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FERMENTATION, CELL CULTURE AND BIOENGINEERING - ORIGINAL PAPER

Enhancement of L-arginine production by increasing ammonium uptake in an AmtR-deficient *Corynebacterium crenatum* mutant

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Abstract

L-Arginine is an important amino acid with extensive application in the food and pharmaceutical industries. The efficiency of nitrogen uptake and assimilation by organisms is extremely important for L-arginine production. In this study, a strain engineering strategy focusing on upregulate intracellular nitrogen metabolism in *Corynebacterium crenatum* for L-arginine production was conducted. Firstly, the nitrogen metabolism global transcriptional regulator AmtR was deleted, which has demonstrated the beneficial effect on L-arginine production. Subsequently, this strain was engineered by overexpressing the ammonium transporter AmtB to increase the uptake of NH_4^+ and L-arginine production. To overcome the drawbacks of using a plasmid to express *amt*B, P_{tac}, a strong promoter with *amt*B gene fragment, was integrated into the *amt*R region on the chromosome in the *Corynebacterium crenatum*/ΔamtR. The final strain results in L-arginine production at a titer of 60.9 g/L, which was 35.14% higher than that produced by *C. crenatum* SYPA5-5.

Keywords *Corynebacterium crenatum* · L-arginine · Nitrogen metabolism · AmtR regulator ammonium transporter AmtB

Introduction

Corynebacterium glutamicum is a gram-positive soil bacterium that was isolated in 1957 [1–3]. The complete genome sequence of the representative wild-type strain, *C. glutamicum* ATCC 13032, has been determined and analyzed, greatly aiding our understanding of the molecular biology and physiology of this organism [4]. This bacterium is frequently used in industry for the production of amino acids, such as L-glutamate, lysine, and L-arginine [5].

Corynebacterium crenatum, a subspecies of *C. glutamicum*, is an L-arginine overproducing strain that was isolated from soil. The mutant strain *C. crenatum* SYPA5-5, which could produce 32.5 g/L of L-arginine, was obtained by UV irradiation and treatment with nitrosoguanidine (NTG) in our previous study [6, 7]. In recent years, owing to the increased understanding of L-arginine biosynthesis and its metabolic regulation, many metabolic engineering strategies aiming to enhance L-arginine production have been carried out in *C. glutamicum* [8–10] and *C. crenatum* [11]. These strategies include removing regulatory repressors of L-arginine operon, optimizing NADPH level, disrupting L-glutamate exporter [8], strengthening the preexisting glucose transporter and developing new glucose uptake system, channeling excess carbon flux from glycolysis into tricarboxylic acid cycle [11].

Nitrogen is an essential component of most complex bacterial molecules, including proteins, nucleic acids and several cofactors [12]. To utilize different nitrogen sources and to survive nitrogen-limiting conditions, prokaryotes have developed several mechanisms to adapt their metabolism as the concentration of nitrogen changes [13–15]. A sufficient supply of nitrogen is essential for L-arginine synthesis, because there are four nitrogen atoms in the L-arginine molecule, corresponding to a nitrogen content of 32.1% [16].

Meijuan Xu and Jing Li contributed equally to this work.

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Ammonium (NH_4^+) is the preferred nitrogen source for most bacterial, fungal and plant cells, although a high concentration of ammonium is toxic to cells. AmtB is a membrane-bound ammonium transporter protein that is present in most bacteria and is responsible for the uptake of NH_4^+/NH_3 by the ammonium channel [17]. The PII signal transduction protein regulates the activities of many different proteins and sensors of cellular nitrogen status, including enzymes and transcription factors, by interacting with proteins [2, 18–20]. The *amt*B gene is always associated with the gene-encoding PII signal transduction protein GlnK in most bacteria and archaea, suggesting a functional relationship between the encoded proteins [21].

In *C. glutamicum*, the ATase/GlnK pathway is responsible for the regulation of ammonium assimilation and metabolism [17, 22], and enzymes that are important for these processes are primarily encoded in the operon *amt*B–*gln*K–*gln*D [23]. NH_4^+ can be assimilated via glutamate dehydrogenase (GDH) or the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway, depending on the availability of ammonium in the medium. At higher NH_4^+ concentrations(>5 mM), ammonium is assimilated by GDH; whereas at low concentrations (≤ 1 mM), it is assimilated by GS/GOGAT into L-glutamate [24]. Furthermore, in *C. glutamicum*, this operon is regulated by the global N-regulatory factor AmtR. The transcriptional regulator AmtR, a member of the TetR family, could regulate at least 35 genes, including those ammonium uptake (*amt*A and *amt*B), ammonium assimilation (*gln*A and *glt*BD), and urea metabolism (*ure*ABCEFGD), as well as those encoding regulatory proteins (GlnK, GlnD), in response to changes in nitrogen levels [17]. Under conditions of intracellular ammonium starvation, when the ammonium concentration is below 5 mM, GlnK is adenylated by GlnD, and the resulting adenylated-GlnK (GlnK-Amp) subsequently interacts with AmtR, which causes the transcriptional upregulation of nitrogen-regulated genes, thereby activating the intracellular transport of ammonium [25]. Then, NH_4^+/NH_3 can enter the cell through the AmtB ammonium transport channel. Conversely, under conditions of nitrogen surplus, de-adenylated GlnK interacts with the ammonium transporter AmtB, blocking the ammonium flux into the cell and therefore resulting in a GlnK-free state, allowing AmtR to be reactivated and bind to the promoter regions of nitrogen-controlled genes, repressing their transcription.

In our previous study on the metabolic engineering of L-arginine production, we focused on carbon flux optimization rather than ammonium uptake and assimilation to improve L-arginine production. Several studies have demonstrated that increasing the expression of genes related to nitrogen metabolism can improve the production of many valuable amino acids [26, 27]. For example, Kim et al. [27] enhanced glutamate production by overexpressing *odh*I in *C. glutamicum*. Similarly, Sindelar et al. [28] improved L-lysine production by overexpressing the *amt*A-*ocd*-*sox*A operon in *C. glutamicum*. Based on these studies, we asked whether an increase in the uptake and assimilation of NH_4^+ in *C. crenatum* can improve L-arginine production. Thus, the objective of this study was to increase L-arginine production via increasing NH_4^+ uptake and assimilation in *C. crenatum* (Fig. 1). We first observed that NH_4^+ had a significant effect on L-arginine production in *C. crenatum*. Furthermore, the effect of knocking out and overexpressing the nitrogen metabolic regulatory factor AmtR on L-arginine production and nitrogen metabolism-related enzyme activities was studied. Based on the qPCR results, we constructed a modified strain that overexpressed the channel protein AmtB, which was beneficial for the uptake of NH_4^+ and for L-arginine production. In addition, the *amt*B gene, under the control of a strong P_{tacM} promoter, was integrated at the *amt*R locus in the Cc–ΔamtR mutant strain, and a subsequent 5-L fermentation assay showed that the L-arginine production was significantly enhanced by increased AmtB expression.

