Original Article

Expression and Characterization of ArgR, An Arginine Regulatory Protein in *Corynebacterium crenatum*[*]

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Abstract

Objective *Corynebacterium crenatum* MT, a mutant from *C. crenatum* AS 1.542 with a lethal *argR* gene, exhibits high arginine production. To confirm the effect of ArgR on arginine biosynthesis in *C. crenatum*, an intact *argR* gene from wild-type AS 1.542 was introduced into *C. crenatum* MT, resulting in *C. crenatum* MT. sp, and the changes of transcriptional levels of the arginine biosynthetic genes and arginine production were compared between the mutant strain and the recombinant strain.

Methods Quantitative real-time polymerase chain reaction was employed to analyze the changes of the related genes at the transcriptional level, electrophoretic mobility shift assays were used to determine ArgR binding with the *argCJBDF*, *argGH,* and *carAB* promoter regions, and arginine production was determined with an automated amino acid analyzer.

Results Arginine production assays showed a 69.9% reduction in arginine from 9.01±0.22 mg/mL in *C. crenatum* MT to 2.71±0.13 mg/mL (P<0.05) in *C. crenatum* MT. sp. The *argC*, *argB*, *argD*, *argF*, *argJ*, *argG*, and *carA* genes were down-regulated significantly in *C. crenatum* MT. sp compared with those in its parental *C. crenatum* MT strain. The electrophoretic mobility shift assays showed that the promoter regions were directly bound to the ArgR protein.

Conclusion The arginine biosynthetic genes in *C. crenatum* are clearly controlled by the negative regulator ArgR, and intact ArgR in *C. crenatum* MT results in a significant descrease in arginine production.

Key words: *Corynebacterium crenatum*; ArgR protein; Arginine biosynthetic genes; Real-time PCR; Electrophoretic mobility shift assay

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INTRODUCTION

L-arginine (L-Arg), a semi-essential amino acid, has recently attracted considerable attention because of its ability to facilitate the synthesis of various immune active materials for preventing the growth of cancer cells[1], promoting urine circulation, and reducing the ammonia content in blood[2]. As a nitric oxide precursor, L-Arg also has a function in relaxing and dilating blood vessels[3].

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Therefore, L-Arg is widely used as an additive in the food and pharmaceutical industries.

Corynebacterium crenatum AS 1.542 is a Gram-positive bacterium isolated from soil[4]. Its mutants are widely exploited in the large-scale production of many amino acids, including L-glutamic acid, L-lysine, L-valine, and so on. In our previous work, we successfully obtained a high-yield strain (*C. crenatum* MT) via mutagenesis screening using nitrosoguanidine. This strain was found to possess a greatly enhanced L-Arg-producing ability compared with the wild-type AS 1.542 strain. The L-Arg yield increased from 0 mg/mL to 9.01±0.22 mg/mL. A point mutation was found in the regulator *argR* gene in *C. crenatum* MT: a C-to-T exchange at position 109 in *argR*, leading to an amino acid replacement of Gln-37 (CAG) by a terminator codon (TAG) and thus a truncated ArgR protein.

ArgR is considered as a regulator in the L-Arg biosynthetic pathway (Figure 1)[4-5]. For example, ArgR cooperating with L-Arg in *Escherichia coli* inhibits the transcription of genes participating in L-Arg biosynthesis at different levels and activates the L-Arg degradation pathway[6]. It also has an important function in regulating the arginine-inducible uptake of L-Arg and ornithine metabolism in *Pseudomonas aeruginosa* under aerobic conditions. ArgR activates the *aotJQMOPargR* operon encoding a protein complex for L-Arg and ornithine uptake. ArgR is also a repressor of three operons (*argF*, *carAB*, and *argG*) in L-Arg biosynthesis and two operons (*gltBD* and *gdhA*) in glutamate biosynthesis[7-8]. The mRNA amounts of *argCJBDFR* and *argGH* are reportedly 10 to 44 times higher in a *Streptomyces clavuligerus* *argR*-disrupted mutant than in the wild type, confirming the existence of an ArgR-mediated gene regulation in L-Arg biosynthesis[9]. These results indicate that the ArgR protein is a vital and multifunctional regulator in L-Arg metabolism.

