arg*B gene coding the *N*-acetyl-L-glutamate kinase was first overexpressed in the strain SYPA5-5, whereas the L-arginine production was narrowly increased by 15.4%. Furthermore, to get a feedback-resistant and robust L-arginine producer, the site-directed mutagenesis was put forward. And then the mutant *arg*B gene was cloned into a newly constructed *E. coli–C. crenatum/C. glutamicum* shuttle expression vector pJC*tac* (5.56 kb, Km^R) (Xu et al. 2011). Following that, the recombinant plasmid $pJCB_{M3}$ was introduced into *C. crenatum* SYPA5-5 to enhance the level of the feedback-resistant NAGK protein and the L-arginine biosynthesis flux and then the fermentation characteristics of the recombinant SYPA-CCB_{M3} were studied.

Materials and methods

Strains, plasmids and oligonucleotide primers

The strains, plasmids, and primers (restriction sites were italicized) used in this study are listed in Table 1.

DNA manipulations and site-directed mutagenesis

Standard protocols were used for the construction, purification and analysis of plasmid DNA, and transformation of *E. coli* (Sambrook and Russell 2001). *C. crenatum* was cultivated at 31°C in LB medium containing 0.5% glucose (LBG) and the competent cell medium was LB medium including 3% glycine and 0.1% Tween. The recombinant plasmids were transformed into *C. crenatum* SYPA5-5 using electroporation methods (Tauch et al. 2002). Site-directed mutagenesis was carried out essentially by the method of overlapping PCR using oligonucleotide three sets of primers for the E19R, H26E and H268N mutations listed in Table 1. When necessary, ampicillin or kanamycin was added with the proper final concentration for the transformants selection. Mutations in individual clones were detected by sequencing.

Protein expression and purification

The recombinant *E. coli* BL21 cells were cultured about 6 h induced with IPTG for another 6 h at 30°C and the *C. crenatum* cells from LBG plates were inoculated into the seed medium to be cultivated for 15 h at 31°C on a rotating shaker at 150 rpm, then collected them by centrifugation. The cells were suspended in binding buffer (sodium phosphate, 20 mM; sodium chloride, 500 mM; pH7.8) and disrupted on ice by sonication. The crude extract of enzyme was precipitated with ammonium sulfate (60% saturation) followed by centrifugation at 10,000 rpm for 15 min at 4°C. The recombinant NAGK proteins were purified by a HisTrap HP affinity column under washing buffer (sodium phosphate, 20 mM; sodium chloride, 500 mM; imidazole, 200 mM, pH 6.0) after they were desalted.

SDS-PAGE and western blot analysis

SDS-PAGE was carried out, using a Mini Protean II vertical tank apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were visualized in gels by staining with Coomassie brilliant blue (Bradford 1976). For western blot analysis, samples (the whole cell proteins of *C. crenatum* and the recombinants) were run on SDS-PAGE (12% gels), blotted onto polyvinylidene difluoride membrane and probed with an anti-His tag monoclonal antibody (Qiagen, Hilden, Germany).

NAGK activity assay

NAGK activity was measured with the hydroxylamine containing colorimetric assay of Haas and Leisinger, which detects at 540 nm the formation of acetylglutamyl hydroxamate (extinction coefficient, 456 M^{-1} cm^{-1}) (Haas et al. 1972; Fernandez-Murga et al. 2004). To prevent enzyme inactivation associated with extreme dilution of the enzyme, 0.02 mg of bovine serum albumin (shown in preliminary tests to prevent dilution inactivation)/ml was added in all assays involving extreme dilutions. The standard enzymatic reaction mixture contained, in a total volume of 3 ml aqueous solution, Tris–HCl, pH 8.0, 0.6 mM; NAG, 0.12 mM; $MgCl_2$, 0.12 mM, ATP sodium, 0.12 mM, $NH_2OH{\cdot}HCl$, 1.2 mM; and enzyme preparation. Reactions were started by the addition of enzyme, and incubations were carried out at 37°C for 1 h; the reactions were stopped by adding 1 ml of N-hydrochloric acid (containing 5% $FeCl_3{\cdot}6H_2O$, 4% trichloroacetic acid) to each mixture. At the same time, the LC-MS experiments were carried out on Waters Platform ZMD 4000 equipped with an electrospray ionization source. A chromatographic method was developed using a Hillic column (2.1 × 150 mm). The column oven was maintained at 25°C. The substrate NAG was ready to be analyzed by LC-MS. The substrate NAG could be detected as two isomerides of *N*-acetylglutamate. But the products *N*-acetylglutamyl oxygen oxime could not be detected. One

