GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS

Controlling the transcription levels of *argGH* redistributed L-arginine metabolic flux in *N*-acetylglutamate kinase and ArgR-deregulated *Corynebacterium crenatum*

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Abstract *Corynebacterium crenatum* SYPA5-5, an L-arginine high-producer obtained through multiple mutation-screening steps, had been deregulated by the repression of ArgR that inhibits L-arginine biosynthesis at genetic level. Further study indicated that feedback inhibition of SYPA5-5 *N*-acetylglutamate kinase (CcNAGK) by L-arginine, as another rate-limiting step, could be deregulated by introducing point mutations. Here, we introduced two of the positive mutations (H268N or R209A) of CcNAGK into the chromosome of SYPA5-5, however, resulting in accumulation of large amounts of the intermediates (L-citrulline and L-ornithine) and decreased production of L-arginine. Genetic and enzymatic levels analysis involved in L-arginine biosynthetic pathway of recombinants SYPA5-5-NAGK$_{H268N}$ (H-7) and SYPA5-5-NAGK$_{R209A}$ (R-8) showed that the transcription levels of *argGH* decreased accompanied with the reduction of argininosuccinate synthase and argininosuccinase activities, respectively, which led to the metabolic obstacle from L-citrulline to L-arginine. Co-expression of *argGH* with exogenous plasmid in H-7 and R-8 removed this bottleneck and increased L-arginine

productivity remarkably. Compared with SYPA5-5, fermentation period of H-7/pDXW-10-*argGH* (H-7-GH) reduced to 16 h; meanwhile, the L-arginine productivity improved about 63.6 %. Fed-batch fermentation of H-7-GH in 10 L bioreactor produced 389.9 mM L-arginine with the productivity of 5.42 mM h^{-1}. These results indicated that controlling the transcription of *argGH* was a key factor for regulating the metabolic flux toward L-arginine biosynthesis after deregulating the repression of ArgR and feedback inhibition of CcNAGK, and therefore functioned as another regulatory mode for L-arginine production. Thus, deregulating all these three regulatory modes was a powerful strategy to construct L-arginine high-producing *C. crenatum*.

Keywords *Corynebacterium crenatum* · L-arginine · Feedback inhibition · *argGH* · L-citrulline · L-ornithine

Introduction

L-arginine, a conditionally essential amino acid which acts as a precursor of nitric oxide (NO), is widely used as an essential ingredient in food flavoring and pharmaceutical industry [2, 17]. Bacterial fermentation from natural carbon sources is the major approach for L-arginine production at the industrial scale [12, 26]. With the development of "omics" technology and swift appearance of various accessible genetic tools, considerable attention has recently been given to construct more efficient production strains, as well as to elucidate the unknown regulatory mechanisms involved the L-arginine metabolism processes [17].

Similar to most of the amino acids, the mutant strains of *Corynebacterium* sp. was employed in industrialized production of L-arginine [26]. *Corynebacterium crenatum*, a facultative anaerobic gram-positive organism, is extensively

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Fig. 1 The pathway and regulatory modes involved in L-arginine biosynthesis in *C. crenatum* SYPA5-5. **a** The de novo L-arginine biosynthetic pathway. Outline of the pathway, gene name, enzyme name, EC number and the co-factor needed in biosynthesis were displayed for each step. The *dash arrow* represents the CcNAGK that was feedback inhibited by terminal product. **b** The *arg* cluster encoding the enzymes for L-arginine biosynthesis including two larger transcripts of *argCJBDFR* and *argGH*. The transcription of *arg* cluster was repressed by ArgR repressor, which was deregulated by introducing nonsense mutation (C109T) in *argR* through an iterative procedure of random mutation and selection in previous study
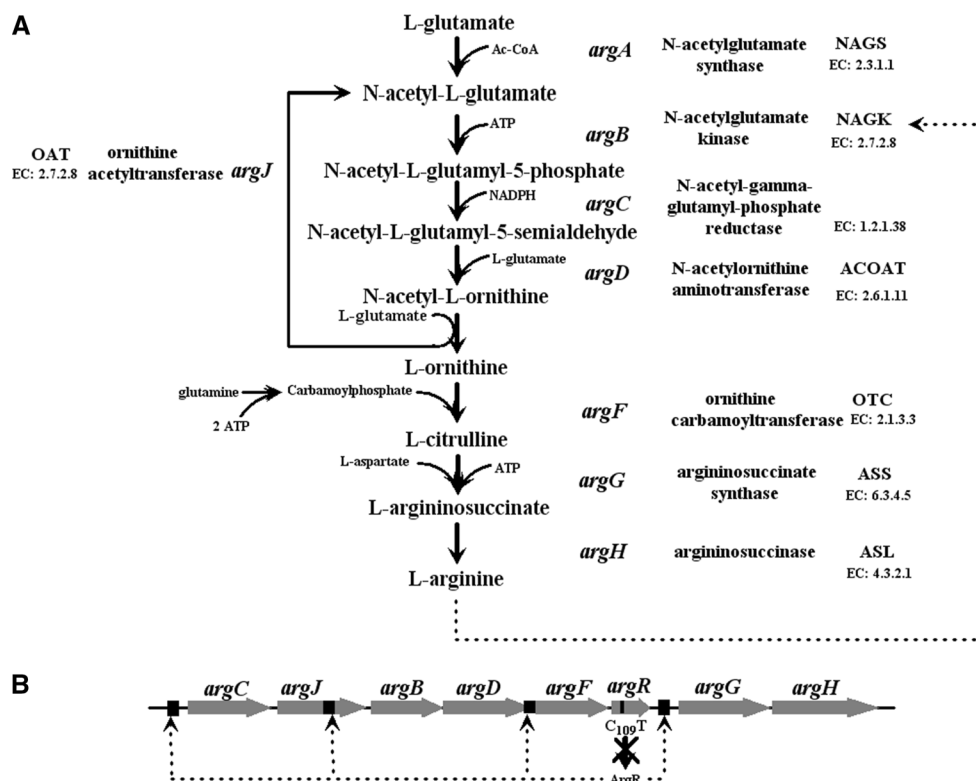
used as a cell factory for amino acids and organic acids production [4, 32]. *C. crenatum* SYPA5-5 was isolated from several mutation-screening steps against L-arginine analogs (e.g., D-arginine, homoarginine and arginine hydroxamate) in our previous study, which could accumulate 30 g L^{-1} L-arginine after cultivated for 96 h in optimized medium using glucose as main carbon source [32].