Methods

Strains and plasmids

The *amt*R (GenBank No: MK034164) and *amt*B (GenBank no: MK034163) genes, encoding a transcriptional regulator of nitrogen metabolism and an ammonium transporter protein, respectively, were identified in *C. crenatum* SYPA5-5 (Cc5-5) and were PCR-amplified. Cc5-5, the L-arginine producer obtained by multiple random mutagenesis, was also called as SYPA5-5 [6, 7], and has been deposited in the China General Microbiological Culture Collection Center (CGMCC) under collection number CGMCC no. 0890. *E. coli* BL21(DE3) was used as the host strain for gene cloning and expression. Cc5-5 was used as the parent strain for the generation of mutants. The shuttle vector pXMJ19 was used for gene expression in Cc5-5 [29]. The suicide vector pK18*mobsac*B, which harbors the sucrose screening marker *sac*B, was used for deleting, inserting, and replacing unmarked genes in *C. glutamicum*, as previously described. All of the strains and plasmids used in this study are listed in Table 1.

Growth medium and culture conditions

Cc5-5 and its recombinant derivatives were routinely cultivated at 30 °C in LBG medium (LB medium supplemented with 7 g/L glucose). For recombinant DNA expression, *E. coli* BL21(DE3) was cultivated at 37 °C in LB medium. Where appropriate, chloramphenicol was added into the medium (12.5 μg/mL). The modified minimal medium

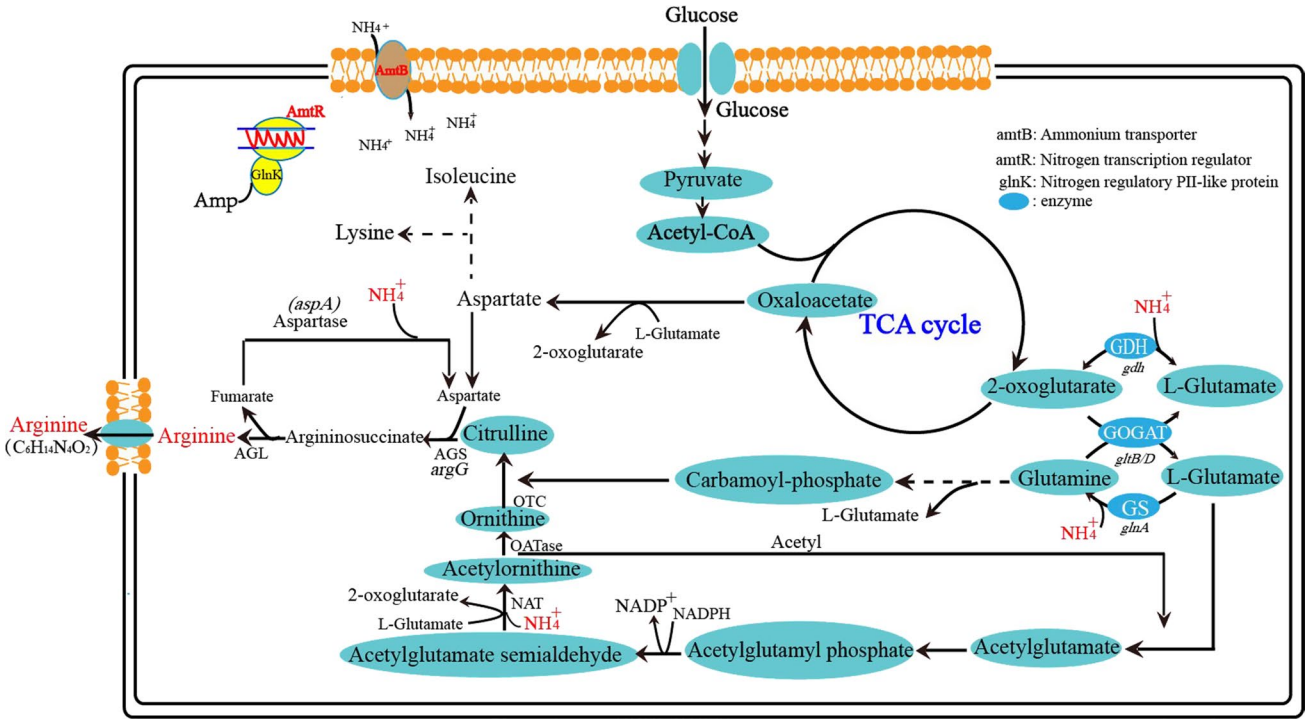

Fig. 1 Schematic representation of the L-arginine biosynthesis pathway and of the ammonium uptake and assimilation pathways of *C. crenatum* SYPA5-5. *GDH* glutamate dehydrogenase, *GS* glutamine synthase, *GOGAT* glutamate synthase

Table 1 Strains and plasmids in this study

Name	Relevant genotype	References
Strains		
E. coli BL21(DE3)	F^- ompT gal dcm lon hsdSB(rB^- mB^-) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Invitrogen
Cc5-5	L-arginine-producing strain	Xu et al. [6, 7]
Cc–ΔamtR	Cc–koamtR with *amt*R deletion	This study
Cc–amtR	Cc–amtR with pXMJ-19-Cc*amt*R	This study
Cc–amtB1	Cc–amtB-1 with pXMJ-19-Cc*amt*B in the Cc–ΔamtR	This study
Cc–amtB2	Cc–amtB-2 with *amt*B and P_{tacM} integration on Cc–ΔamtR chromosome	This study
Plasmids		
pK18*mobsac*B	Mobilizable vector allows for selection of double crossover in *C. glutamicum*, Km, *sac*B	This study
pXMJ19	Shuttle vector between *E. coli* and *C. glutamicum*	This study
pXMJ19–*amt*R	pXMJ19 harboring Cc*amt*R	This study
pXMJ19–*amt*B	pXMJ19 harboring Cc*amt*B	This study
pK18–*amt*B_2	A derivative of pK18*mobsac*B, harboring *amt*B, P_{tacM} and Δ*amt*R fragment	This study

CGXII (containing glucose 50 g, $FeSO_4{\cdot}7H_2O$·10 mg, $MnSO_4{\cdot}H_2O$·10 mg, KH_2PO_4·1 g, $ZnSO_4{\cdot}7H_2O$·1 mg, K_2HPO_4·1 g, $CuSO_4$·0.2 mg, $MgSO_4{\cdot}7H_2O$·0.25 g, $NiCl_2{\cdot}6H_2O$·0.02 mg, biotin 0.2 mg, $CaCl_2$·10 mg, 3-(*N*-morpholino) 42 g, protocatechuic 0.03 mg, and $CaCO_3$ 10 g) was used to culture *C. crenatum* strains for growth, enzyme activity and qPCR experiments. For the L-arginine shake flask fermentation, a single clone of the mutant was cultured on LBG plate for 24 h. Subsequently, a fresh bacterial colony was inoculated into 25 mL of seed medium in a standard 250-mL shake flask at 30 °C for 16 h. Next, 1.5 mL of the seed culture was transferred to 25 mL of the fermentation medium in a standard 250-mL shake flask and was cultured for 72 h at 30 °C with shaking at 200 r/min on a reciprocating shaker. The fermentation medium used for shake fermentation contained the following (per liter): 100 g glucose, 8 g yeast extract, 1.5 g·KH_2PO_4, 0.5 g·$MgSO_4{\cdot}7H_2O$, 0.02 g $FeSO_4{\cdot}7H_2O$, 0.02 g $MnSO_4$,