Xu et al. reported that overepression of the arginine biosynthetic *argC-H* cluster from *C. crenatum* with a lethal *argR* gene could improve the production of L-Arg. However, the characterization of *argR* had not been elaborated in detail[10]. This study aims to elucidate further the function of ArgR in L-Arg biosynthesis in *C. crenatum* and to provide basis for the future genetic engineering of L-Arg-producing bacterial strains. Our study focuses on characterizing the *C. crenatum* ArgR protein and its role in the transcriptional regulation of L-Arg biosynthetic gene expression in *C. crenatum* by reconstructing the wild-type *argR* gene in *C. crenatum* MT.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

The bacterial strains and plasmids used in this work were described in Table 1. LB medium was used for cultivation of *E. coli* DH5α *and* BL21 (DE3) strains. Seed medium (per liter) for cultivation of *C. crenatum* consisted of 30 g glucose, 20 g cornsteep liquor, 20 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 1.5 g urea. Fermentation medium (per liter) for L-Arg production was composed of 120 g glucose, 40 g cornsteep liquor, 20 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 2H_2O$, 8×10^{-5} g biotin, 5×10^{-4} g histidine, and 30 g $CaCO_3$.

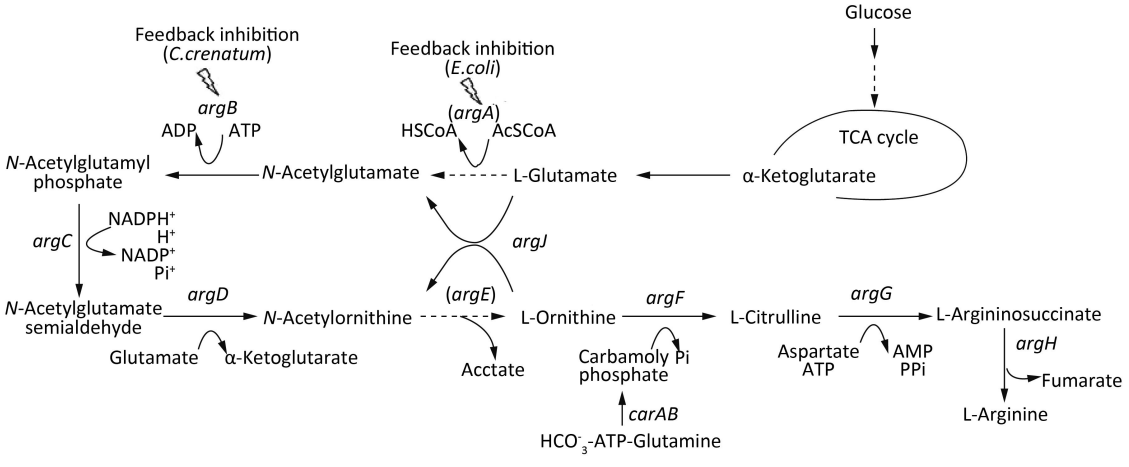

Figure 1. Illustration of the arginine biosynthetic pathway. The difference between the linear pathway and the cycle pathway is that the *argE* gene encodes acetylornithine deacetylase in the linear pathway, whereas the *argJ* gene encodes acetylornithine transferase in the circular pathway.

Recombinant DNA Techniques

ArgR gene fragments were amplified as described previously[11] (Jiao et al. 2011), and the DNA fragments were ligated into pET-22b (+) and pXMJ19[12] (Jakoby et al. 1999) to generate pET-argR and pXMJ19-argR, respectively. Overexpression and purification of His-tagged ArgR was used in electrophoretic mobility shift assays (EMSAs) or in preparing anti-ArgR polyclonal antibody by immunizing mice.