As well as *Corynebacterium glutamicum*, *C. creanatum* possesses the so-called economical cyclic L-arginine biosynthetic pathway [5, 37]. Figure 1a shows the de novo biosynthetic pathway which initiates by acetylation of glutamate and proceeds via three enzymatic steps which form further acetylated intermediates (*N*-acetyl-ornithine). The ornithine acetyltransferase (OAT) could use acetyl group of *N*-acetyl-ornithine to acetylate glutamate with L-ornithine released. Three more reactions (OTC, ASS, ASL) are followed to finish the assembly of the guanidine group on L-arginine from carbamoylphosphate and the amino group of L-aspartate. Feedback inhibition on *N*-acetylglutamate kinase (NAGK) by L-arginine appears to be a rate-limiting step in this cyclic pathway [39]. Meanwhile, the genes encoding these enzymes are clustered in chromosome including two large transcripts corresponding to *argCJBDFR* and *argGH,* and their transcription levels are regulated by ArgR repressor encoding by *argR* gene (Fig. 1b) [14, 21, 38].

Previously, chemical mutagenesis and selection with L-arginine analogs led to isolation of L-arginine producer in which NAGK activity was released from the feedback inhibition [39]. In the post-genomic era, "genome-based strain reconstruction" was an efficient method to identify mutations benefit to amino acids production and breeding efficient producers, which was also successfully used for construction of L-arginine producer [19]. The mutations in *argB* (A26V, M31V) of classical L-arginine producers was identified by sequence analysis to deregulate feedback inhibition of *C. glutamicum* NAGK, and introducing these mutations into *C. glutamicum* ATCC13032 could make it accumulate large amount of L-arginine and L-citrulline when combined with deletion of *argR* gene [13]. Recently, a new in vivo ultrahigh-throughput screening approaches by using metabolite sensors could also yielded feedback-resistant NAGK variants [23]. In our previous studies, sequence analysis of *arg* cluster of SYPA5-5 showed that the nonsense mutation in *argR* (C109T) made *arg* cluster release from ArgR repression through random mutagenesis [34]. Further study by introducing the point mutation (E19R, H26E, R209A, H268N or G287D), respectively, in CcNAGK increased 50 % inhibitory L-arginine concentration (I$_{0.5}$) significantly in vitro [33], which deregulated the feedback inhibition of CcNAGK by L-arginine in SYPA5-5 during the fermentation [35]. However, moderate expression of a feedback-resistant enzyme, rather than its overexpression by multicopy plasmid, has been proven more useful for increasing amino acids production [7]. Therefore, two of the positive mutations (R209A or H268N)

of CcNAGK were introduced into the chromosome of SYPA5-5, respectively, in this study. Unexpectedly, the recombinants accumulated large amount of L-citrulline and L-ornithine as intermediate metabolites of L-arginine biosynthetic pathway.

Similarly, Park et al. proposed effective and systematic metabolic engineering strategies to improve L-arginine production by *C. glutamicum* ATCC 21831, involving random mutagenesis, removal of regulatory repressor (ArgR and FarR), optimization of NADPH level, disruption of L-glutamate exporter, reverting back of mutated *argF* (G166C) to its original gene sequence and over-expression of *carAB* [20]. However, during construction of the L-arginine high-producer, L-citrulline, a simultaneously secreted by-product of L-arginine, was accumulated. In view of revealing the metabolic block between L-ornithine, L-citrulline and L-arginine and its synthetic mechanism, this work analyzed the genetic and enzymatic levels involved in L-arginine biosynthesis. The results showed that transcription levels of *argG* and *argH* genes and the corresponding specific enzyme activities of ASS and ASL in recombinants were all decreased compared with SYPA5-5. Then, the metabolic obstacle of accumulating L-citrulline and L-ornithine, caused by the down-regulation of *argGH* transcription levels after deregulation of the feedback inhibition of NAGK in ArgR deficient *C. crenatum*, was removed by co-expression of *argGH*, and L-arginine productivity increased greatly.

Materials and methods

Bacterial strains, plasmids, oligonucleotides

All bacterial strains, plasmids and oligonucleotides used in this study and their relevant characteristics and sources are listed in Table 1 and Table S1 in the supplementary material. *C. crenatum* SYPA5-5 [32], deposited in CGMCC with the No. 0890, was chosen as the parental strain.

Culture conditions

Escherichia coli was cultured aerobically on Luria–Bertani (LB) [24] at 37 °C as 10 mL cultures in a 50-mL shake flask on a rotary shaker at 220 rpm. SYPA5-5 and its recombinant derivatives were cultivated aerobically at 30 °C on a reciprocating shaker at 120 rpm.

Before L-arginine fermentation, cells were pre-activated in plate with broth medium (containing 20 g L^{-1} glucose) at biochemical incubator for 24 h, and then transformed into 30 mL seed medium (30 g L^{-1} glucose, 10 g L^{-1} yeast extract, 20 g L^{-1} (NH$_4$)$_2$SO$_4$, 1 g L^{-1} KH$_2$PO$_4$, 0.5 g L^{-1} MgSO$_4$·7H$_2$O, 1.5 g L^{-1} urea) in a 250-mL

shake flask cultured for about 15 h (OD$_{562nm}$ = 15–18). For batch fermentation in shake flask, 1 mL seed culture was inoculated into 20 mL fermentation medium (150 g L^{-1} glucose, 10 g L^{-1} yeast extract, 50 g L^{-1} (NH$_4$)$_2$SO$_4$, 1.5 g L^{-1} KH$_2$PO$_4$, 0.5 g L^{-1} MgSO$_4$·7H$_2$O, 0.02 g L^{-1} FeSO$_4$·7H$_2$O, 0.02 g L^{-1} MnSO$_4$·H$_2$O, 8 × 10^{-5} g L^{-1} biotin, 5 × 10^{-4} g L^{-1} L-histidine, 30 g L^{-1} CaCO$_3$) in a 250-mL shake flask. For fed-batch fermentation of recombinant strains in 10-L bioreactor, 300 mL seed culture was transformed to 6 L optimized fermentation medium [100 g L^{-1} glucose, 14 g L^{-1} yeast extract, 20 g L^{-1} (NH$_4$)$_2$SO$_4$, 1.5 g L^{-1} KH$_2$PO$_4$, 0.5 g L^{-1} MgSO$_4$·7H$_2$O, 0.02 g L^{-1} FeSO$_4$·7H$_2$O, 0.02 g L^{-1} MnSO$_4$·H$_2$O, 8 × 10^{-5} g L^{-1} biotin, 5 × 10^{-4} g L^{-1} L-histidine, pH 7.0]. The agitation speed was kept at 600 rpm, pH was maintained at 7.0 by flowing aqueous ammonia and the glucose was fed when residual concentration below 10 g L^{-1}. The plasmid-carrying strains were cultured with kanamycin (25 µg mL^{-1}) or ampicillin (100 µg mL^{-1}) depending on the characteristics of plasmids.