20 g $CaCO_3$, and 1–400 mM $(NH_4)_2SO_4$ (optimized nitrogen concentration). To produce L-arginine via fermentation in 5-L bioreactors, the seed culture (150 mL) was transferred into 5-L stirred fermenters (BIOTECH-5BG, Baoxing Co., China) containing 2.5 L of fermentation medium. The seed medium contained the following (per liter): 50 g glucose, 20 g yeast extract, 20 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, and 0.5 g $MgSO_4{\cdot}7H_2O$. The 5-L fermentation medium contained the following (per liter): 100 g glucose, 20 g yeast extract, 1.5 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.02 g $FeSO_4{\cdot}7H_2O$, 0.02 g $MnSO_4$, and 40 g $(NH_4)_2SO_4$, (≈300 mM).

DNA manipulation and strain construction

To construct the recombinant strains, genomic DNA from strain Cc5-5 was isolated using a genomic DNA extraction kit (Sangon Biotech, Shanghai, China) and used for PCR amplification. The PCR products and vectors were obtained using a PCR product purification kit and a mini-plasmid isolation kit (Takara, Dalian, China), respectively. The primers used in this study are listed in Table 2. The suicide vector pK18*mobsac*B was used to introduce modifications into the chromosome of Cc5-5 [29, 30], as described previously.

To generate the *amt*R knockout strain, upstream and downstream DNA fragments (approximately 1500 bp) flanking *amt*R were amplified and fused by PCR. Subsequently, the fused fragments were inserted into the *Eco*RI/*Xba*I sites of the pK18*mobsac*B plasmid using T_4 DNA ligase, generating the recombinant plasmid pK18–*amt*R. In addition, the *amt*R and *amt*B genes were inserted into the *Eco*RI/*Hin*dIII sites of the expression plasmid pXMJ19, generating the plasmids pXMJ19–*amt*R and pXMJ19–*amt*B. All recombinant plasmids were transformed in Cc5-5. The knockout strains obtained after two rounds of homologous recombination were confirmed by colony PCR.

The *amt*B gene under the control of the strong promoter P_{tacM} was integrated into the Cc5-5 chromosome at the *amt*R locus. The upstream and downstream regions of *amt*R using the primers are listed Table 2, and the template was the recombinant plasmid pXMJ19–*amt*B. Subsequently, the upstream region and the downstream region of *amt*R were amplified, and the P_{tacM}–*amt*B fragment was joined with the partial *amt*R fragment through overlapping PCR to obtain the target segment that generated the integrated gene construct P_{tacM}–*amt*B. Subsequently, the P_{tacM}–*amt*B fragment was inserted into the *Eco*RI/*Hin*dIII sites of the pK18*mobsac*B plasmid using T_4 DNA ligase, generating the recombinant plasmid pK18–*amt*B_2. Next, pK18–*amt*B_2 was transferred into the Cc5-5 strain by electroporation. After double crossover recombination, the positive recombinant strain was detected by colony PCR to identify the Cc–amtB2 isolates.

Expression of the AmtR and AmtB proteins in *C. crenatum*

Recombinant *E. coli* BL21(DE3) cells were cultured in LB medium at 37 °C to an OD_{562} of 1.0 (approximately 3 h), after which the cells were induced with 1 mM IPTG and incubated for 12 h at 24 °C. The recombinant *C. crenatum* strain was inoculated into LBG medium and cultured at 30 °C for 5 h with shaking 180 r/min, after which the culture was induced with 1 mM IPTG, and then the culture was incubated for 12 h. The cells were harvested by

Table 2 Primers used in this study

Name	Sequence (5′–3′)	Restriction sites
KO–*amt*RF1	CCGGAATTCCGGCGATCGCTGAAGTAGATGACAAAGC	*Eco*RI
KO–*amt*RRm	CGCATTTCGACCCATCCACTAAACTTAAACAATGCGTGGAGGTTGTTGCGAAGCC	–
KO–*amt*RFm	CAAGGCTTCGCAACAACCTCCACGCATTGTTTAAGTTTAGTGGATGGGTCGAAATGC GTCGCAACGAC	–
KO–*amt*RR1	CCAAGCTTACTAGATGGCCTCAACATTATCGCCATG	*Hin*d III
pXMJ19–*amt*RF	CCCAAGCTT**AAAGGAGGACAACC**ATGGCAGGAGCAGTGGGACGC	*Hin*d III
pXMJ19–*amt*RR	CCGGAATTCTTAGTGGTGGTGGTGGTGGTGTTTCGCGTCAGCCTGC	*Eco*R I
pXMJ19–*amt*BF	CCCAAGCTT**AAAGGAGGGAAATC**ATGAGCGCAGATCAAATCGC	*Hin*d III
pXMJ19–*amt*BR	CCGGAATTCTTAGTGGTGGTGGTGGTGGTGACGAATTTCCGGCCCG	*Eco*R I
P_{tacM}–*amt*BF1	CCGGAATTCCGGCGATCGCTGAAGTAGATGACAAAGC	*Eco*R I
P_{tacM}–*amt*BR1	CCGTGCAGTC GATAAGCTCC CAGCGAGGCT TGGCGGATTC CCAC	–
P_{tacM}–*amt*BF2	GTGGGA ATCCGCCAAG CCTCGCTG GGAGCTTATC GACTGCACGG	–
P_{tacM}–*amt*BR2	GTTCTGCGCG GGGGTCGTCA CCTTAACGAA TTTCCGGCCC TGTG	–
P_{tacM}–*amt*BF3	CACAGGGCCGG AAATTCGTTA AGGTGACGACCCCCGCGCAGA AC	–
P_{tacM}–*amt*BR3	CCCAAGCTTACTAGATGGCCTCAACATTATCGCCATG	*Hin*d III

The underlined sequences were restriction sites, ribosomal binding sites are in boldface

centrifugation and then were resuspended in phosphate buffer (pH 7.2) and lysed on ice by sonication to obtain the crude enzyme fraction. The concentrations of the recombinant AmtR and AmtB proteins were determined by SDS–PAGE (12% acrylamide) [31], and the proteins were used for activity assays.

Enzyme activity assays

GDH activity was measured using a Glutamate Dehydrogenase Activity Colorimetric Assay Kit (Solarbio, China) following the manufacturer's instructions. The Cc–amtR, Cc–ΔamtR, Cc–amtB1 and Cc–amtB2 strains were harvested by centrifugation at 8000 r/min for 10 min after 48 h of cultivation in CGXII medium. One unit of GDH activity was defined as the amount of enzyme that generated one micromole of NADH per minute under the assay conditions. The GS activity was measured using a Glutamine Synthetase Assay Kit (Solarbio, China) following the manufacturer's directions. One unit of the GS activity was defined as the amount of enzyme that resulted in a change in the absorbance of 0.01 (at 540 nm) per minute under in a 1-mL reaction. The GOGAT activity was measured as described by Tesch et al. [32].