Table 1 Strains, plasmids and primers in this study

	Characteristics	Source
Strains		
E. coli JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) e14$^-$ [F' traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17($r_K^- m_K^+$)	Invitrogen
E. coli BL21(DE3)	F$^-$ ompT gal dcm lon hsdS_B(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen
C. crenatum SYPA5-5	L-Arginine producer	Our labs
Plasmids		
pET-28a	*E. coli* expression vector, Km^R	Novagen
pJC*tac*	*E. coli–C. crenatum* shuttle expression vector, Km^R	Our labs
28a-*arg*B	A derivative of pET-28a, harboring the WT *arg*B gene	This study
28a-*arg*B_{H268N}	A derivative of pET-28a, harboring mutant *arg*B gene (H268N)	This study
28a-*arg*B_{H26E}	A derivative of pET-28a, harboring mutant *arg*B gene (H26E)	This study
28a-*arg*B_{E19R}	A derivative of pET-28a, harboring mutant *arg*B gene (E19R)	This study
28a-*arg*B_{M2}	A derivative of pET-28a, harboring mutant *arg*B gene (H268N/H26E)	This study
28a-*arg*B_{M3}	A derivative of pET-28a, harboring mutant *arg*B gene (H268N/H26E/E19R)	This study
pJCB	A derivative of pJC*tac*, harboring WT *arg*B gene	This study
pJCB_{M3}	A derivative of pJC*tac*, harboring mutant *arg*B gene (H268N/H26E/E19R)	This study
pJCB_{M3H}	A derivative of pJC*tac*, harboring mutant *arg*B gene (H268N/H26E/E19R) and 6× his tag gene	This study
Primers 5′ → 3′		
P*arg*B F	5′-CGCGAATTCATGAATGACTTGATCAAAG-3′ (*Eco*RI)	
P*arg*B R	5′-CGCGTCGACTTACAGTTCCCCATCCTTG-3′ (*Sal*I)	
P*arg*B_{H268N} F	5′-GGAGTAAGTGCTGCT*A*ATGTCATTGACGGCCG-3′	
P*arg*B_{H268N} R	5′-CGGCCGTCAATGACAT*T*AGCAGCACTTACTC-3′	
P*arg*B_{H26E} F	5′-TTCCATGGTTGCAG*GAA*TTCCGCGACAAG-3′	
P*arg*B_{H26E} R	5′-TCTTGTCGCGGAA*TTC*CTGCAACCATGGC-3′	
P*arg*B_{E19R} F	5′-AAATGTCCTCGCT*CG*GGCGTTGCCATG-3′	
P*arg*B_{E19R} R	5′-CAACCATGGCAACGCC*CG*AGCGAGGAC-3′	
P*arg*B-his6 R	5′-CGCGTCGAC*GTGGTGGTGGTGGTGGTG*CAGTTCCCCATCCTTG-3′ (*Sal*I)	

Amp^R ampicillin-resistant phenotype, *Km^R* kanamycin-resistant phenotype

unit of NAGK is defined as the requirements of the enzyme that generate 1 μmol NAG oxygen oxime every minute or consume 1 μmol NAG. Thus the absorbance (light path 0.5 cm) of the resulting solution (clarified by centrifugation) was determined at A_{540}. And we calculated the reduction of the substrate NAG every minute.

L-Arginine feedback inhibition experiments

$I_{0.5}^R$ is the concentration of L-arginine that yields 50% inhibition. The same program was used for hyperbolic fitting of the substrate saturation data and for estimating V_{max} and K_m values from secondary hyperbolic plots of apparent V_{max} values for one substrate versus the concentration of the other substrate. In this way, the K_m value given for NAG ($K_{m(NAG)}$) has been estimated at infinite concentration of the substrate ATP, and the catalytic constant K_{cat} is a measure of V_{max}/E_t expressed in units of inverse time whether the 1 mM L-arginine was added or not.

Homology modeling

The amino acid sequences in the multiple alignments were extracted from the SWISS-PROT and EMBL databases of the ExPASy Molecular Biology Server (http://www.expasy.org/sprot/), including *E. coli* NAGK (1gs5), *Pseudomonas aeruginosa* NAGK (2buf), *Thermotoga maritime* NAGK (2bty), and *Mycobacterium tuberculosis* NAGK (2ap9). Based on the alignment produced by ClustalW2 (http://www.ebi.ac.uk/clustalw/), the crystal structure of Mtub_NAGK (PDB ID, chain: 2ap9, E), with a resolution of 2.80 Å, was used as the template to model the Ccre_NAGK by Automatic Modelling Mode. Algorithm used to produce a tree from given distances (or dissimilarities) between

sequences by ClustalW2 (http://www.ebi.ac.uk/clustalw/). Evolutionary distance between two sequences modeled as expected fraction of amino acid substitutions per site given the fraction of mismatched amino acids in the aligned region.

Growth medium and conditions for L-arginine production

Corynebacterium crenatum SYPA5-5 and its transformants are auxotrophic for L-histidine (Xu et al. 2009). A stock culture was maintained on agar slants containing (in g/l) peptone 10, beef extract 10, yeast extract 5, NaCl 5, and agar 20. The seed culture medium (in g/l) was consisted of glucose 30, corn steep liquor 20, $(NH_4)_2SO_4$ 20, KH_2PO_4 1, $MgSO_4{\cdot}7H_2O$ 0.5 and urea 1.5. The seed was inoculated from agar slants, and cultured at 31°C for 14–16 h in a shake flask. The shake flask culture was then transferred into a 5 L bioreactor (BIOTECH-5BG, Baoxing Co., China) containing 3 L fermentation medium A (in g/l) which consists of glucose 150, corn steep liquor 40, $(NH_4)_2SO_4$ 20, KH_2PO_4 1.5, $MgSO_4{\cdot}7H_2O$ 0.5, $FeSO_4{\cdot}7H_2O$ 0.02, $MnSO_4{\cdot}H_2O$ 0.02, biotin 8×10^{-5}, L-histidine 5×10^{-4} (pH 7.0). Glucose and chalk were sterilized separately. And the aeration rate was controlled at 1 vvm for all the experiments. Studies in the bioreactor were carried out at the agitation rate of 600 rpm.

Assays of cell concentration, glucose and L-arginine

Cell concentration was first monitored at 562 nm, and the dry cell weight (DCW) was determined by a pre-calibrated relationship (1 OD = 0.375 g/l DCW). Glucose concentration in the media assayed using a glucose analyzer (Biosensor SBA-50, Shandong, China). Concentrations of amino acids were measured by an Agilent 1100 HPLC, under the following conditions: column Hypersil ODS-C18 4 × 125 mm, temperature 40°C, flow rate 1.0 ml/min, detection fluorescence detector, Ex 340 nm Em 450 nm, eluent A 20 mM Na-acetate, eluent B 20 mM of Na-acetate:methanol:acetonitrile = 1:2:2 (*v/v*). All of the measurements, particularly the most important state variables, such as the concentrations of cells, L-arginine and glucose were measured in three parallels.