Site-directed mutagenesis

Site-directed mutagenesis was carried out essentially via overlap extension PCR [1]. The partial fragments on each side of the mutant site were separately amplified with two pairs of primers (*argJBD*F-RM209/268, FM209/268-*argJBD*R) using the genome of SYPA5-5 as template. There was some overlap between primers FM209/268 and RM209/268, respectively. The 5′-end fragment was amplified by forward primer *argJBD*F as well as reverse primer RM209/268, and the 3′-end fragment by forward primer FM209/268 as well as reverse primer *argJBD*R (Table 1). The two PCR fragments were purified and used as the templates of another round of PCR with *argJBD*F and *argJBD*R as the primers to obtain the fragments with corresponding point mutations for homologous recombination.

Construction of plasmids and strains

SYPA5-5 with chromosomal mutations were performed by the suicide vector pK18mob*sacB* which could fulfill markerless replacement of genes through homologous recombination [22]. The fragments with point mutations of H268N or R209A were verified by sequence and ligated with pK18mob*sacB* to obtain the recombinant plasmids pK18mob*sacB-argB*$_{H268N}$ and pK18mob*sacB-argB*$_{R209A}$. After transforming them into SYPA5-5, respectively, by electroporation [27], the desired mutants were firstly selected on sorbitol plates containing kanamycin (25 µg mL^{-1}) and finally selected in LB plate with 10 % (w/v) sucrose. Then, the recombinants were screened and confirmed by PCR and DNA sequencing.

Table 1 Strains, plasmids or oligonucleotides used in this study

Strains, plasmids or oligonucleotides	Relevant characteristics or sequences	Sources, references or purposes
Strains		
E. coli JM109	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lac-proAB*)/F' [*traD36 proAB*+ *lacI*q *lacZ* Δ M15]	Invitrogen
C. crenatum SYPA5-5	L-arginine-producing strain screened from random mutagenesis	[29]
C. crenatum H-7	SYPA5-5 derivative with chromosomally integrated mutations into *argB* coding for amino acid exchanges H268N	This work
C. crenatum R-8	SYPA5-5 derivative with chromosomally integrated mutations into *argB* coding for amino acid exchanges R209A	This work
C. crenatum 5-GH	Kan^r, SYPA5-5 derivative with *argGH* over-expressed by pDXW-10-*argGH*	This work
C. crenatum H-7-GH	Kan^r, H-7 derivative with *argGH* over-expressed by pDXW-10-*argGH*	This work
C. crenatum R-8-GH	Kan^r, R-8 derivative with *argGH* over-expressed by pDXW-10-*argGH*	This work
Plasmids		
pK18mob*sacB*	Kan^r, vector for allelic exchange in *C. crenatum*	
pK18mob*sacB*-*argB*$_{H268N}$	Kan^r, pK18mob*sacB* derivative containing *argB* recombinant fragment with the point mutation of H268N	This work
pK18mob*sacB*-*argB*$_{R209A}$	Kan^r, pK18mob*sacB* derivative containing *argB* recombinant fragment with the point mutation of R209A	This work
pDXW-10	Amp^r, Kan^r, *tac-M* promoter, *E. coli/C. crenatum* shuttle	[28]
pDXW-10-*argGH*	pDXW-10 derivative containing *argGH* gene	This work
Oligonucleotides		
FM209	GATTGGTGCAGAA<u>GCA</u>CTGCTGGTTC	Mutation of *argB*
RM209	GAGAACCAGCAG<u>TGC</u>TTCTGCAC	
FM268	GTAAGTGCTGCT<u>AAC</u>GTCATTGAC	Mutation of *argB*
RM268	GTCAATGAC<u>GTT</u>AGCAGCACTTAC	
*argJBD*F	GC<u>GTCGAC</u>ATGAATGACTTGATCAAAG	Mutation of *argB*, *SalI*
*argJBD*R	CT<u>GGATCC</u>CTCGAGGAAGATAGCAGC	Mutation of *argB*, *BamHI*
argGH-F	GA<u>GCTAGC</u>AGAAGGAGATATACCATGACTAACCGCATCGTTCTTG	Expression of *argGH*, *NheI*
argGH-R	GC<u>AAGCTT</u>TTATCGACGTACCCCCGCAC	Expression of *argGH*, *HindIII*

The restriction enzyme sites and mutation sites were marked with underline and double underline, respectively

Kan^r kanamycin-resistance, *Amp^r* ampicillin-resistance, *Cm^r* chloromycetin-resistance

The *argGH* fragment was amplified via PCR using *argGH*-F and *argGH*-R as primers (Table 1) and the genome of SYPA5-5 as template. The fragment was purified, ligated with pMD-19-T simple vector and transformed in *E. coli* JM109. The strain with recombinant plasmid was verified by sequencing. Correct fragment was digested and inserted into pDXW-10 [31] to obtain the recombinant plasmid of pDXW-10-*argGH*. After transforming it into SYPA5-5, H-7 and R-8, respectively, by electroporation, positive clones were separately picked out in LB plate containing kanamycin (25 μg mL^{-1}).

Determination of enzyme activities

Corynebacterium crenatum from exponential phase was harvested by centrifugation (10 min, 8000×*g*, 4 °C), washed and suspended with 50 mM Tris–HCl pH 7.5,

and then broken at 4 °C by ultrasonication with the ultrasonic cell disruptor (Scientz Biotechnology, JY92-II, Ningbo, China) for 15 min at 300 W. After centrifugation (15 min, 20000×*g*, 4 °C), the supernatant was used for enzyme assay. Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method of Bradford with bovine serum albumin as the standard [3].

NAGK activity was measured with the hydroxylamine-containing colorimetric assay of Haas and Leisinger, which detected the formation of acetylglutamyl hydroxamate (extinction coefficient 456 M^{-1} cm^{-1}) at 540 nm [11]. The final assay system (total volume 1 mL) contained 200 mM Tris–HCl (pH 8.0), 40 mM *N*-acetylglutamate, 40 mM MgCl$_2$, 40 mM ATP, 400 mM NH$_4$OH·HCl. The mixture was incubated at 37 °C for 30 min, terminated by addition of 2 mL stopping solution [4 % (w/v) FeCl$_3$ and 5 % (w/v)

trichloroacetic acid in 1 M HCl], and followed by detecting at 540 nm after centrifugation. One unit of the enzyme activity was defined as the amount of enzyme generating one micro mole acetylglutamyl hydroxamate per minute under the condition described above.