Determination of ammonium concentrations by ion chromatography

A Thermo Scientific ICS-5000$^+$ ion chromatograph(Thermo Scientific,USA) was used to detect the concentration of extracellular NH_4^+ at 0, 12, 24, 36, 48, 60 and 72 h in the fermentation broth of the Cc–amtB1, Cc–amtB2 and Cc5-5 cultures. Ion chromatography including an IonPac CG12A guard column (4 × 50 mm i.d.) and an IonPac CS12A separation column (4 × 250 mm i.d.). The eluent was obtained using a CH_3SO_3H eluent generator, and suppression was achieved using a CERS500 4-mm self-regenerating suppressor. The column temperature was set at 30 °C, the eluent flow rate was set at 1.00 mL/min, and the injection volume was 25 µL. To prepare the samples, 50 mL of broth was centrifuged at 1000 r/min for 10 min to remove $CaCO_3$, after which the supernatant was centrifuged at 8000 r/min for 10 min. Next, a high-pressure homogenizer was used to lyse the cells, and cell debris was subsequently removed by centrifugation at 10,000 r/min for 10 min. The supernatants were filtered twice through 0.22-µm filters, and the organic substances in the sample were removed using a Thermo OnGuard II RP column. The instrument control and data collection were performed by a Dionex Chromeleon 6.5 [33].

Real-time PCR

For RNA analysis, 500-µL samples of strains cultured to the exponential phase were collected during batch fermentation in shake flasks for mRNA isolation. RNA was extracted from the collected cells using an RNAiso Plus reagent (Takara, Dalian, China) following the manufacturer's instructions. cDNA was synthesized using a PrimeScript RT reagent kit (Takara, Dalian, China). Cc5-5 16S rRNA was used as an internal standard [30, 34]. The Ct values of the 16S rDNA gene and those of the *amt*B, *gln*K, and *gln*D genes were obtained by RT-qPCR using a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Hercules, CA) with SYBR Premix Ex Taq™ II (Takara, Dalian, China). The primer sequences used for RT-qPCR and RT-PCR for assess the integration of P_{tacM} are shown in Table S1. Each assay was replicated three times.

Analytical procedures

Cell growth was monitored by measuring the OD_{562} (1 OD_{562} = 0.375 g/L Dry cell weight, DCW) using a spectrophotometer (UNICOTM-UV2000, Shanghai, China) after dissolving $CaCO_3$ with 0.125 M HCl. To quantify substrate consumption and production, 1.5-mL samples of the fermentation culture were harvested and centrifuged. The supernatant was analyzed for glucose using an SBA-40C biosensor analyser (developed by the Biology Institute of the Shangdong Academy of Sciences). The sample supernatants were processed using 0.22-µm filters and centrifuged (10,000 r/min for 10 min at 4 °C). L-Arginine concentrations were determined by HPLC (high-pressure liquid chromatography) on an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) following the procedure described by Xu [6]. All assays were performed in triplicate.

Results

High nitrogen concentrations are essential for L-arginine production

Most microorganisms use ammonium (NH_4^+) as a preferred nitrogen source, and the nitrogen content in the L-arginine molecule is as high as 32.15%. There are four processes to assimilate the NH_4^+ in L-arginine biosynthesis (Fig. 1). Therefore, detection of the effect of different concentrations of NH_4^+ on L-arginine production by Cc5-5 during shake flask fermentation. When 1, 10, 100, 200, 300 and 400 mM $(NH_4)_2SO_4$ were added into the fermentation medium with an initial glucose concentration of 100 g/L, the cell growth and glucose consumption rate increased as the concentration of NH_4^+ increased. As shown in Table 3, when an NH_4^+

Table 3 L-arginine shake flask fermentation parameters of different $(NH_4)_2SO_4$ concentrations

$(NH_4)_2SO_4$ (mM)	L-arginine (g/L)	Residual glucose (g/L)	Dry cell weight (g/L)	Specific arginine yield (g/g DCW)	Productivity (g/L h)
1	2 ± 0.5	14 ± 1.5	12.31 ± 1.1	0.15 ± 0.12	0.02 ± 0.01
10	5.3 ± 0.8	13 ± 2	12.83 ± 1.3	0.42 ± 0.15	0.07 ± 0.02
100	15 ± 1.5	12 ± 2.1	13.79 ± 0.6	1.09 ± 0.14	0.21 ± 0.02
200	25.3 ± 1.4	10 ± 1.8	14.04 ± 1.6	1.80 ± 0.17	0.35 ± 0.04
300	31.8 ± 1.8	9 ± 1.7	13.85 ± 1.9	2.30 ± 0.15	0.44 ± 0.05
400	28.2 ± 1.3	15 ± 1.5	11.71 ± 1.5	2.41 ± 0.12	0.39 ± 0.05

concentration of 300 mM was used, the increase in L-arginine production and glucose consumption was the greatest compared with the production in other samples in the 250-mL shake flask cultivation assay. After 72 h of cultivation, the strain cultured with 300 mM NH_4^+ exhibited an L-arginine production of 31.8 ± 1.8 g/L with a productivity of 0.44 ± 0.05 g/(L·h). For an NH_4^+ concentration of 400 mM, the observed L-arginine production was 28.2 ± 1.3 g/L, and while the cell growth and glucose consumption rate decreased, the specific L-arginine production increased. As expected, this result indicated that a rich supply of NH_4^+ is essential for high-efficiency L-arginine biosynthesis. In addition, in fermentation medium, lower amounts of added NH_4^+ hardly affected strain growth, but excessive NH_4^+ was unfavourable for strain growth.

Effect of AmtR deletion or overexpression on L-arginine production

AmtR is a global transcriptional regulator of nitrogen metabolism in *C. glutamicum* that regulates the transcription of at least 35 genes. When nitrogen sources are sufficient, the expression of most of these genes is downregulated [35]. To investigate the effect of AmtR on L-arginine production in *C. crenatum* SYPA5-5 (Cc5-5), the AmtR deletion and overexpression AmtR mutant strains Cc–ΔamtR and Cc–amtR were constructed, as shown in Table 1; the AmtR deletion mutant strain is verified in Fig. S1. The growth of the constructed strains was normal in LBG medium, similar to the Cc5-5 strain (data not shown). Next, the Cc–ΔamtR, Cc–amtR and Cc5-5 strains were cultivated in the 250-mL shake flask to investigate the effect of AmtR deletion or overexpression on L-arginine production. As shown in Fig. S2, almost no L-arginine was produced during the first 10 h; while after 72 h of cultivation, the production of L-arginine by the Cc–amtR strain (12 ± 1.2 g/L) was only half that observed for the Cc5-5 strain, and the Cc–ΔamtR strain exhibited a 16.67% increase in L-arginine production (30 ± 1.5 g/L) compared to that of the Cc5-5 strain. These results indicated that the deletion of the nitrogen transcriptional regulator AmtR is beneficial to L-arginine production.