Results and discussions

Cloning and expression of the WT Ccre_*arg*B in *E. coli* BL21

The *arg*B gene fragment from *C. crenatum* SYPA5-5 was obtained by PCR amplification and analysis of the 0.95-kb nucleotide sequence revealed 99.47% homologous to the *arg*B gene of *C. glutamicum* ATCC13032. The *arg*B nucleotide sequence of *C. crenatum* SYPA5-5 had been submitted to GenBank Nucleotide Databases with the accession no. HQ602711. The DNA fragment was subcloned into pET28a, resulting in the recombinant expression plasmid 28a-*arg*B. When the recombinant *E. coli* BL21/28a-*arg*B cells were induced with 0.5 mM isopropyl-*β*-*D*-thiogalactopyranoside (IPTG) at 30°C, SDS-PAGE analysis showed a predominant band corresponding to the expected size (approximately 36 kDa), which was a fusion hybrid protein and was the same size as estimated from the deduced amino acid sequence of the fusion region including the 6× His tag in pET-28a vector (Fig. 2a, lane 1). And the target recombinant enzyme was observed a high NAGK activity (4.64 U/mg) in the recombinant cell extracts (shown in Table 2).

Effects of L-arginine on WT Ccre_NAGK activity

The WT Ccre_NAGK with N-terminal 6× His tag were purified from the cell extract of *E. coli* transformants using a HisTrap HP affinity column. As proposed, the purified Ccre_NAGK was potently inhibited by L-arginine. Similar to what have been observed with the NAGK from *P. aeruginosa* (Benkert et al. 2008), *T. maritime* (Fernandez-Murga et al. 2004; Ramón-Maiques et al. 2006) and *B. subtilis* (Sun et al. 2009) involving the cycle L-arginine pathway. Whereas, the WT Ccre_NAGK showed an even lower $I^{R}_{0.5}$ which is only 0.4 mM. These results illuminated a strong feedback inhibition of WT Ccre_NAGK with L-arginine.

Homology model and proposed 3D structure of Ccre_NAGK

Homology structural alignment of the NAGKs was carried out in this study between *C. crenatum* (Cglu_NAGK), *M. tuberculosis* (Mtub_2ap9) *P. aeruginosa* (Paer_2buf), *T. maritime* (Tmar_2bty), *E. coli* (Ecol_1gs5) and the distances between them were 0.18346, 0.19782, 0.23889, 0.25048 and 0.45419, respectively (Fig. 3). With the 64.0% similarity of Ccre_NAGK and Mtub_NAGK, the crystal structure of Mtub_NAGK (PDB ID: 2ap9, chain E), with a resolution of 2.80 Å, was used as the template to model the Ccre_NAGK by SWISS-MODEL Automatic Modelling Mode (Fig. 4a).

Construction of Cre_NAGK mutants

Based on the study of the structural bases of feedback control of arginine biosynthesis in *T. maritima* and *P. aeruginosa* and the feedback resistant forms of Ecol_NAGK the NAGKs were compared to propose the

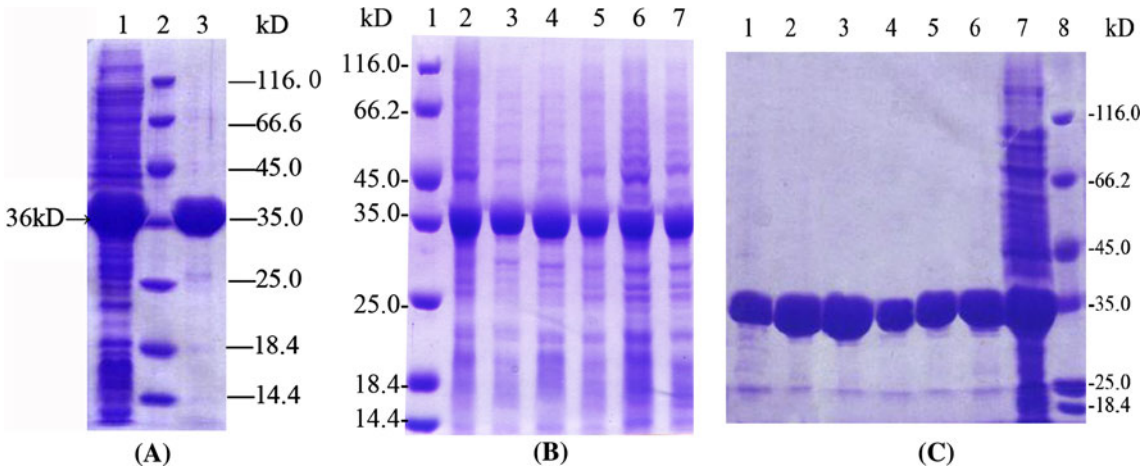


Fig. 2 SDS-PAGE of wild-type and mutated NAGK proteins. **a** *Lane 1* Whole cell proteins of BL21/28a-*arg*B, *lane 2* molecular mass markers, *lane 3* NAGK purified by a HisTrap HP affinity column. **b** *Lane 1* Molecular mass markers (kDa), *lane 2–7* whole cell proteins of BL21/28a-*arg*B, 28a-*arg*B_{H268N}, 28a-*arg*B_{H26E}, 28a-*arg*B_{E19R}, 28a-*arg*B_{M2} and 28a-*arg*B_{M3}, respectively. **c** *Lane 1* Purified $NAGK_{M3}$, *lane 2* purified $NAGK_{M2}$, *lane 3* purified $NAGK_{E19R}$, *lane 4* purified $NAGK_{H26E}$, *lane 5* purified $NAGK_{H268N}$, *lane 6* WT Ccre_NAGK, *lane 7* whole cell proteins of BL21/28a-*arg*B, *lane 8* molecular mass markers