Measurement of argininosuccinate synthase (ASS) activity was carried out in two steps [15]. Phosphate was released from ATP hydrolysis catalyzed by ASS in first step with a final system (total 0.9 mL) containing 20 mM Tris–HCl (pH 7.8), 2 mM ATP, 2 mM citrulline, 2 mM aspartate, 6 mM $MgCl_2$, 20 mM KCl, 0.2 U pyrophosphatase. The mixture was incubated at 37 °C for 15 min, and followed by the second step with addition of 6 % H_2SO_4, 7.5 mM ammonium molybdate, 30 mM ascorbic acid. After incubating at 30 °C for 30 min, the supernatant was analyzed for absorption at 820 nm. Aliquots of solution containing 1–10 µg of Pi were used to make the standard curve. One unit of the enzyme activity was defined as the amount of enzyme generating 1 µmol of Pi per minute under the condition described above.

Measurement of argininosuccinase (ASL) activity was depending on L-arginine synthesis. The final system contained 50 mM Tris–HCl (pH 7.5), 2 mM argininosuccinate, and moderate enzyme. The mixture was incubated at 37 °C for 20 min and product of L-arginine was analyzed by HPLC. One unit of the enzyme activity was defined as the amount of enzyme generating 1 µmol of L-arginine per minute under the condition described above.

Quantitative real-time PCR (qRT-PCR)

SYPA5-5 and its recombinant derivatives were harvested by centrifugation (10 min, $8000\times g$, 4 °C) in the exponential growth phase during fermentation and prepared total RNA by RNA extract kit (Thermo Scientific, USA). The first strand cDNA was synthesized by PrimeScript®RT reagent Kit (Takarra, Japan), and qRT-PCR was performed using SYBR®Premix *Ex* TaqTM II (TliRNaseH Plus) on a Bio-Rad CFX96 Manager PCR system (Bio-Rad, USA) using the cycling parameters: 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s and extension in 72 °C for 20 s. The primers of qRT-PCR are listed in Supplementary Table S1. The method of $2^{-\triangle\triangle Ct}$ was applied to analyze the data and normalized to the transcription level of 16S rRNA [16].

Analysis of fermentation parameters

Cell growth was monitored by measuring the optical density at 562 nm (OD_{562nm}). Cell dry weight (CDW) was calculated as a ratio of 0.375 g L^{-1} per OD_{562nn} [32].

For quantification of substrate consumption and product formation, about 0.5 mL of samples were taken from the fermentation culture every time and centrifuged ($12000\times g$, 5 min). The resulting supernatants were used for determination of glucose, $(NH_4)_2SO_4$ and amino acids concentration. Glucose was measured by the method of DNS according to Miller [18]. Phenol-hypochlorite method was used to determine the concentration of $(NH_4)_2SO_4$ [29]. The concentration of amino acids were detected by HPLC (DIONEX, UltiMate 3000, America) equipped with a venusil AA column (Agela technologies, China). Before analysis of amino acids, the samples were derived by phenyl-isothiocyanate and detected at 254 nm (UV) [8]. All assays were performed by triplicate cultures.

### Detection of intracellular L-arginine concentration

Cells were harvested every 24 h during fermentation, mixed with pre-cooling quenching agent (glycerol: 13.5 g L^{-1} NaCl = 3:2) immediately [28], centrifugated at 4 °C ($8000\times g$, 10 min), washed twice with 0.9 % NaCl, and then subjected to sonication. The supernatant was harvested by centrifugation ($8000\times g$, 10 min, 4 °C) and mixed with isometric 10 % trichloroacetic acid to subside the intracellular soluble protein. Then amino acids concentration of the supernatant was analyzed by HPLC as described above. The intracellular L-arginine concentration was calculated considering a cytoplasmic volume of 1.7 µL mg^{-1} dry cells [25].

Results

Construction of feedback inhibition deregulated H-7 and R-8

The favorable mutations (H268N or R209A) in CcNAGK, which significantly decreased the sensitivity to L-arginine and kept the specific activity [33], was integrated in SYPA5-5, respectively, through substituted *argB* gene by its mutant in SYPA5-5. Recombinants of SYPA5-5-CcNAGK$_{H268N}$ (designated as H-7) and SYPA5-5-CcNAGK$_{R209A}$ (designated as R-8) were screened on sucrose agar plate after twice homologous recombination.

To examine the expression levels of CcNAGK$_{H268N}$ and CcNAGK$_{R209A}$ in the recombinants, CcNAGK activities with different concentrations of L-arginine were determined (Fig. 2). As expected, the CcNAGK mutants showed constant specific activities compared to the wild type and remained about 98.2 and 94.3 % activities, respectively, in presence of 15 mM L-arginine (the maximum intracellular L-arginine concentration was 14.5 mM over the fermentation course, Supplementary Fig S1). These results indicated
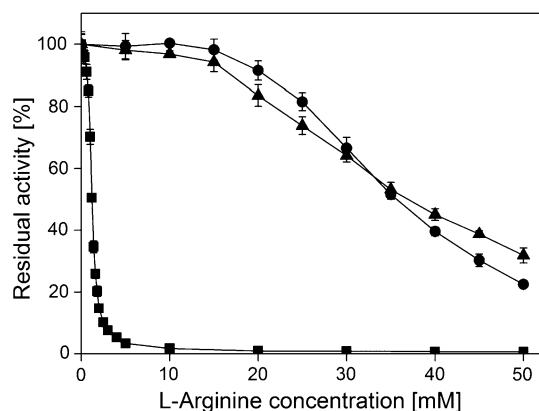
Fig. 2 Residual activities of CcNAGK in SYPA5-5 (*filled squares*), H-7 (*filled circles*) and R-8 (*filled triangles*) were analyzed by adding different concentrations of L-arginine. The specific activities were 0.62 ± 0.021, 0.79 ± 0.024, and 0.77 ± 0.046 U mg^{-1}, respectively, without adding L-arginine. (All assays were performed by triplicate cultures, standard deviations of the biological replicates were represented by *error bars*.)

that feedback inhibition of CcNAGK in H-7 and R-8 was successfully deregulated.

Deregulation of CcNAGK feedback inhibition increases L-ornithine and L-citrulline accumulation

The recombinants H-7 and R-8 were investigated for shake flask fermentation using SYPA5-5 as control. The fermentation profiles over the experimental course were monitored and shown in Fig. 3. Unexpectedly, L-arginine yields of recombinants were lower than SYPA5-5 (Fig. 3a), accompanied by accumulation of large amounts of L-citrulline and L-ornithine as the intermediates of L-arginine biosynthetic pathway (Fig. 3c, d). Meanwhile, glucose and $(NH_4)_2SO_4$ consumption rates of recombinants were decreased (Fig. 3e, f), which resulted in lower biomass (Fig. 3b) and L-arginine yields (Fig. 3a). After culturing for 88 h, H-7 and R-8 produced 139.4 and 129.9 mM L-arginine, 41.7 and 47.7 mM L-citrulline, 16.8 and 15.0 mM L-ornithine, respectively. Compared with SYPA5-5, H-7 and R-8 showed the reduction of L-arginine production about 16.2 and 21.9 %, the increase of L-citrulline concentration about 3.9-fold and 4.6-fold and the increase of L-ornithine concentration about 1.5-fold and 1.2-fold, respectively. Furthermore, compared with SYPA5-5, cumulative reduction of the major by-product of L-lysine was 67.8 and 75.2 % in H-7 and R-8, respectively (Supplementary Fig. S2). These results suggested that deregulation of feedback inhibition of CcNAGK in SYPA5-5 significantly affected the metabolic flux redistribution involved in L-arginine biosynthetic pathway.

