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ORIGINAL PAPER

Purification and characterization of an arginine regulatory protein, ArgR, in *Corynebacterium glutamicum*

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Abstract *Corynebacterium glutamicum*, a Gram-positive bacterium, has been widely used for industrial amino acid production. We previously showed that, in *C. glutamicum*, *argCJBDFRGH* arginine biosynthesis genes are clustered but independently transcribed from *argC* and *argG* promoters, leading to the generation of two transcripts corresponding to *argCJBDFR* and *argGH*. In this report, we show the effect of the *C. glutamicum* ArgR repressor on *argC* and *argG* promoters by overexpressing or disrupting the *argR* gene. Gel filtration assay results indicate that native ArgR is a hexamer of equal subunits with molecular mass of 110 kDa. Protein sequence analysis revealed the presence of an "SR" (Ser_{57}-Arg_{58}) motif for the DNA binding site at the N-terminal region and the "GTIAGDDTV" motif for arginine binding and its oligomerization at the C-terminal region. An *argC* or *argG* promoter–*lacZ* fusion reporter assay and *argR* mutational analysis showed that transcription of the *argCJBDFR* arginine biosynthesis genes is regulated from the *argC* promoter by ArgR in cooperation with L-arginine in *C. glutamicum*. This finding was supported by the gel mobility–shift assay showing direct binding of hexameric ArgR to the *argC* promoter in the presence of L-arginine. Unexpectedly, *argGH* transcription was not responsive to the level of ArgR repressor and/or arginine. In a further study, a *C. glutamicum argR* mutant was constructed by disrupting the chromosomal *argR* gene to manufacture an improved arginine-producing strain. Arginine productivity was increased in the *C. glutamicum argR* mutant strain under conditions of both limited and excessive arginine.

Keywords *Corynebacterium glutamicum* · Arginine biosynthesis genes · Transcriptional regulation · ArgR transcriptional repressor

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Introduction

Corynebacterium glutamicum, a high-GC Gram-positive bacterium, is widely used as an industrial producer of amino acids [2, 22]. Because of its economic value, much effort has been made to elucidate the molecular biology of this organism [16, 18, 20, 25]. In particular, the regulatory systems of amino acid biosynthesis genes have been targeted to design engineered strains with improved amino acid production. In spite of extensive studies on transcriptional regulation of arginine biosynthesis genes in many bacteria, little information is available regarding *C. glutamicum*.

Arginine biosynthesis genes have been characterized in many microorganisms. In prokaryotes, arginine is synthesized from glutamate through eight enzymatic steps (Fig. 1a) [7]. The first four steps involve *N*-acetylated intermediates, beginning with acetylation of glutamate and ending with deacetylation of *N*-acetylornithine. Two