Table 2 Crude enzyme activities for WT Ccre_NAGK and its variants in *E. coli* and *C. crenatum*

Crude enzyme activity	Total activity (U/ml)	Total protein content (mg/ml)	Specific enzyme activity (U/mg)
E. coli BL21	0.27	3.92	0.07
BL21/28a-*arg*B	26.95	5.81	4.64
BL21/28a-*arg*B_{M3}	25.83	5.64	4.58
C. crenatum SYPA5-5	1.65	3.23	0.51
SYPA-CCB	5.14	3.89	1.32
SYPA-CCB_{M3}	5.18	3.87	1.38
SYPA-CCB_{M3H}	5.53	3.99	1.34

All values are means the average value of three independent experiments

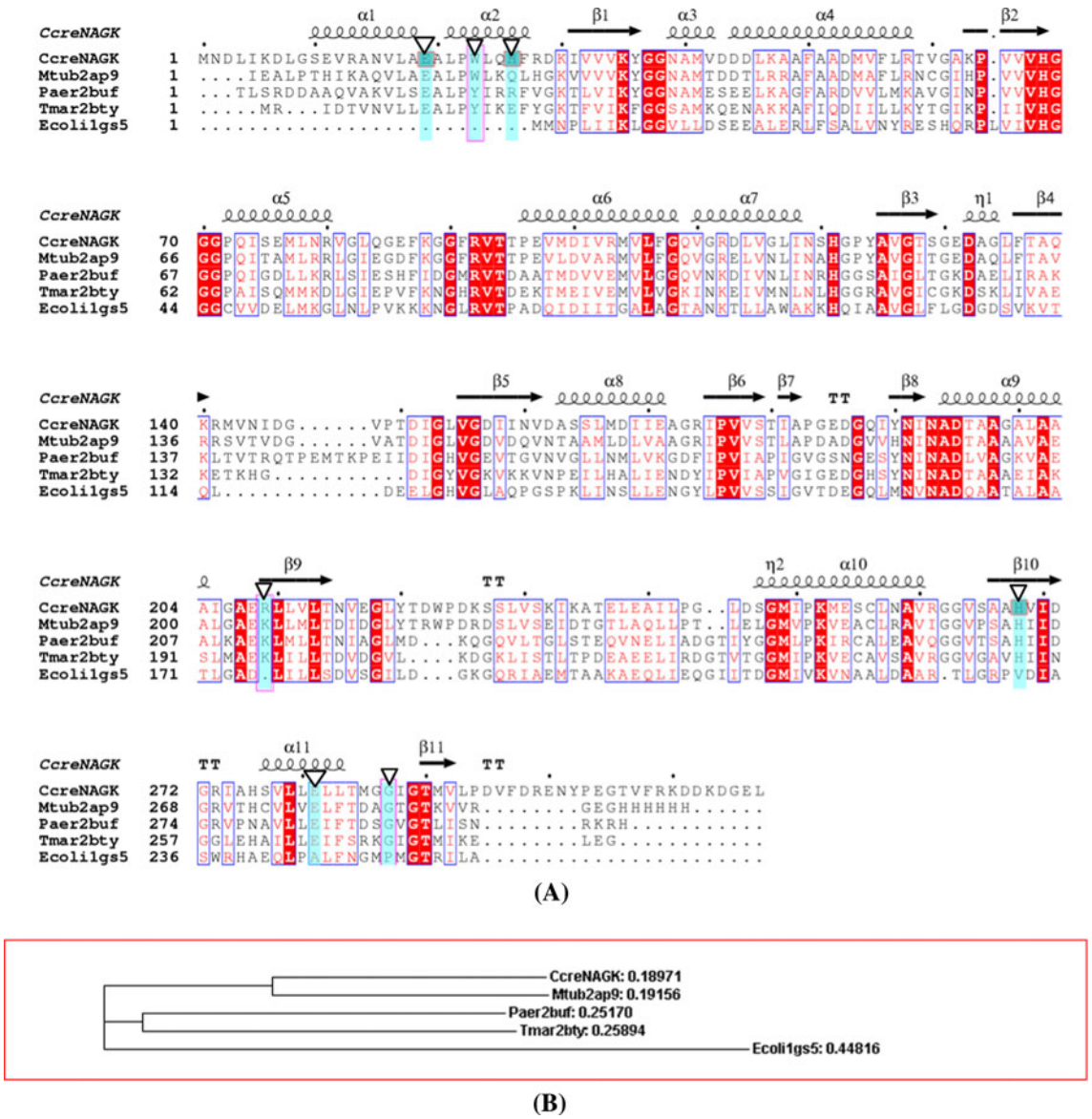
arginine sites here. Meanwhile, with the study of the site-mutagenesis lining or close to the L-arginine site of Paer_NAGK, the putative arginine-binding site of Ccre_NAGK was proposed on the basis of the structural alignment of the NAGKs. These residues (E19, W23, H26, R209, H268, E281 and G287) of the proposed arginine-sites in Ccre_NAGK were selected and site-directed (the sites of arginine signatures were labeled with inverse triangle in Fig. 3a).

However, the three point variants $NAGK_{E19R}$, $NAGK_{H26E}$ and $NAGK_{H268D}$ could increase the $I^R_{0.5}$ 50–60-fold efficiently (shown in Table 3, and the other mutant data were not shown here). The mutants behaved similarly to the wild-type enzyme in SDS-PAGE and the proteins were purified to apparent homogeneity (Fig. 2b, c). Additionally, the specific enzyme activities of the mutant Ccre_NAGKs were at a consistent level.