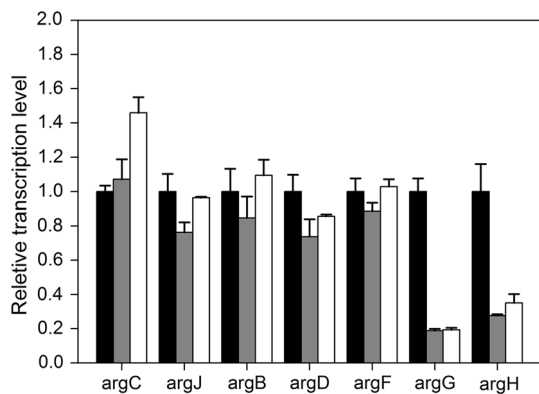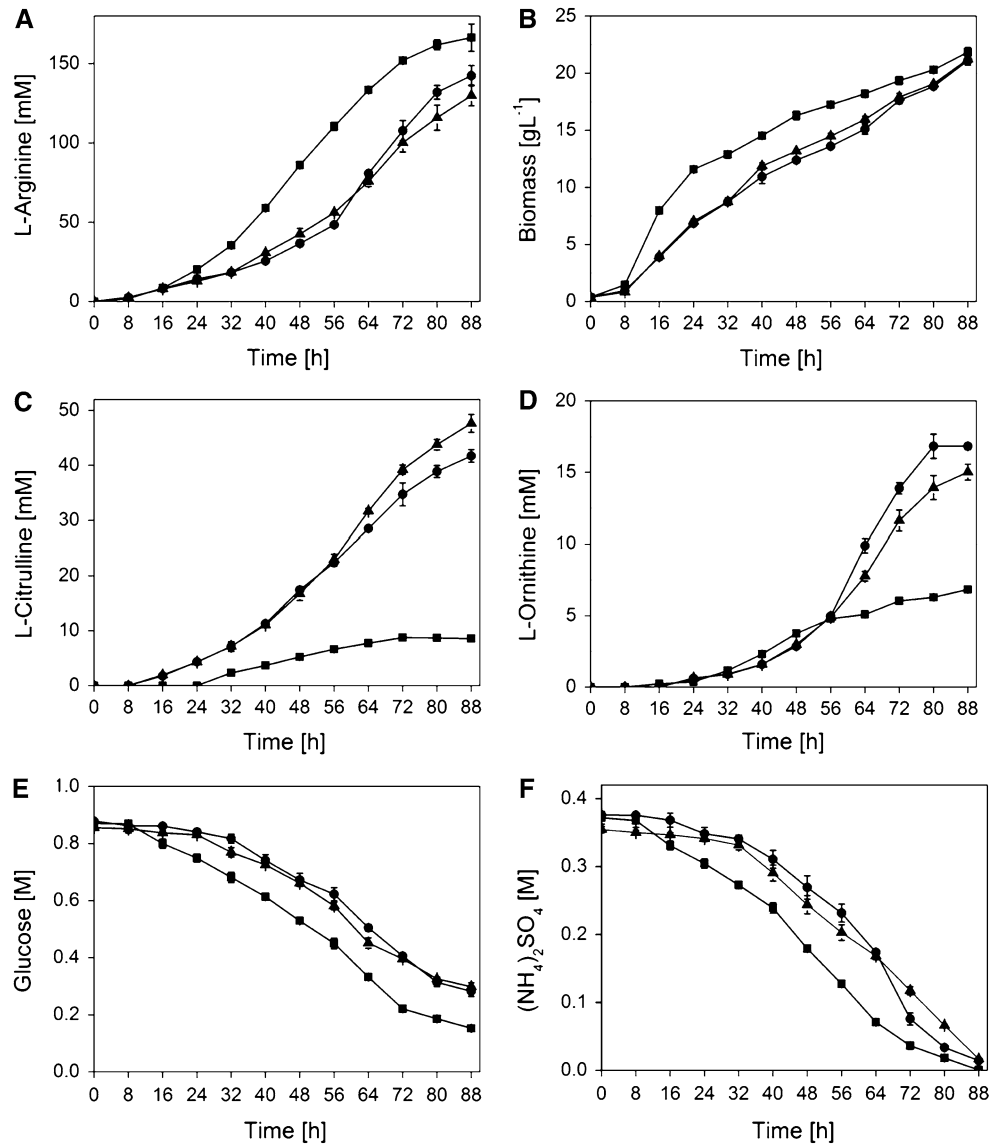## Transcription analysis of genes involved L-arginine biosynthetic pathway of H-7 and R-8

To reveal the generative mechanism of L-citrulline and L-ornithine accumulation and L-arginine production reduction, the transcription levels of *arg* gene cluster were determined in two recombinants, using SYPA5-5 as control. The results showed that the transcription levels of *argCJBDF* transcript did not show obvious differences (Fig. 4). However, transcription levels of *argG* and *argH* decreased more than 80 and 65 % (Fig. 4; Table 2), respectively. In accordance with that, the specific activities of ASS and ASL, encoded by *argG* and *argH*, decreased more than 50 % (Table 2). However, transcription levels of central metabolism genes, such as TCA cycle (*gltA*, *icd*, *kgd*, *acn*, *fumC* and *gdh*), anaplerotic pathway (*pyc*), glycolytic (*pgi*, *pfk*, *fda*, *gap*, *pgk*, *pyk*, *aceE*), glucose uptake system (*ptsG*) and pentose phosphate pathway (*zwf* and *gnd*), were all up-regulated (Supplementary Table S2). These results indicated that the reduction of transcription levels of *argGH* transcript, which affected the enzyme expression responsible for the downstream metabolism of L-ornithine and L-citrulline, was the reason for accumulation of the intermediates.