ArgR Expression in C. crenatum MT and Western Blotting Assays

pXMJ19-argR was transferred into *C. crenatum* MT using the electroporation method as previously described[13]. The transformant *C. crenatum* MT. sp was selected by chloramphenicol (10 μg/mL) resistance. The expression of ArgR in *C. crenatum* MT. sp was induced by 0.5 mmol/L isopropyl beta-D-thiogalactopyranoside (IPTG) for 8 h and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a discontinuous system including a 5% (w/v) stacking gel and a 12% (w/v) running gel. The gels were stained with silver nitrate after electrophoresis. For Western blot assays, the proteins in the gels were blotted to polyvinylidene fluoride membranes by electroblotting. 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Sigma, USA) was used to detect antigen-antibody complexes[14].

L-Arg Production Assays

A sample (1.5 mL) of *C. crenatum* MT from the overnight seed culture was inoculated into 20 mL of fermentation medium in a 250 mL flask and cultivated at 30 °C for 120 h with shaking at 200 rpm. For *C. crenatum* MT. sp, a similar procedure was conducted, except that IPTG (final concentration of 0.5 mmol/L) was added to the fermentation culture after 12 h of cultivation. The concentration of free amino acids including L-Arg in the fermentation culture was determined with a L-8800 automated amino acid analyzer (Hitachi High-Technologies, Tokyo, Japan). Before analysis, 200 μL of sample solution was centrifuged at 15 000 rpm for 10 min at 4 °C. The supernatant was diluted with 0.02 mol/L of HCl and used for analysis. The concentrations of L-Arg

Table 1. Bacterial Strains, Plasmids, and Primers Used in This Work

Strains, Plasmids, and Primers	Relevant Characteristics	Source, Reference, or Note
Strains		
C. crenatum AS 1.542	The wild-type strain	China general microbiological culture collection center
C. crenatum MT	derived through nitrosoguanidine mutagenesis from *C. crenatum* AS 1.542, His^-, SG^R, $D\text{-}Arg^R$, $H\text{-}Arg^R$	This lab
C. crenatum MT. sp	derived from *C. crenatum* MT containing a plasmid of pXMJ19 insert carrying *argR* gene	This Work
Plasmids		
pET-22b(+)	Amp^R, f1 origin, 6×histidylfusion expression vector	Novagen
pET-argR	pET-22b(+) with 520 bp *Nde I-Hind III* fragment carrying *argR*, Amp^R	This work
pXMJ19	Shuttle expression vector (Cm^R ptac laclq pBL1 or i $V_{C.glutamicum}$ pK18 ori$V_{E.coli}$)	gift from Dr. Andreas Burkovski
pXMJ19-argR	pXMJ19 with 516 bp *Sac I-Hind III* fragment carrying *argR* from *C. crenatum* AS 1.542, Cm^R	This work
pMD18-T	Cloning of PCR products	TaKaRa
Primers 5′ to 3′		
PargG-F	biotin -AGCTGCCCAGTACCTGGCAAG	Amplification of *argG* promoter or operator region for EMSA, 384 bp
PargG-R	TCAGGTATGGAATTGCCACAG	
PargC-F	biotin -TCCAGTCGAGGCACCTGTT	Amplification of *argC* promoter or operator region for EMSA, 362 bp
PargC-R	TATCCACTGGCTCCTGCGATT	
PcarAB-F	biotin -CCAATCGCAGAAGGTGAGCCAG	Amplification of *carAB* promoter or operator region for EMSA, 365 bp
PcarAB-R	GAACCAGGTATGCCGGAACGGAT	

were measured in triplicate.

Quantitative Real-time PCR Analysis

The total RNA from *C. crenatum* MT. sp, *C. crenatum* MT, and *C. crenatum* AS 1.542 after 20 h incubation was extracted using a TRIzol kit according to the manufacturer's instructions (Invitrogen, USA) and then digested using RNAse-free DNAse I (Takara, Japan). Approximately 3 μg of total RNA was reverse-transcribed into complementary DNA (cDNA) by a reverse-transcription (RT)-PCR kit (Invitrogen, USA). The primer pairs reported previously by Chen et al.[15] were used for the amplification of the *argC*, *argB*, *argD*, *argF*, *argJ*, *carA*, *argG*, and 16S rRNA (internal standard) genes. All primers were assured for specificity by PCR to produce only a single PCR band of the right size. The cDNA levels were quantified by real-time quantitative PCR (qPCR) using SYBR green PCR master mix (Takara, Japan). The thermal cycling conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. A control reaction without template was implemented to evaluate primer dimer formation. Each assay was performed in triplicate. Relative transcription values for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method[16].