The resistant feedback inhibition NAGK mutants

In this study, to obtain the feedback-resistant NAGK, the 951-bp fragments containing the multiple points mutations (associated E19R/H26E/H268D) in *arg*B were amplified by using two or three times over-lapping PCR and then cloned into pET28a. The WT Ccre_NAGK and variants were abundantly expressed in the soluble form and then purified similarly (Fig. 2b, c). The E19R/H26E/H268D variants induced no changes in protein folding or conformational stability. And the specific activities of NAGK with the mutations were determined and they showed the same level as the WT Ccre_NAGK. The $I^R_{0.5}$ of the variants was evidently increased (data shown in Table 3). Consequently, the $NAGK_{M3}$ could be considered a resistant-feedback inhibition mutant for a high $I^R_{0.5}$ value. The kinetic constants of enzyme activity were determined in comparison with WT Ccre_NAGK and their activities were indistinguishable (data shown in Table 3). These kinetic parameters for the substrate NAG of the reaction in the absence of arginine were little changed in the mutants. In the presence of the WT kinase, the activity was fully inhibited by 10 mM L-arginine, while 10 mM L-arginine could not yet inhibit the mutant $NAGK_{M3}$ activity. When the L-arginine concentration was increased to 20 mM, the enzyme activities were decreased only by 5.85% (Fig. 5).

Site-directed mutagenesis of the three residues of the Ccre_NAGK domain arginine signatures (portrayed in Fig. 4) drastically increased the $I^R_{0.5}$, with 50–60-fold $I_{0.5}$



(A)

(B)

Fig. 3 Multiple-sequence alignment of NAGK homologies. **a** Levels of homology between the NAGK amino acid sequences. The sequences used were NAGK homologies from *Escherichia coli* (Ecol_1gs5), *Pseudomonas aeruginosa* (Paer_2buf), *Thermotoga maritime* (Tmar_2bty), *M. tuberculosis* (Mtub_2ap9) and *Corynebacterium crenatum* (Ccre_NAGK). The NAGK homologies were aligned by using Clustal W2 programs. The identical residues in the five prokaryotic NAGK sequences are shaded and homologous residues detected by the PAM250 matrix of amino acid similarity. The mutations are indicated above the sequence (Labeled with *highlighted* and *inverse triangle*). **b** Phylogenetic tree showing the evolutionary relatedness. The estimated genetic distance between sequences is proportional to the lengths of the horizontal lines connecting one sequence to another

increases triggered by the E19R, H26E and H268N mutations. The proposed 3D structures of Ccre_NAGK illustrated that the E19 and H26 residues may belong to part of the sites in the N-helix, and the H268 residue may be located to the βJ-αK-βK loop (Figs. 3a, 4a). Thereby, these mutations might result in the interaction with the N-helix at its entry into the enzyme body, conceivably hampering arginine inhibition by altering the N-helix position (Fig. 4b). The results indicated that the ring-like hexamer of the arginine combination might have been modified by the three mutated resides in the Ccre_NAGK.

Construction of the recombinant *C. crenatum* SYPA-CCB, SYPA-CCB$_{M3}$ and SYPA-CCB$_{M3H}$

The introduction of copies with the *arg*B enhanced the arginine production and the modulation of gene expression and metabolism of *C. crenatum* proved an efficient tool for