Co-expression of *argGH* transcript in H-7 and R-8 improved L-arginine production

The fermentation profiles and relative genes transcription analysis of recombinants H-7 and R-8 indicated that a bottleneck of the carbon flux from L-citrulline to L-arginine in biosynthetic pathway was a crucial node needed to break through. To increase the expression of *argGH*, we constructed the recombinant plasmid pDXW-10-*argGH* and transformed it into H-7, R-8 and SYPA5-5, resulting in H-7/pDXW-10-*argGH* (H-7-GH), R-8/pDXW-10-*argGH* (R-8-GH), and SYPA5-5/pDXW-10-*argGH* (5-GH), respectively. As expected, the transcription levels of *argGH* and the specific activities of ASS and ASL were increased remarkably in all recombinants (Table 2).

As shown in Fig. 5, after culturing for 64 h, fermentation was terminated when the nitrogen source $(NH_4)_2SO_4$ was completely consumed by H-7-GH and R-8-GH (Supplementary Table S3), and the yields of the major amino acids were detected. Compared with SYPA5-5, 5-GH showed about 9.5 % increase of L-arginine production, 78.6 % reduction in L-citrulline formation, and a lower L-ornithine formation. Compared with H-7 and R-8, H-7-GH and R-8-GH showed about 1.4–1.6 folds increase of L-arginine production, about 77–80 % reduction in L-citrulline formation and about 76–80 % reduction in L-ornithine formation, respectively. As expected, co-expression of *argGH* significantly decreased the accumulation of L-citrulline and L-ornithine simultaneously,

Fig. 3 L-arginine (**a**), biomass (**b**), L-citrulline (**c**), L-ornithine (**d**), glucose consumption (**e**), and (NH$_4$)$_2$SO$_4$ consumption (**f**) of SYPA5-5 (*filled squares*), H-7 (*filled circles*) and R-8 (*filled triangles*), respectively, during a representative shake flask batch fermentation. (All assays were performed by triplicate cultures, standard deviations of the biological replicates were represented by *error bars*.)


Fig. 4 Transcription analysis of the genes involved in L-arginine biosynthetic pathway of recombinants H-7 (*white bars*) and R-8 (*dark gray bars*) using SYPA5-5 (*black bars*) as control. (All assays were performed by triplicate cultures, standard deviations of the biological replicates were represented by *error bars*.)

which disrupted the metabolism bottleneck of H-7 and R-8 and increased their L-arginine yields dramatically. Significantly, in H-7-GH and R-8-GH, the increased $Y_{p/x}$ (amino acids yield on biomass) of L-arginine (about 7.3–7.5 mM $g_{(DCW)}^{-1}$, L-arginine yields on biomass of H-7-GH or R-8-GH minus that of H-7 or R-8, respectively) were dramatically higher than the decrease of total $Y_{p/x}$ of L-ornithine and L-citrulline (about 2.9–3.1 mM $g_{(DCW)}^{-1}$, total of L-citrulline and L-ornithine yields on biomass of H-7 or R-8 minus that of H-7-GH or R-8-GH, respectively), while $Y_{p/x}$ of 5-GH only increased 1.32 mM $g_{(DCW)}^{-1}$ (about 0.68 mM $g_{(DCW)}^{-1}$ from L-ornithine and L-citrulline) compared to SYPA5-5 (Table 3). These results revealed that co-expression of *argGH* in H-7 and R-8 significantly strengthened L-arginine biosynthetic pathway, which showed the positive effect of feedback-resistant CcNAGK on L-arginine production.

Table 2 Transcription levels and specific activities analysis of ASS, ASL in recombinants and their parental strains

Strains	Relative transcription level[a]		Specific activity (U mg^{-1})	
	argG	*argH*	ASS	ASL
SYPA5-5	1 ± 0.076	1 ± 0.16	0.12 ± 0.012	0.029 ± 0.002
5-GH	3.13 ± 0.15	2.90 ± 0.16	0.24 ± 0.014	0.102 ± 0.002
H-7	0.19 ± 0.01	0.28 ± 0.008	0.059 ± 0.002	0.014 ± 0.003
H-7-GH	4.89 ± 0.25	3.61 ± 0.20	0.25 ± 0.021	0.12 ± 0.004
R-8	0.19 ± 0.012	0.35 ± 0.051	0.067 ± 0.004	0.010 ± 0.003
R-8-GH	5.08 ± 0.19	3.15 ± 0.11	0.34 ± 0.023	0.13 ± 0.005

[a] Relative transcription levels of genes in recombinants H-7, H-7-GH, R-8, R-8-GH were calculated by method of $2^{-\triangle\triangle Ct}$ using SYPA5-5 as control. (All assays were performed by triplicate cultures, standard deviations of the biological replicates were represented by *error bars*.)
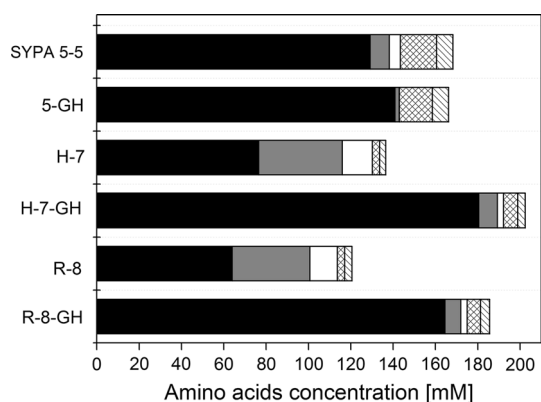
Fig. 5 The concentration of L-arginine (*black bars*), L-citrulline (*dark gray bars*), L-ornithine (*white bars*), L-lysine (*grid shed bars*) and L-isoleucine (*sparse shed bars*) produced by recombinants and their parental strains after culturing for 64 h in shake flask fermentation. The values were averages of at least three independent measurements and the standard deviations were within $\pm 10 \%$

Table 3 Amino acids yield on biomass ($Y_{p/x}$) of recombinants and their parental strains

Strains	Biomass[a] (g L^{-1})	Amino acids yield on biomass ($Y_{p/x}$)[b] (mM g$_{(DCW)}^{-1}$)		
		L-arginine	L-citrulline	L-ornithine
SYPA5-5	17.76 ± 0.26	7.28 ± 0.22	0.51 ± 0.03	0.29 ± 0.05
5-GH	16.39 ± 0.70	8.60 ± 0.52	0.12 ± 0.01	0.00
H-7	13.80 ± 0.74	5.54 ± 0.15	2.86 ± 0.12	1.04 ± 0.05
H-7-GH	14.05 ± 0.49	12.85 ± 0.33	0.62 ± 0.03	0.21 ± 0.006
R-8	13.30 ± 0.35	4.81 ± 0.25	2.76 ± 0.15	0.98 ± 0.03
R-8-GH	13.38 ± 0.28	12.30 ± 0.21	0.56 ± 0.03	0.23 ± 0.006