To further investigate the function of AmtR in Cc5-5, growth e-related enzymes (GSxperiments and activity assays for nitrogen metabolism, GDH, and GOGAT) regulated by AmtR were carried out in modified CGXII minimal medium containing $(NH_4)_2SO_4$ at concentrations of 1, 10, and 100 mM. As shown in Fig. 2a, in the presence of different concentrations of $(NH_4)_2SO_4$, the growth of all three strains increased steadily in medium containing 1 and 10 mM. However, after 36 h of cultivation, cultures containing 1 mM $(NH_4)_2SO_4$ grew significantly slower than those containing 10 mM $(NH_4)_2SO_4$. Interestingly, in cultures containing 100 mM $(NH_4)_2SO_4$, strains grew slowly over the first 24 h; after 24 h, the growth of strains increased steadily. This result suggested that a sufficient concentration of a nitrogen source is essential for the growth of Cc5-5 strain, but excessive NH_4^+ is detrimental to the growth of the Cc5-5 strain in CGXII medium.

Glutamate and glutamine are the two major donors of NH_4^+ during the biosynthesis of L-arginine (Fig. 1), and NH_4^+ assimilation proceeds via glutamate dehydrogenase (GDH) or glutamine synthetase/glutamate synthase (GS/GOGAT), which are regulated by AmtR. The assimilation of NH_4^+ was observed by comparing the activity of these enzymes in the above three strains. In Fig. 3b, c, when the concentration of $(NH_4)_2SO_4$ was 1 mM, the GOGAT and GS activities in Cc–ΔamtR strain were two–threefold higher than those observed for the Cc5-5 strain. At 100 mM $(NH_4)_2SO_4$, the activity of GDH was the highest, 3.5 times higher than that observed for the Cc5-5 strain (Fig. 3a). However, the overexpression of AmtR caused a sharp decrease in the activities of the three assayed enzymes compared with those observed in the Cc5-5 strain. These results show that AmtR plays an important role in the production of L-arginine and in nitrogen metabolism-related enzyme activity in Cc5-5.

Comparative analysis of the transcriptional levels of AmtR-related genes

Deletion of the transcriptional regulator AmtR can increase the activity of NH_4^+ assimilation-related enzymes and promote L-arginine synthesis when the nitrogen source

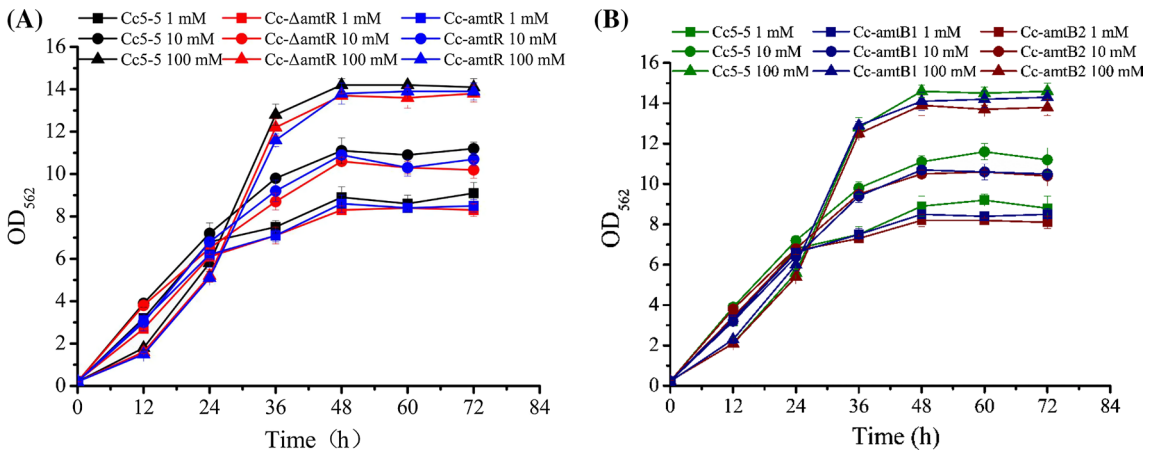

Fig. 2 Growth curves for the Cc–ΔamtR, Cc–amtR and Cc5-5 strains (**a**) and the Cc–amtB1 and Cc–amtB2 strains (**b**). Strains were cultivated at 30 °C and 200 r/min for 72 h in 250-mL flasks in CGXII minimal medium [supplemented with 1, 10, and 100 mM $(NH_4)_2SO_4$]. The data represent the mean values and standard deviations obtained from at least three independent measurements