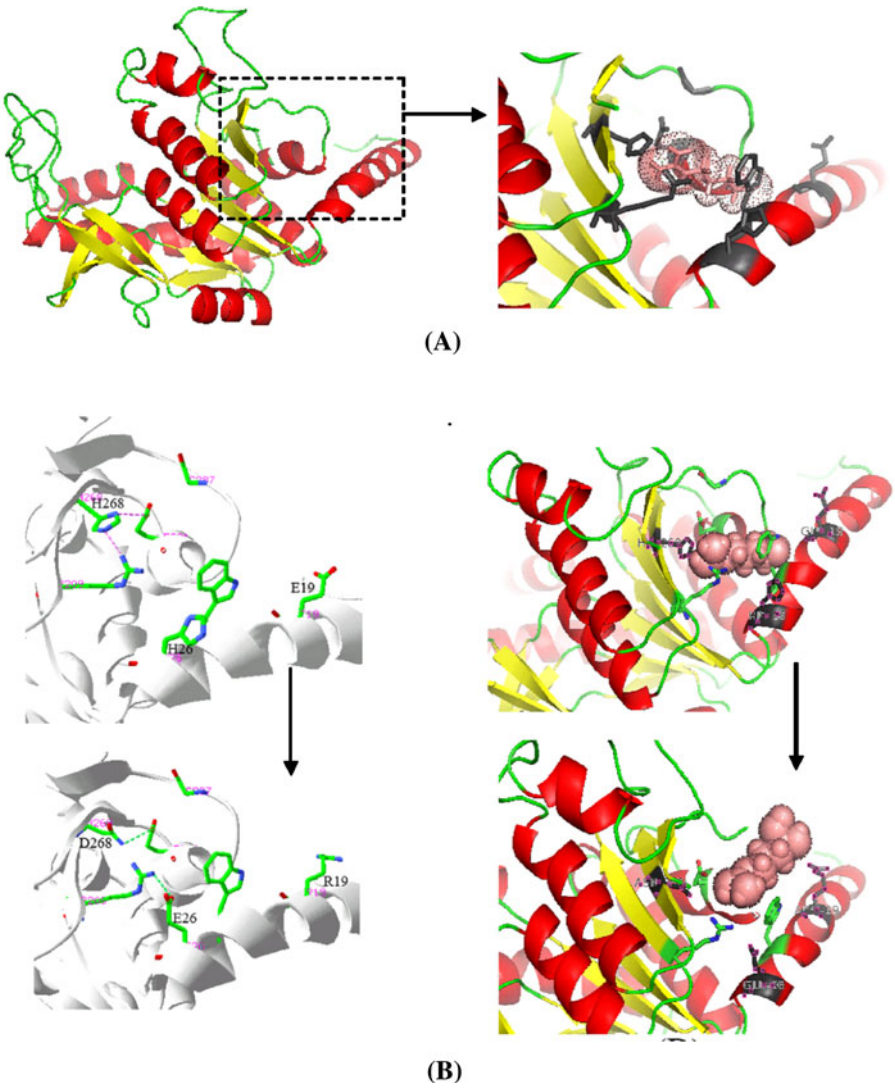


Fig. 4 Predicted 3D structure of Ccre_NAGK. **a** An overall view of NAGK and the sphere view of the proposed arginine-binding sites (E19, H26, R209, H268, E281, G287) in the predicted 3D structure of Ccre_NAGK. **b** A view of the origin resides (E19, H26, R209, H268, E281, G287) and mutant resides (R19, E26, R209, N268, E281, G287) binding with L-arginine

Table 3 Enzyme activities, kinetic parameters and $I^{R}_{0.5}$ for WT Ccre_NAGK and its variants

NAGKs	Source	Mutation sites	Expression level	Enzyme activity (U/mg)	$K_{m(NAG)}$ value (mM)	K_{cat} value (1/s)	$I^{R}_{0.5}$ value (mM)	K_{cat} value with 1 mM Arginine (1/s)
WT Cre_NAGK	BL21/28a-*argB*	n.a.	+++	19.05*	3.42 ± 0.06*	45.1 ± 2.1	0.4	31.4 ± 1.5
$NAGK_{H268N}$	BL21/28a-$argB_{H268N}$	H268 N	+++	18.54	3.45 ± 0.03	45.3 ± 1.1	22	41.1 ± 0.5
$NAGK_{H26E}$	BL21/28a-$argB_{H26E}$	H26E	+++	22.56	3.29 ± 0.05	44.4 ± 2.2	21	41.0 ± 3.1
$NAGK_{E19R}$	BL21/28a-$argB_{E19R}$	E19R	+++	19.41	3.33 ± 0.08	45.8 ± 0.9	24	42.1 ± 2.8
$NAGK_{M2}$	BL21/28a-$argB_{21}$	H268 N; H26E	+++	17.78	3.42 ± 0.11	46.1 ± 2.4	350	45.6 ± 1.0
$NAGK_{M3}$	BL21/28a-$argB_{M3}$	H268 N; H26E; E19R	+++	18.65	3.36 ± 0.05	46.5 ± 1.0	784	46.0 ± 1.5

* All values are means the average value of three independent experiments; *n.a.* not applicable. Data are the mean value ± SD of two separate experiments with duplicate determinations

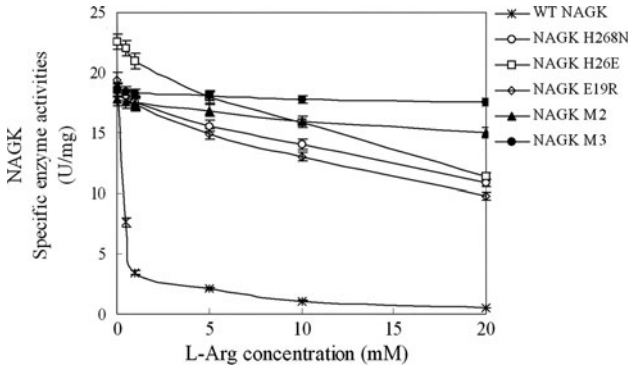


Fig. 5 Feedback inhibition by L-arginine of purified NAGK and variants from the recombinant BL21/28a-*arg*B, 28a-*arg*B_{H268N}, 28a-*arg*B_{H26E}, 28a-*arg*B_{E19R}, 28a-*arg*B_{M2} and 28a-*arg*B_{M3}, respectively

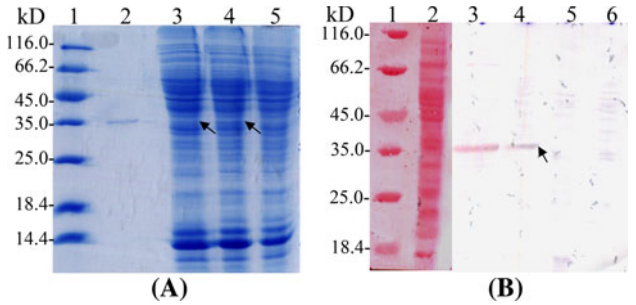


Fig. 6 SDS-PAGE and western blot analysis of $NAGK_{M3}$ from the *C. crenatum* recombinants. **a** SDS-PAGE of the whole cell proteins from SYPA 5-5, SYPA-CCB_{M3}, SYPA-CCB_{M3H} and the purified $NAGK_{M3H}$ underwent electrophoresis on a standard 12% gel and were stained with Coomassie Brilliant Blue. *Lane 1* Molecular mass markers, *lane 2* $NAGK_{M3H}$ purified from SYPA-CCB_{M3H}, *lane 3* SYPA-CCB_{M3}, *lane 4* SYPA-CCB_{M3H}, *lane 5 C. crenatum* SYPA5-5. **b** Western blot analysis for the $NAGK_{M3H}$ of the recombinant SYPA-CCB_{M3H} stained with Ponceau S or HRP-DAB. *Lane 1* Molecular mass markers, *lane 2* SDS-PAGE (12%) analysis of *C. crenatum* SYPA5-5 stained with Ponceau S, *lane 3* positive control (*E. coli* BL21/28a-*arg*B stained with HRP-DAB), *lane 4* His-tagged $NAGK_{M3H}$ (*C. crenatum* SYPA-CCB_{M3H} stained with HRP-DAB), *lane 5, 6* negative control (*C. crenatum* SYPA 5-5 stained with HRP-DAB)