[a] Biomass of recombinants and their parental strains after culturing for 64 h

[b] Amino acids yield on biomass ($Y_{p/x}$) was amino acid concentration normalized to the biomass, which represented the ability of amino acids synthesis of every cell. (All assays were performed by triplicate cultures, standard deviations of the biological replicates were represented by *error bars*.)
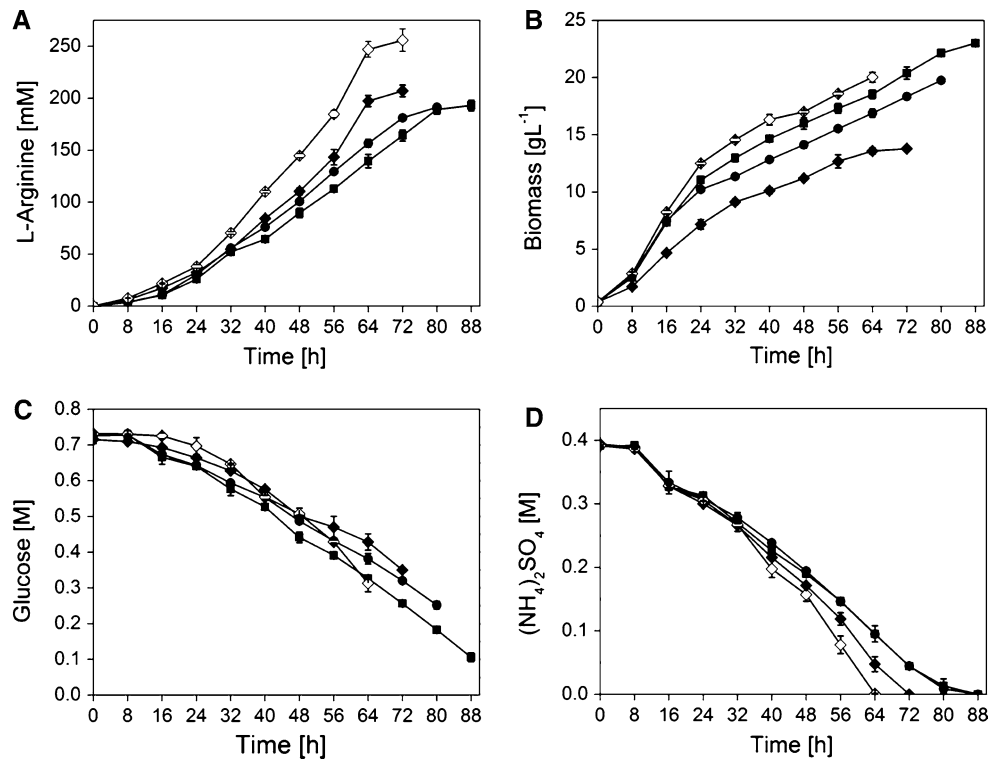
Batch fermentation of H-7-GH

In above results, recombinants H-7-GH and R-8-GH both improved their L-arginine production; however, H-7-GH showed the preferable ability to produce this target amino acid. Thus, the fermentation characteristics of H-7-GH were studied in the original fermentation medium by this work and shown in Fig. 6 and Table 4. The biomass of H-7-GH was 13.6 g L^{-1}, decreased about 26.4 % compared to SYPA5-5 after culture for 64 h (Fig. 6b; Table 4). Due to its lower growth rate, the glucose consumption rate of H-7-GH was also slower (Fig. 6c). However, $(NH4)_2SO_4$ consumption rate of H-7-GH was higher than SYPA5-5 (Fig. 6d), which resulted in higher L-arginine synthetic rate (Fig. 6a) and decreased fermentation duration (Table 4). After culturing for 64 h, only 45 % of the added glucose was consumed by H-7-GH; however, the $Y_{p/s}$ (L-arginine yield on glucose, about 0.69 mmol mmol$_{(glucose)}^{-1}$) and L-arginine productivity (3.08 mmol h^{-1}) were greatly improved, and were 97.7 and 54.2, 30.5 and 22.2 % higher than SYPA5-5 and 5-GH, respectively (Table 4).

The L-arginine productivity of H-7-GH was obviously limited by the lower growth rate. Then, we optimized the concentration of yeast extract which acts as the most important factor for growth in fermentation medium (Supplementary Fig. S3). When it was raised from 10 to 14 g L^{-1}, the biomass, L-arginine production and L-arginine productivity of H-7-GH were 20.0 g L^{-1}, 247.0 mM and 3.86 mM h^{-1}, increased 47.0, 25.1, 20.2 % compared to culture in original medium (Fig. 6; Table 4). Meanwhile, biomass of H-7-GH in the optimized medium was comparable to SYPA5-5 in the original medium which acted as the optimal condition for its L-arginine production; however, the L-arginine productivity and $Y_{p/s}$ were increased 63.6 and 69.5 %, respectively (Table 4). The $Y_{p/s}$ of H-7-GH in the optimized medium was lower than in the original medium, probably due to higher biomass need large amount of glucose. These results strongly suggested that co-expressing of *argGH* and deregulating feedback-regulation of CcNAGK

Fig. 6 L-arginine production (**a**), biomass (**b**) glucose consumption (**c**) and $(NH_4)_2SO_4$ consumption (**d**) of SYPA5-5 (*filled squares*), 5-GH (*filled circles*), H-7-GH in original culture (*filled diamonds*), H-7-GH in optimized culture (*open diamonds*), respectively, during shake flask batch fermentation. (All assays were performed by triplicate cultures, standard deviations of the biological replicates were represented by *error bars*.)

**Table 4** Process parameters of SYPA5-5, 5-GH and H-7-GH during shake flask batch fermentation

Process parameters	Original medium			Optimized medium
	SYPA5-5	5-GH	H-7-GH	H-7-GH
Fermentation duration (h)	80	72	64	64
Biomass (g L^{-1})	22.1	18.3	13.6	20.0
Glucose consumption (mM)	543.9	406.3	286.7	418.5
$(NH4)_2SO_4$ consumption (mM)	378.0	347.3	346.4	391.0
L-arginine production (mM)	189.2	181.1	197.4	247.0
L-arginine productivity (mmol h^{-1})	2.36	2.52	3.08	3.86
L-arginine yield on glucose ($Y_{p/s}$) [mmol $mmol_{(glucose)}^{-1}$]	0.348	0.446	0.688	0.590

could efficiently improve L-arginine productivity in SYPA 5-5. In summary, breaking through the metabolic bottleneck of *argGH* after deregulating repression of ArgR and feedback inhibition of CcNAGK should be the priority on metabolic engineering of L-arginine-producing strains.