Electrophoretic Mobility Shift Assays (EMSAs)

The DNA fragments were amplified by PCR using the primers listed in Table 1, with the forward primers being tagged with biotin. For specificity assays, the labeled and excess unlabeled fragments (40-fold molar excess) were incubated with His_6-ArgR in the binding buffer (20 mmol/L HEPES (pH 7.6), 1 mmol/L EDTA, 10 mmol/L $(NH_4)_2SO_4$, 1 mmol/L dithiothreitol, 0.2% (w/v) Tween-20, and 30 mmol/L KCl) for 30 min at room temperature. DNA-protein separation by 1.5% agarose gel electrophoresis was carried out at 50 V with 0.5×tris-acetate-EDTA buffer. Subsequently, the DNA-protein complex was blotted onto a Hybond-N+ nylon membrane by vacuum blotting. Streptavidin-abeled horseradish peroxidase and DAB were used to detect biotin-labeled DNA fragments.

Statistical Analysis

All of the data are reported as means±S.E.M., with n indicating the number of experiments. Results were analyzed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Probability values of $P \leq 0.05$ indicated significance.

RESULTS AND DISCUSSION

ArgR Expression in C. crenatum Strains

An intact *argR* from the wild-type strain was introduced into this strain with an IPTG-inducible promoter to investigate whether the truncated ArgR causes the high L-Arg yield in *C. crenatum* MT strain. The introduction of this intact *argR* resulted in a *C. crenatum* MT. sp strain. The ArgR expression in *C. crenatum* strains was analyzed by SDS-PAGE and Western blot. ArgR (approximately 18.4 kDa) was expressed in *C. crenatum* MT. sp with IPTG induction (arrow in lane 2 of Figure 2A). By contrast, no ArgR was observed in cells of *C. crenatum* MT containing an empty pXMJ19 vector with IPTG induction (lane 3 of Figure 2A), uninduced or induced cells of *C. crenatum* AS 1542 (lanes 4 and 5 of Figure 2A), and uninduced cells of *C. crenatum* MT. sp (lane 1 of Figure 2A). This result was confirmed by Western blot assay (Figure 2B).

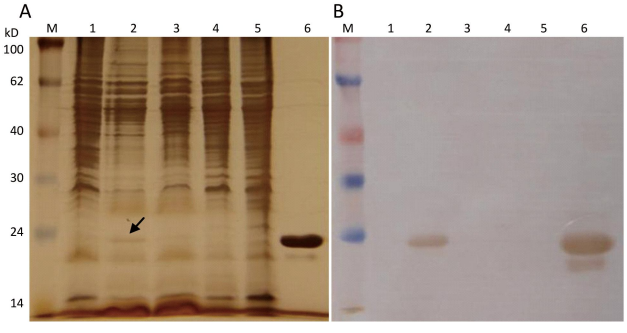

Figure 2. Analysis of ArgR expression using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (A) and Western blot (B). Lane M, Blue plus II protein marker; lane 1, uninduced cells of *C. crenatum* MT. sp; lane 2, cells of *C. crenatum* MT. sp induced by 0.5 mmol/L IPTG; lane 3, cells of *C. crenatum* MT containing an empty pXMJ19 vector with induction of 0.5 mmol/L IPTG; lane 4, uninduced cells of *C. crenatum* AS 1.542; lane 5, cells of *C. crenatum* AS 1.542 induced by 0.5 mmol/L IPTG; and lane 6, purified His-tagged ArgR.