constructing amino acid-producing strains. However, the L-arginine production of the recombinant SYPA-CCB was only increased by 15.4%, for the overexpression NAGK was severely inhibited by the product L-arginine. In other words, the recombinant CCB possessed the feedback inhibition with L-arginine as well as the wild-type L-arginine producer SYPA5-5. To achieve a higher yield of L-arginine, the constructed strain *C. crenatum* SYPA-CCB_{M3} (combinations of the mutants NAGK-E19R/H26E/H268N) with NAGK which is resistant to inhibition by L-arginine was used as a basis to develop a new type of arginine producer by genetic engineering. The combination with the three residues mutations described above *arg*B_{M3} used for the substitution of the identical wild-type *arg*B gene cloned in vector pJC*tac*. The activity of the NAGK mutant was up-modulated by over-expression in the *C. crenatum* via the plasmid of pJC*tac*.

Additionally, to testify the overexpression level of the mutant NAGK, the pJCB_{M3H}, which directed the expression product of the C-terminal 317 amino acid residues of the Ccre_NAGK α subunit with the 6× His tag at the C-terminal end was constructed and transformed into *C. crenatum*. The expressions of *arg*B_{M3} and *arg*B_{M3H} in *C. crenatum* by using the plasmid pJC*tac* were firstly checked using SDS-PAGE (Fig. 6a, lanes 3, 4). The specific enzyme activities in the recombinant SYPA-CCB_{M3} and CCB_{M3H} were evaluated by comparing with those in the control SYPA5-5. As a result, it was found that the specific enzyme activities in CCB_{M3} and CCB_{M3H} displayed 2.63 and 2.76 times those in the original strain SYPA5-5, respectively (Table 2). Typically, the $NAGK_{M3H}$ was purified from the recombinant CCB_{M3H} (Fig. 6a, lane 2). Its specific enzyme activity was 19.34 ± 0.08 U/mg and the residual activity was 13.41 ± 0.05 U/mg when 500 mM L-arginine added in the enzymatic reaction. Enzyme assays revealed that the resulting $NAGK_{M3}$ enzyme was expectedly less sensitive to end product inhibition than the original enzyme and the previous mutants. At the same time, the $NAGK_{M3H}$ from the recombinant CCB_{M3H} was further confirmed by western blot analysis (Fig. 6b). The objective protein band with a 6× His tag could be observed on the western blot, indicating that the *arg*B_{M3} had been overexpressed in *C. crenatum* SYPA carried by a multi-copy plasmid pJC*tac*.

Improved production of L-arginine by feedback inhibition-resistant SYPA-CCB_{M3}

Since the $I^{R}_{0.5}$ of $NAGK_{M3}$ had been remarkably increased, the feedback regulation was insensitive to the presence of intracellular L-arginine. The engineered strains SYPA-CCB_{M3}, which carried the *arg*B_{M3} mutation were studied for L-arginine fermentation, the highest level of L-arginine production was 45.6 g/l, about 41.7% higher than the WT *C. crenatum* SYPA. The analysis of fermentation characteristics showed that the growth of cells of SYPA was a little better than the recombinant CCB_{M3} from beginning to 24 h due to the kanamycin contained in the medium of the recombinant, however, the growth of the two strains provided the similar appearance in their growth curves after the early logarithmic phase. It was illustrated that the over expression of *arg*B gene effected the growth of cells slightly in the *C. crenatum*. The consumption of glucose did not have any differences until 36 h. The recombinant did not showed the advantage of producing L-arginine till logarithmic phase, and about 23.4% higher than SYPA at the end of the fermentation (Fig. 7a, b). Compared with the