Fed-batch fermentation of H-7-GH in 10-L bioreactor

To study the relevance for an industrial application, we established fed-batch fermentation with H-7-GH in optimized fermentation medium which improve the concentration of yeast extract and decrease of initial concentration of glucose and $(NH_4)_2SO_4$ to reach higher cell densities. As

shown in Fig. 7, fed-batch culture of the H-7-GH resulted in higher biomass of 26.2 g L^{-1}, higher L-arginine production of 389.9 mM, and higher productivity of 5.42 mM h^{-1} after culturing for 72 h when compared with the shake flask fermentation (Fig. 6; Table 4). However, the $Y_{p/s}$ (0.35 mmol $mol_{(glucose)}^{-1}$) was lower than shake flask fermentation, probably because of higher biomass requiring large amount of glucose, which was also found in the fermentation of pyruvate [30]. These results demonstrated that metabolically engineered H-7-GH was capable of efficiently producing L-arginine at appropriate medium and fed-batch fermentation.
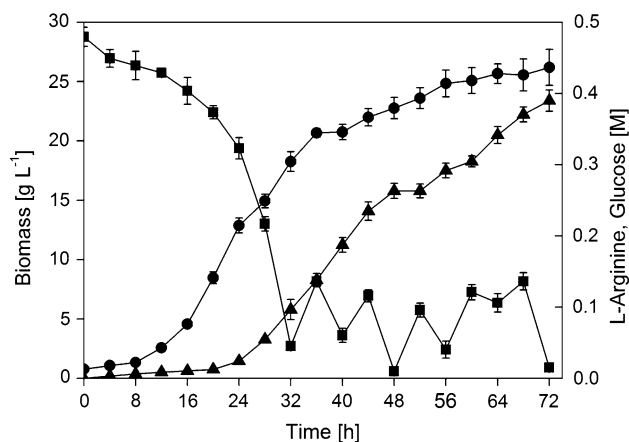
Fig. 7 Growth (*filled circles*), glucose consumption (*filled squares*), and L-arginine production (*filled triangles*) of H-7-GH during a fed-batch fermentation in a 10-L bioreactor. At least three independent fermentations were performed, showing comparable results

Discussion

A variety of regulatory mechanisms are imposed to control the metabolic flux into L-arginine biosynthesis at the genetic and enzymatic levels [17]. Two main regulatory modes, operating at the genetic level via ArgR repressor and at enzymatic level via allosteric regulation of CcNAGK, have been reported to be performed in *Corynebacterium* sp. [34, 35]. Breeding of L-arginine producers through random mutation against L-arginine analogs as well as using rational design based on genomics and proteomics databases were expected to disrupt these regulatory modes [13, 39].

Undergoing an iterative procedure of random mutation and selection, nonsense mutation (A109T) in *arg*R was identified in SYPA5-5 which makes the L-arginine biosynthesis operon (*arg* cluster) release from the repression of ArgR. After deregulating allosteric inhibition of CcNAGK by introducing either one of the point mutation (H268N or R209A) into its chromosome, the two main regulatory modes involved in L-arginine biosynthesis were removed simultaneously, and then led to accumulation of L-citrulline and L-ornithine and decrease of L-arginine production. Interestingly, during reconstructed L-arginine high-producer based on comparative sequence analysis by Ikeda et al., after introducing feedback-resistant CgNAGK into the chromosome of *C. glutamicum* Δ*argR*, the resulting strain could accumulate total amount of L-arginine and L-citrulline about 80 mM at the ratio of 63:37 [13]. Meanwhile, when point mutation A26V was introduced in NAGK of *C. glutamicum* AR4 (Δ*argR*, *argB*$_{M31V}$) to further alleviate the feedback inhibition by L-arginine, resulted in slower consumption of carbon sources and decrease of L-arginine production. These

phenomena, similar with our results, were not cause enough concern. In present study, the metabolic flux redistribution in L-arginine biosynthesis pathway prompted us to investigate the mechanisms in detail.

The decrease of transcription levels of *argGH* accompanied by the reduction of ASS and ASL activities resulted in a metabolic obstacle from L-citrulline to L-arginine, and reasonably explained the accumulation of intermediates. In addition, after fermenting for 64 h, the total yield of L-arginine, L-citrulline and L-ornithine on biomass ($Y_{p/x}$) of recombinants H-7 and R-8 (8.5–9.4 mM g$^{-1}_{(DCW)}$) compared to SYPA5-5 (8.1 mM g$^{-1}_{(DCW)}$) did not show advantages (Table 3), which strongly suggested that the total metabolic flux towards L-arginine was also controlled after deregulating repression by ArgR and feedback inhibition of CcNAGK. Taking these phenomena together, we presumed that another regulatory mode involved in L-arginine biosynthesis works after breaking these two main regulatory modes by regulation of *argGH* transcription. The results of further over-expressing *argGH* through exogenous plasmid pDXW-10 with an unregulated promoter (*tac*-M) [31] to remove this regulatory mode proved the vital function of regulation of *argGH* transcription for L-arginine biosynthesis.

Recent studies showed that FarR, one of the transcription regulators belongs to GntR family, involves in ribosomal protein, carbon and energy metabolism, transport system and also participates in regulation of *arg* cluster, which has a weak binding site upstream of *argC* and strong binding site upstream of *argG* [10]. Moreover, deletion of FarR was proved to benefit L-arginine production in *C. glutamicum* [20]. The transcription of *argGH* cluster was a common regulatory node and must have been relevant to global regulation depending on some global regulatory factors. Accordingly, the comparative genes expression analysis of central metabolism (Supplementary Table S2) of recombinants H-7 and R-8 indicated that global response was also induced, which explained the changes of their growth rates. However, different type of factors involved in L-arginine biosynthesis might induce the global response through multilevel complex regulatory network in response to the growth and metabolism [9, 17]. Therefore, the regulatory mechanisms involved in control of the transcription of *argGH* might be various and complicated that remain unclear, and its interaction with global response were unknown yet.

These studies demonstrated that down-regulation of *argGH* transcription levels was another regulatory mode for controlling L-arginine biosynthesis after deregulating the repression of ArgR and allosteric inhibition of CcNAGK, and disrupting all three regulatory modes could improve L-arginine productivity remarkably. Thus, this work provided a fresh perspective in exploring new

regulatory mechanisms involved in L-arginine metabolism process. Further studies can be performed for elucidating repressors that participated in regulation of *argGH* operon transcription and constructing the higher producer by assembling the other positive strategies, such as overexpression of bifunctional *argJ* [6], L-arginine transporter *lysE* [36] and *acb* cluster or improved the supplement of NADPH and carbamoylphosphate by strengthening the pentose phosphate pathway and overexpressed *carAB*, respectively [20].

Compliance with ethical standards

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