L-Arg Production

The effect of ArgR on cell growth and L-Arg production in *C. crenatum* strains was investigated. The results indicated that cell growth did not differ among the three strains (data not shown). The level of amino acid production of *C. crenatum* MT, *C. crenatum* MT. sp, and wild-type AS 1.542 was determined every 12 h during a 120 h fermentation time. The results showed that L-Arg production did not differ (P>0.05) between *C. crenatum* MT and *C. crenatum* MT. sp in early fermentation (0-24 h). After 120 h of cultivation, L-Arg production showed a significant decrease (P<0.05) from 9.01±0.22 mg/mL in *C. crenatum* MT to 2.71±0.13 mg/mL in *C. crenatum* MT. sp (Figure 3). The expression of the *argR* gene in *C. crenatum* MT. sp significantly inhibited L-Arg production. One of the possible interpretations for this result is the down-regulation at the transcript level of the genes involved in the L-Arg biosynthetic pathway. However, compared with the wild type strain, the L-Arg production of the recombinant strain was higher. This finding indicated that some mutations occurred in other related genes in the MT strain. These additional mutations also had a positive effect on L-Arg accumulation.

The analysis results of of other amino acid produ-ion significantly changing in different *C. crenatum* trains after 120 h of fermentation were shown in Table 2. A high alanine production was detected in the wild-type strain, whereas the MT and the MT. sp strains exhibited a significant decrease in alanine production from 2.40±0.06 mg/mL to 0.69±0.03 mg/mL ($P<0.05$) and 0.48±0.02 mg/mL (P<0.05), respectively, which was analyzed to be beneficial to accumulate arginine because of decrease of alanine production for reducing the consumption of glutamate and pyruvate. Four amino acids including glycine, lysine, glutamate, and proline increased significantly in the MT strain compared with the wild-type strain (P<0.05). Among the above produced amino acids, glutamate production in *C. crenatum* MT. sp exhibited a remarkable decrease (P<0.05) compared with MT strain and showed no difference (P>0.05) with the wild-type strain, which indicated that the *argR* disruption in *C. crenatum* strain was beneficial to the glutamate accumulation. Lysine production in *C. crenatum* MT. sp and *C. crenatum* MT increased by over more than 10-fold from 0.15±0.02 mg/mL in the wild-type strain to 1.68±0.05 mg/mL and 2.06±0.06 mg/mL, respectively. The MT strain also exhibited a remarkable increase (P<0.05) in proline production from 0.63±0.03 mg/mL in the wild-type strain to 5.24±0.13 mg/mL. In the amino acid biosynthetic network, glutamate is the common precursor of indicate

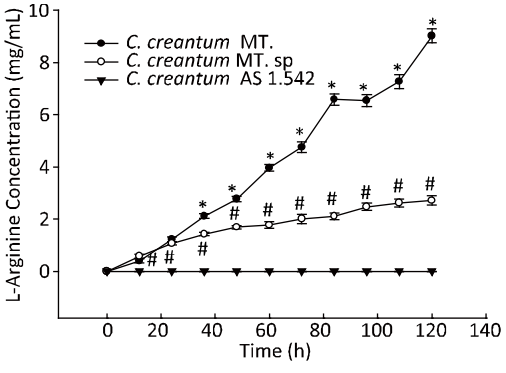

Figure 3. Growth characterization of *Corynebacterium crenatum* strains. Values are means±S.E.M., n=3. Cell growth did not differ ($P>0.05$) between *C. crenatum* MT and *C. crenatum* MT. sp during a 120 h fermentation. *: L-Arg production differed ($P<0.05$) between *C. crenatum* MT and *C. crenatum* MT. sp at the same fermentation time point (36-120 h). #: L-Arg production differed ($P<0.05$) between *C. crenatum* MT. sp and *C. crenatum* AS 1.542 at the same fermentation time point (12-120 h).

Table 2. The Extracellular Amino Acids Concentrations in the Fermentation of *C. crenatum* MT, *C. crenatum* MT. Sp, and *C. crenatum* AS 1.542

Amino Acid (mg/mL)	*C. crenatum* MT	*C. crenatum* MT. sp	*C. crenatum* AS 1.542
Ala	0.69±0.03	0.48±0.02[c]	2.40±0.06[a]
Lys	2.06±0.06	1.48±0.05[c]	0.15±0.02[a]
Pro	5.24±0.13	5.03±0.10	0.63±0.03[a]
Glu	0.90±0.04	0.12±0.02[c]	0.19±0.02[b]

Note. Values are means±S.E.M., n=3. The extracellular amino acids concentrations were analyzed in the 120 h fermentation time. [a]Amino acid production differed ($P<0.05$) between *C. crenatum* AS 1.542 and other two strains. [b]Amino acid production in *C. crenatum* AS 1.542 differed ($P<0.05$) with that in *C. crenatum* MT, while amino acid production in *C. crenatum* AS 1.542 did not differ ($P>0.05$) with that in *C. crenatum* MT. sp. [c]Difference ($P<0.05$) did exist in amino acid production between *C. crenatum* MT and *C. crenatum* MT. sp.