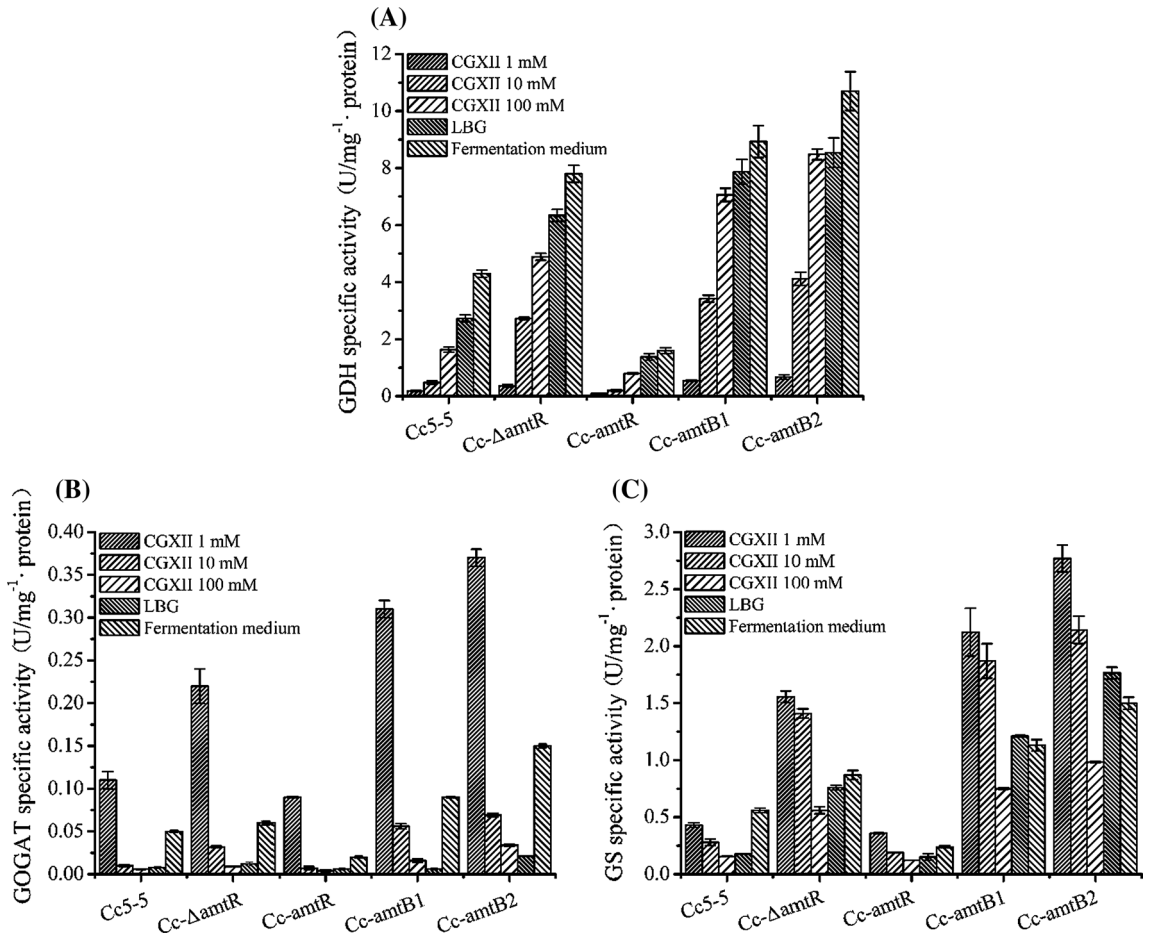

Fig. 3 Measurements of glutamate dehydrogenase (GDH) (**a**), glutamate synthase (GOGAT) (**b**), and glutamine synthetase (GS) (**c**) for the Cc–ΔamtR, Cc–amtR, Cc–amtB1, Cc–amtB2 and Cc5-5 strains. Strains were cultivated at 30 °C, 200 r/min for 72 h in 250-mL flasks in CGXII minimal medium [supplemented with 1, 10, 100 mM $(NH_4)_2SO_4$], LBG medium and fermentation medium. The data represent the mean values and standard deviations obtained from at least three independent measurements

is sufficient. To investigate the mechanism by which the deletion of the gene-encoding AmtR increased L-arginine production, the transcriptional profiles of genes related to the uptake and assimilation of NH_4^+ were measured in the Cc–ΔamtR, Cc–amtR and Cc5-5 strains at different concentrations of NH_4^+. First, the strains were grown in modified CGXII minimal medium containing 1 or 100 mM $(NH_4)_2SO_4$ as the sole nitrogen source, with cells harvested at an OD_{562} of between 4 and 6. To accurately evaluate the RT-qPCR results, a series of oligonucleotide primers were tested to determine the PCR efficiency (Table S1), and RiboGreen was used to accurately determine the total amount of RNA used in the real-time PCR experiments. This method allowed for the use of the 16S rRNA gene to standardize the results obtained without the need for additional control genes. As shown in Fig. 4, in cultures containing 100 mM $(NH_4)_2SO_4$, a comparison of the transcriptional response of the Cc–ΔamtR and Cc5-5 strains revealed that the *amt*B gene were increased 31.2-fold, while those of the *gln*K and *gln*D genes were increased two-fold, respectively. In addition, nitrogen assimilation systems were investigated by measuring the transcriptional level of the genes encoding the nitrogen metabolism-related enzyme GS, GDH, and GOGAT (*gln*A, *gdh*, and *glt*BD). The transcription of the *gdh* gene in Cc–ΔamtR strain was observed to be increased 20.5-fold compared to that of the Cc5-5 strain; the transcription of the other genes was not significantly changed. In contrast, comparing the Cc–amtR and Cc5-5 strains in the presence of 100 mM $(NH_4)_2SO_4$, the expression of the *amt*B gene in the Cc–amtR strain was downregulated 64.2-fold, and that of the *gln*K and *gln*D genes was downregulated four-fold, respectively, while that of the *gdh* gene was downregulated 30.3-fold. In cultures containing 1 mM $(NH_4)_2SO_4$, the trend was consistent with an insufficient nitrogen supply, but not notably so. The qPCR results indicated that AmtR gives an obvious influence on the ammonium uptake and assimilation.

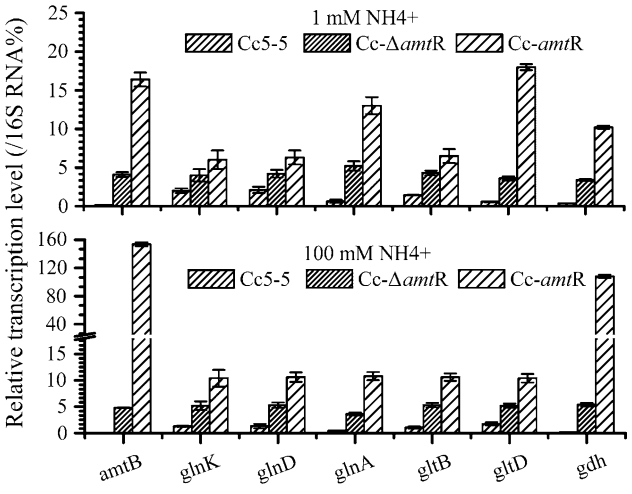

Fig. 4 The transcriptional regulation of AmtR for the *amt*B–*gln*K–*gln*D operon and the genes encoding L-arginine synthesis-related enzymes (GS, GDH, and GOGAT) in different strains. Relative expression levels of *amt*B, *gln*K, *gln*D, *gln*A, *gdh*, and *glt*BD at 1 and 100 mM $(NH_4)_2SO_4$ concentrations were calculated based on the quantified real-time qPCR results. The error bars denote the standard deviations, and $n = 3$

Effect of AmtB overexpression on ammonium uptake and L-arginine production

AmtB is an ammonium channel protein involved in ammonium transport, and the *amt*B gene sequence was compared and analyzed in BLAST searches. The results showed that the Cc5-5 *amt*B gene sequence was identical to that of *C. glutamicum* SCgG2 (GenBank: CP004048.1), with the alignment results for *C. glutamicum* ATCC 13032 showing that the alignment identity of *amt*B was 81%; while the amino acid sequence alignment showed that the AmtB sequence was 89.95% identical in the two strains. These results showed little difference between the *C. glutamicum* and Cc5-5 *amt*B genes. Based on the comparison results and qPCR (Fig. 4), we speculated that the overexpression AmtB would have a great influence on nitrogen assimilation in Cc5-5 strain. First, AmtB was overexpressed in the Cc–ΔamtR strain, resulting in the Cc–amtB1 strain. Next, a strong promoter, P_{tacM} [36], was integrated with the *amt*B gene at the *amt*R locus to increase AmtB expression in the mutant strain Cc–ΔamtR. As shown in Fig. S3, compared to that in the Cc5-5 strain, the transcriptional level of the *amt*B gene in strain Cc–amtB2 increased by 38.3-fold and increased sevenfold compared to that in the Cc–ΔamtR strain. The results suggested that the recombinant strain was successfully constructed and that the expression of the *amt*B gene was increased by the insertion of the stronger P_{tacM} promoter.