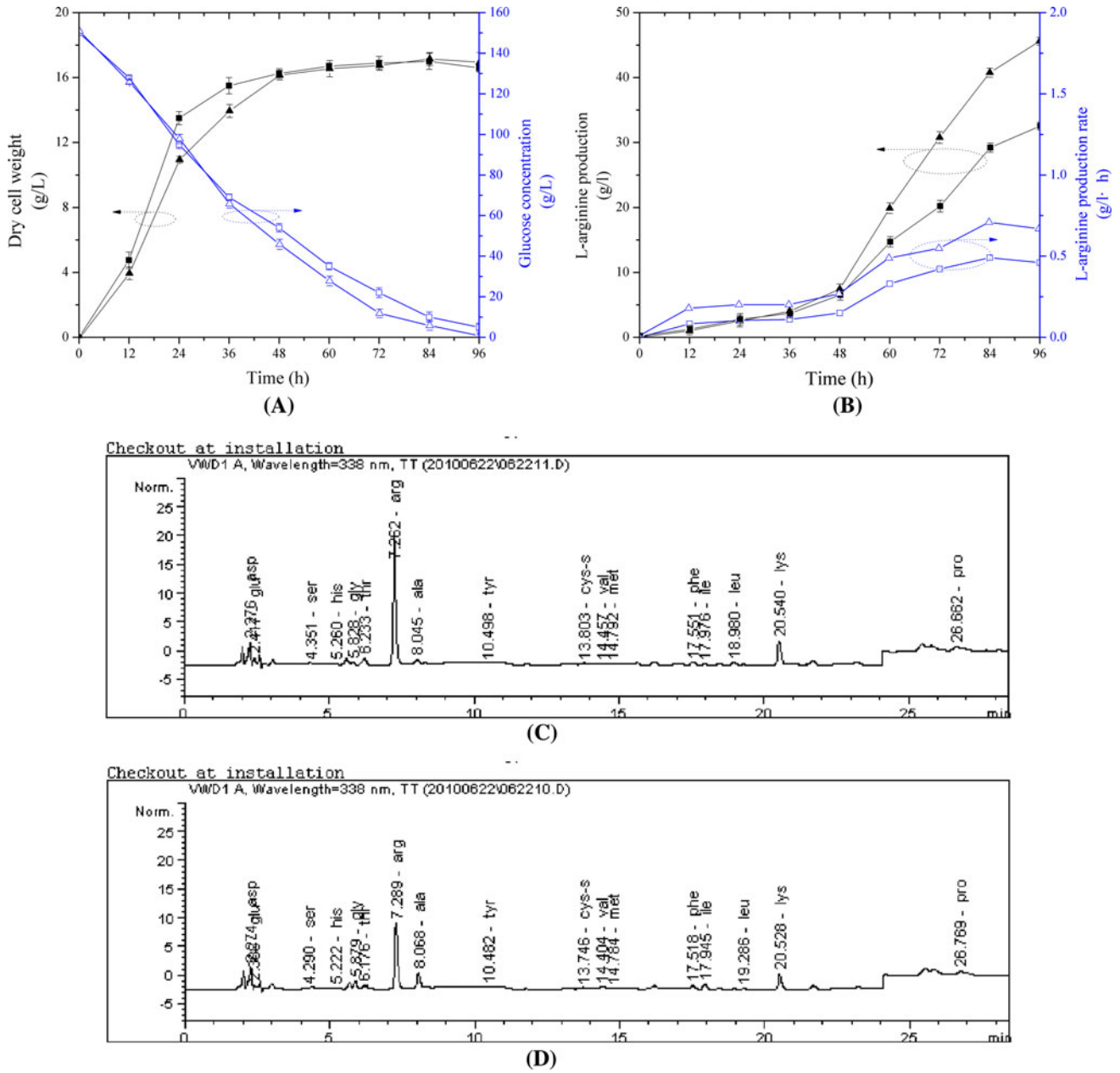


Fig. 7 Comparison of L-arginine production between the *C. crenatum* SYPA-CCB$_{M3}$ and *C. crenatum* SYPA5-5. **a** Cell concentration and glucose concentration, **b** L-arginine concentration and L-arginine production rate. *Open and filled triangles* SYPA-CCB$_{M3}$ recombinant, *Open and filled squares C. crenatum* SYPA5-5. **c** Concentrations of amino acids analysis of the SYPA-CCB$_{M3}$ recombinant fermentation at 96 h. **d** Concentrations of amino acids analysis of the wild-type SYPA5-5 fermentation at 96 h. *All values are means standard deviations of at least three independent experiments

initial strain and the recombinant SYPA-CCB, introducing *arg*B mutations rendered that intracellular L-arginine could not feedback inhibit by L-arginine. Thus, as a result of feedback-resistant inhibition, the increased L-argininie flows of carbon metabolism may be the main reason for the difference of L-arginine production in the CCB$_{M3}$. Amino acids analysis of the fermentation liquor showed the concentration of glutamate, proline, alanine, isoleucine were decreased obviously (Fig. 7c vs. d; Table 4). It proved that more NAGK enhanced the utilization rate of glutamate, and at the same time it weakened the synthesis of proline and some other amino acids. These results explained that the L-arginine synthesis had been largely enhanced due to the overexpression of the *arg*B$_{M3}$, which is feedback-resistant. The constructed strain SYPA-CCB$_{M3}$ with NAGK, resistant to inhibition by L-arginine, was used as a basis to develop a new type of arginine producer by genetic engineering. And the activity of the NAGK was

Table 4 The 12 extracellular amino acids concentrations in the fermentation of SYPA and SYPA-CCB$_{M3}$ (concentration, g/l)

Amino acids	SYPA	SYPA-CCB$_{M3}$	Amino acids	SYPA	SYPA-CCB$_{M3}$
Ala	2.22	0.77	Thr	0.19	0.62
Ile	1.35	0.42	Asp	4.42	3.11
Lys	2.49	2.63	Val	0.54	0.19
Pro	1.55	0.98	Glu	1.49	0.26
Arg	32.32	45.60	Leu	0.25	0.71
Ser	0.34	0.30	Phe	1.24	1.02

Fermentation time was 96 h

up-modulated by site-directed mutagenesis and over-expression in the *C. crenatum*.

Conclusion

The main strategy was to modulate expression of the genes involved in the biosynthesis of arginine. The findings indicate that an overexpression of NAGK$_{M3}$ could not be the only explanation for the over-production of arginine, as a high concentration of arginine could have not inhibited its activity and further increased metabolic products formation in the arginine-production strain. The newly engineered strain, SYPA-CCB$_{M3}$, is resistant to L-arginine feedback inhibition, which could be clearly reflected on the difference in the fermentation profiles between the new strain and the WT strain. This resistant-feedback inhibition led to a not-restricted L-arginine biosynthesis flux and decreased formation of by-products. Thereby, the engineered strain SYPA-CCB$_{M3}$ could achieve a higher level of production above the classical producer *C. crenatum* SYPA5-5. This is an example of up-modulation of the flux through the L-arginine metabolic pathway by deregulating the key enzyme of the pathway. As a result, the increased gene dosage does bring about an equally increased gene expression and synthesis of the final products. However, the other enzymes involved in the arginine biosynthesis may have the original activities as in WT *C. crenatum* SYPA and this may become a new limiting factor in the arginine production. Thus we shall moderate or controlled strengthening of *arg*CJBDFGH gene cluster expression to further improve L-arginine production. And the other most efficient L-arginine producer may be improved by further steps, including improvement of L-arginine export and increasing the substrate glutamate or ATP pools in the cells. Furthermore, many beneficial mutations would be studied within individual *Corynebacterium* sp. strains and their exploitation is now progressing in our laboratories. In near future, most of them will be regenerated for useful knowledge that is widely available for the amino acid industry. This work studies on L-arginine fermentation could be a paradigm for future the strain development in fermentation industries.

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