L-Arg synthesis and proline synthesis branch[17]. Lysine synthesis begins with oxaloacetate of the tricarboxylic acid (TCA) cycle and glutamate from α-ketoglutarate of the TCA cycle[18]. Therefore, blocking the proline and lysine biosynthetic pathways may be beneficial to further improve L-Arg production.

Variation of Genes in the Arginine Biosynthetic Pathway

The point mutation found in the *argR* gene of *C. crenatum* MT is the replacement of Gln-37 by a stop codon. This variation made the mutational *argR* gene express a truncated protein containing only 36 amino acid residues, which was not able to form a functional domain for DNA binding. Therefore, the incomplete ArgR protein was unable to bind to the regulatory regions of the genes regulated by ArgR.

A RT-qPCR assay was employed to determine whether the related genes involved in the arginine biosynthetic pathway were regulated at the transcriptional level in different *C. crenatum* strains. As reported previously, the genes involved in the arginine biosynthetic pathway in *C. crenatum* were located in the *argCJBDFR*, *argGH*, and *carAB* operons[4]. The *argGH* operon is situated adjacent but not linked to the major cluster of the *argCJBDFR* operon, whereas the *carAB* operon is not near to the major clusters[19]. The adjacent genes *carA* and *carB*, which constitute an operon oriented from A to B, encode the small and large subunits, respectively. The former subunit carries a glutamate-binding site, whereas the latter subunit contains the catalytic site of carbamate-phosphate synthase, which catalyzes the synthesis of carbamoyl phosphate from NH_3. Carbamoyl phosphate is one of the precursors of the arginine pathway[20]. Thus, seven genes from the aforementioned three operons were selected for our gene transcription study: *argC*, *argJ*, *argB*, *argD*, *argF*, *argG*, and *carA*. The transcriptional levels of these genes in wild-type AS 1.542 were used as the baseline. They were found to be down-regulated by 117.3-, 132.5-, 119.0-, 100.7-, 96.3-, 4.9-, and 4.5-fold, respectively, in *C. crenatum* MT. sp compared with those in *C. crenatum* MT (Figure 4). As expected, the expression level of the house-keeping gene 16S rRNA was unchanged in the three strains. These results further confirm that the ArgR protein regulates the transcription of genes participating in arginine biosynthesis and acts as a negative regulator in the arginine biosynthetic pathway, which coincide with the phenotype of improved L-Arg production in *C. crenatum* MT with truncated ArgR protein.

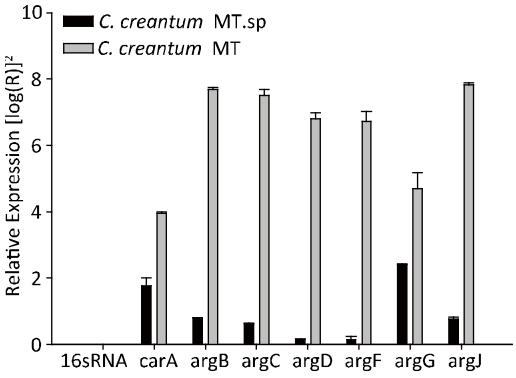

Figure 4. Regulation of genes involved in arginine biosynthesis in *Corynebacterium crenatum* strains. Transcriptional levels of the 16S rRNA (internal control), *argB*, *argC*, *argD*, *argF*, *argJ*, *argG*, and *carA* genes were determined by real-time qPCR analysis (values are means±S.E.M., *n*=3) and differed (*P<0.05*) between *C. crenatum* MT and *C. crenatum* MT. sp at 20 h incubation time point.