The L-arginine production of the Cc–amtB1, Cc–amtB2 and Cc5-5 strains was compared under shake flask fermentation conditions. As shown in Fig. S2, during the first 48 h, the production of L-arginine was almost the same, but as the fermentation progressed, the Cc–amtB1 and Cc–amtB2 strains exhibited greater L-arginine production than the Cc5-5 strain. After cultivation for 72 h, the Cc–amtB1 strain showed a 23.8% increase in L-arginine production (32.8 ± 1.1 g/L) compared to that of the Cc5-5 strain. The Cc–amtB2 strain produced approximately 36.5 ± 1.2 g/L L-arginine, which was 12.4% more than was produced by the Cc–amtB1 strain. The results suggested that modulation of AmtB overexpression resulted in an improvement in L-arginine production under similar fermentation conditions.

By inserting the P_{tacM} promoter and the *amt*B gene segment into the *amt*R chromosomal locus, we overcame deficiencies observed in the plasmid-based AmtB

overexpression systems that hampered the generation of derivative strains. This study highlighted the importance of AmtB overexpression in the development of L-arginine-producing strains. Furthermore, in this study, as shown in Fig. 5, the extracellular NH_4^+ levels in the Cc–amtB1 and Cc–amtB2 strains were notably more quickly reduced than that was observed in the Cc–ΔamtR strain, especially, after 36 h of fermentation. Furthermore, the NH_4^+ levels were slightly lower in the Cc–amtB2 strain than in the Cc–amtB1 strain. The result indicated that enhancing the ammonium transporter expression can improve the efficiency of NH_4^+ utilization. Moreover, the effect was stronger with the P_{tacM} promoter integrated into the chromosome than with AmtB overexpression in the Cc–ΔamtR strain.

Further investigations of growth and nitrogen metabolism-related enzyme activity (GS, GDH, and GOGAT) were carried out in the Cc–amtB1, Cc–amtB2 and Cc–ΔamtR strains, which were cultured in different NH_4^+ concentrations in CGXII minimal medium. The growth rate was almost the same in the presence of 1 and 10 mM NH_4^+ (Fig. 2b), although the final OD_{562} values were found to depend directly on the amount of NH_4^+ added. At 100 mM NH_4^+, the Cc–amtB2 strain maintained a certain growth advantage over the Cc–ΔamtR strain throughout the growth process. As described above, in the growth experiment, it can be seen that the enhanced expression of the ammonium transporter improved NH_4^+ consumption. After ammonium is transported into the cells, assimilation proceeds via GDH and GS/GOGAT. As shown in Fig. 3a, as the NH_4^+ concentration increased, the GDH activity gradually improved. When NH_4^+ was present at 100 mM, the GDH enzyme activity of the Cc–amtB2 strain was increased 5.2-fold compared to control group, and 1.2-fold compared to the Cc–amtB1 strain. The GS/GOGAT activity was slightly higher at 1 mM NH_4^+. Furthermore, the three enzyme activities were higher in the fermentation medium than in the CGXII minimal medium, especially for the Cc–amtB2 strain, which had the highest enzyme activity and explained the increase in L-arginine production.

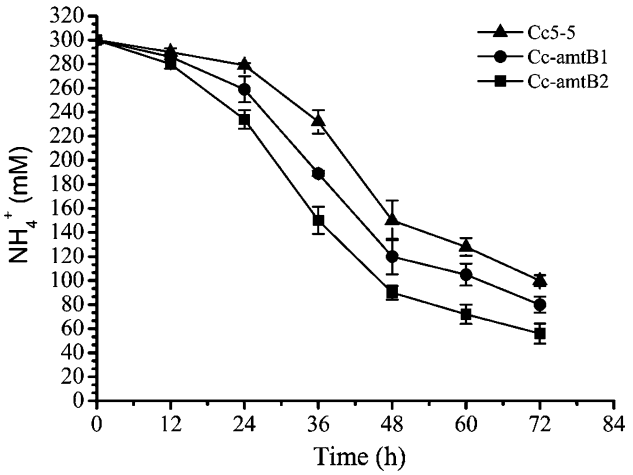

Fig. 5 Comparison of residual NH_4^+ concentrations in the Cc–amtB1, Cc–amtB2 and Cc5-5 strains under shake flask fermentation. Strains were cultivated at 30 °C and 200 r/min for 72 h in 250-mL flasks in fermentation medium. The data represent the mean values and standard deviations obtained from at least three independent measurements

Fed-batch fermentation of Cc5-5 and Cc–amtB2 strains

To maintain the NH_4^+ concentration at a relatively steady level and relieve the inhibition of growth by a high concentration of nitrogen, a strategy was investigated by continuously adding 25% $NH_3{\cdot}H_2O$ in 5-L fed-batch fermentation. Fed-batch cultures were performed to examine the performance of the mutant strains for L-arginine production. Time profiles of L-arginine fed-batch cultures of the Cc5-5 and Cc–amtB2 strains are shown in Fig. 6a, b, respectively. The cell growth of the two strains was similar throughout the whole fermentation period, indicating that the knockout of AmtR and the insertion of the P_{tacM} with the *amt*B gene segment into the *amt*R chromosomal locus had no effect on Cc5-5 growth. The Cc–amtB2 strain was also observed to consume glucose more quickly before the first feeding (36 h after incubation) than the Cc5-5 strain (42 h after incubation). At the end of the fed-batch fermentations, the Cc–amtB2 strain produced 60.9 ± 1.31 g/L of L-arginine with a yield of 0.36 ± 0.018 g/g glucose and a productivity of 0.634 g/L/h. In comparison, the fed-batch fermentation of the Cc5-5 strain resulted in 39.5 ± 0.57 g/L of L-arginine with a yield of 0.25 ± 0.02 g/g glucose and a productivity of 0.411 g/(L·h), while the L-arginine production from glucose by the Cc–amtB2 strain was approximately 30.56% higher than that by the Cc5-5 strain in fed-batch fermentation. Amino acids and organic acids analysis of the fermentation broth showed the concentration of lactate, lysine, isoleucine compared with the Cc5-5 strain was decreased slightly and proline was undetectable; specific data are shown in Table S2. This result indicated that the L-arginine yield can be markedly increased by knocking out the nitrogen regulator AmtR and that expressing the ammonium transporter AmtB by integrating a strong promoter on the chromosome in the Cc–ΔamtR strain is a useful strategy to increase the L-arginine yield.

Discussion

With the increased research on nitrogen metabolism in *C. glutamicum*, it is clear that *C. glutamicum* has a specific strategy to regulate this process. The most crucial step to

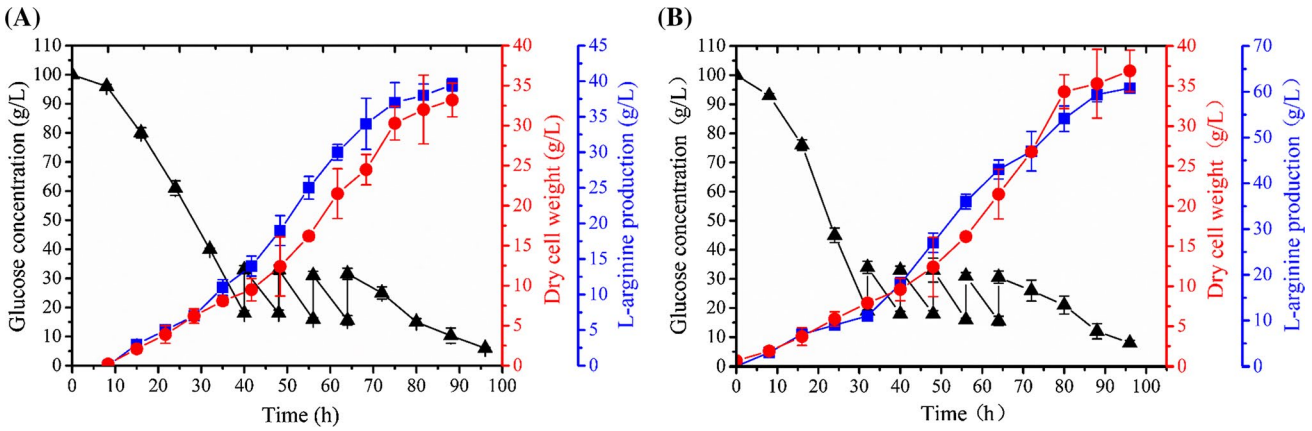

Fig. 6 Time course of L-arginine fed-batch fermentations of the Cc5-5 (**a**) and Cc–amtB2 (**b**) strains in 5-L fermenters. The filled triangle represents glucose, the filled square represents L-arginine, and the filled circle represents dry cell weight (DCW). The total glucose concentrations in the fed-batch fermentations of the Cc5-5 and Cc–amtB2 strains were 160 and 175 g/L, respectively. The data represent the mean values and standard deviations obtained from three independent cultures

increase nitrogen metabolism in this bacterium involved the discovery of the global repressor AmtR, which blocks the transcription of many genes in *C. glutamicum* during cultivation in medium with sufficient nitrogen [35]. In this study, we explored the effect of the deletion of AmtR and the overexpression of the ammonium transporter AmtB on nitrogen metabolism in Cc5-5, especially with respect to L-arginine production.

To study the function of AmtR in Cc5-5 strain, we constructed *amt*R gene deletion and overexpression strains. Growth assays revealed that the growth of the Cc–ΔamtR and Cc–amtR strains was slightly slower than that of the Cc5-5 strain before 36 h but resulted in the same biomass in the stationary phase. However, the growth of the strain in the presence of low nitrogen source concentrations (1 and 10 mM $(NH_4)_2SO_4$) in the early stages of the exponential period was significantly faster than in the presence of a high nitrogen concentration [100 mM $(NH_4)_2SO_4$]. Obviously, Cc5-5 can avoid the effects of ammonium limitation on the growth rate using intracellular stored nitrogen sources such as glutamate and glutamine [37].

L-Arginine is a basic amino acid, and nitrogen is required for L-arginine biosynthesis [16]. By comparing the enzyme activities of the strains Cc–ΔamtR, Cc–amtR and Cc5-5 at three NH_4^+ concentrations, we observed that the GDH activity at a high nitrogen source concentration was significantly higher than that at 1 mM $(NH_4)_2SO_4$, and that GOGAT had almost no activity in the presence of 100 mM $(NH_4)_2SO_4$. Thus, the high expression of GDH could improve the accumulation of glutamate [38]. Furthermore, glutamate provides nitrogen for the synthesis of the precursor L-arginine [39]. We suspected that this is the reason for the more favorable L-arginine production when the nitrogen source is sufficient. Beckers and Schulz observed that GDH is preferentially used in ammonium-rich medium [40, 41]; while the transcription of *glt*BD, coding for GOGAT, is completely repressed in this situation, and the transcription of *gln*A, coding for GS, is maintained at a basal level to provide L-glutamine for growth [42]. When bacterial cells under the limitation of nitrogen, the ammonium assimilation via GDH is not sufficient due to the low affinity of the enzyme. In this situation, the GS/GOGAT pathway is preferentially used for ammonium assimilation [43]. Thus, the AmtR deletion strain exhibited increased ammonium assimilation. In the shake flask fermentation experiment, the final L-arginine production of the Cc–ΔamtR strain was increased by 16.67% compared to that of the Cc5-5 strain.

In a previous study, AmtR repressed the transcription of many genes, including those encoding transporters and genes for ammonium assimilation (*amt*B, *gln*K, and *gln*D) when bacterial cells were grown in nitrogen-rich standard minimal medium. To fully evaluate the performance of the engineered strains Cc–ΔamtR and Cc–amtR strains, the effect of AmtR on the expression of the *amt*B–*gln*K–*gln*D operon and the ammonium assimilation system genes *gln*A, *glt*BD, *gdh* was verified by RT-qPCR. The transcriptional level of the *gdh* gene increased 20.5-fold in the Cc–ΔamtR strain when cells were grown with 100 mM $(NH_4)_2SO_4$, a result consistent with the observed enzyme activity. However, it was determined that the overexpression of AmtR had an obvious effect on the ammonium transporter AmtB, resulting in a 64.2-fold downregulation of its expression.

From these results, we concluded that the knockout of the *amt*R gene could relieve the transcriptional repression of extracellular nitrogen metabolism protein-encoding genes. Furthermore, the overexpression of the ammonium transporter protein AmtB promoted the uptake of NH_4^+, resulting in a significant increase in L-arginine production. This result

indicated that the overexpression of the ammonium transporter AmtB improved the efficiency of nitrogen source utilization. We can also infer from the cell growth results that the strain grows rapidly from 36 to 48 h and that more nitrogen is needed during exponential growth. Considering the instability of the free plasmid, the promoter P_{tacM} was integrated into the *amt*B gene in the Cc–ΔamtR strain, enhancing the expression of AmtB. Finally, the Cc–amtB2 and Cc5-5 strains were investigated in a fed-batch process. Fed-batch fermentation of the Cc–amtB2 strain resulted in the production of 60.9 ± 1.31 g/L of L-arginine with a yield of 0.36 ± 0.018 g/g glucose and a productivity of 0.634 g/(L h). The fermentation results demonstrated that the high NH_4^+ concentration of 400 mM is essential for high-efficiency L-arginine production. By deleting the AmtR regulator, which represses the ammonium assimilation corresponding genes under the high NH_4^+ Cc5-5, the transcriptional level of ammonium transporter AmtB was significant increased. Extracellular NH_4^+ concentration analysis indicated that the NH_4^+ consumed more in the engineered strains.

In this study, we have created an environment rich in supply of nitrogen for the L-arginine production, improved the efficiency of nitrogen uptake and assimilation in Cc5-5. Along with the advances made via the continued study of nitrogen metabolism complexities, further enhancement of L-arginine production would be achieved by fine-tuning the adjustment of the expression of ammonium absorption and assimilation genes and through the rebalance of carbon and nitrogen fluxes.

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