Our data suggest that the *argC*, *argJ*, *argB*, *argD*, and *argF* genes located in the same operon have approximately equally high transcription levels in *C. crenatum* MT. The depression of this gene cluster was clearly evident with induction greater than 95-fold upon ArgR disruption. Although the *argG* gene, as well as *carA* gene, was affected to a lesser degree by ArgR, they were still down-regulated by approximately fivefold (>threefold) in *C. crenatum* MT. sp This result is different from that in *C. glutamicum*, in which the *argG* gene did not respond to ArgR overexpression[21].

Verification of ArgR Binding with Promoters of Genes in the Arginine Biosynthetic Pathway

EMSAs were conducted to study the interaction between ArgR and the *argCJBDFR*, *argGH*, and *carAB* operons, to verify whether the regulation of ArgR on arginine biosynthesis was direct or indirect at the transcriptional level, and to identify the specificity of this regulation. Three probes carrying putative 'ARG boxes'[22] in the promoter regions of the *argCJBDFR*, *argGH*, and *carAB* operons in *C. crenatum* were separately amplified by PCR and subjected to gel retardation experiments. As shown in Figure 5, hints for strong ArgR binding with the upstream of *argCJBDFR* and relatively weak ArgR binding with the upstream of *argGH* and *carAB* were found. The results

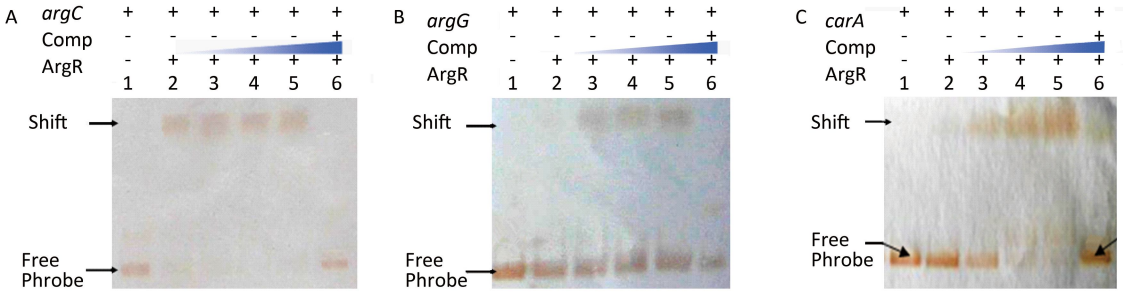

Figure 5. Binding of the ArgR protein to the promoter regions of operons involved in arginine biosynthesis in *Corynebacterium crenatum* by electrophoretic mobility shift assays (EMSAs). (A) EMSA with the upstream region probe of the *argC* gene; (B) EMSA with the upstream region probe of the *argG* gene; (C) EMSA with the upstream region probe of the *carA* gene. (Lane 1, control assay without ArgR protein; lane 2-6, DNA band shift assay with ArgR protein, and 'Comp' added in lane 6 indicates the unlabeled promoter fragments for competition with the labeled probes).

indicate that the *argCJBDFR*, *argGH*, and *carAB* promoters can be directly bound by the ArgR protein. Arginine as corepressor should be added in EMSAs for helping ArgR binding with DNA, but we found that whether or not agrinine added in binding buffer did not affect the binding ability of ArgR, which was consistant with the results reported by Ghochikyan et al.[23]. This study is first to show that ArgR binds the upstream of *argC*, *argG,* and *carA* in *C. crenatum*, although specific ArgR-binding sequences on the *arg* operon were not identified.

Compared with random mutagenesis, a notable characteristic of pathway engineering is its directionality with remarkable advantages in modification of target of interest, experiment design, and data analysis. However, the special qualities of wild-type strains isolated from nature and species with excellent genetic traits screened by traditional mutagenesis program are the important information resources that provide theoretical basis for pathway design and target selection. In the present study, *C. crenatum* AS 1.542 via random mutagenesis was changed significantly to accumulate L-Arg. These changes in protein properties involved in L-Arg biosynthesis will provide useful information and guidance for employing genetic engineering approaches to improve the performance of *C. crenatum*.

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