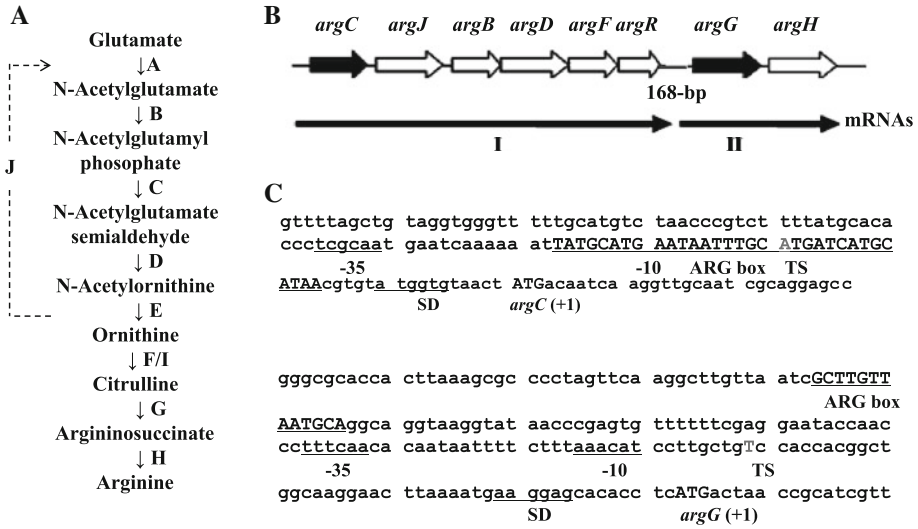

Fig. 1 Characterization of arginine biosynthetic genes. **a** L-Arginine biosynthesis pathway. The genes encode the following enzymes: *A*, *N*-acetylglutamate synthetase (*argA*). *B*, *N*-acetylglutamate kinase (*argB*). *C*, *N*-acetylglutamate 5-semialdehyde reductase (*argC*). *D*, *N*-acetylornithine aminotransferase (*argD*). *E*, *N*-acetylornithinase (*argE*). *F/I*, ornithine transcarbamylase (*argF*, *argI*). *G*, argininosuccinate synthetase (*argG*). *H*, argininosuccinate lyase (*argH*). *J*, ornithine acetyltransferase (*argJ*). The biosynthesis steps from glutamate to arginine are shown in order. **b** Genetic organization of the arginine biosynthetic genes in *C. glutamicum*. Open reading frames (ORFs) are indicated by *open* and *closed arrows*. *Thick black solid arrows* correspond to the transcripts of the arginine biosynthesis genes. **c** Genomic structure of the *argC* and *argG* promoter regions. DNA sequences show the upstream and downstream regions of the *argC* and *argG* translational start site (ATG). TS represents transcription start site in *argC* and *argG* promoter regions, which are indicated with *gray color*. The putative ArgR repressor binding region, an "*ARG box*," is represented with *upper-case letters*. The putative ribosome-binding sites (SD) are *underlined*. Translation start codons (ATG) are indicated in *upper and boldface type*. *Numbers* indicate the relative positions of the nucleotides upstream or downstream of the *argC* and *argG* genes

alternative routes have evolved in subsequent steps. Some bacteria (e.g., *Enterobacteriaceae*, *Vibrionaceae*, *Myxococcus xanthus*, and the archaebacterium *Sulfolobus solfataricus*) use a linear pathway characterized by acetylornithinase that hydrolyzes *N*-acetylornithine to acetate and ornithine [7, 13, 41, 42]. In these organisms, *N*-acetylglutamate synthase, the first enzyme in the pathway, is the target of feedback inhibition by arginine. Other bacteria (e.g., *Neisseria* spp. and *Streptomyces* spp.) including *Corynebacterium* use a more economical *N*-acetyl cycle that involves ornithine acetyltransferase [40]. This enzyme catalyzes the transfer of the acetyl group from *N*-acetylornithine to glutamate, and the resulting *N*-acetylglutamate is cycled back into arginine biosynthesis (Fig. 1a). Thus, ornithine acetyltransferase allows microorganisms to bypass the first step that acetylates glutamate from acetyl-CoA by *N*-acetylglutamate synthetase in a linear pathway [32, 40] (Fig. 1a).

Regarding the genetic organization of arginine biosynthesis genes in chromosomal DNA, the arginine biosynthesis genes are scattered throughout the chromosome in some bacteria (e.g., *Pseudomonas* spp. [12] and *Cyanobacterium* spp. [9]), while they are clustered in other bacteria (e.g., *M. tuberculosis* [6] and *C. glutamicum* [43]). Despite this difference in genetic organization of arginine biosynthetic genes among microorganisms, their transcriptional regulation is surprisingly similar. First, there is no evidence for transcription attenuation control of arginine biosynthetic genes, which is different from most amino acid biosynthesis [7]. Second, the arginine biosynthesis pathway is controlled by the end-product arginine through feedback inhibition [7, 31]. Third, the transcription of arginine biosynthetic genes is generally mediated by the ArgR protein, an L-arginine-dependent DNA binding protein that acts as a transcriptional repressor of the arginine regulon [1, 23]. The ArgR repressors have been extensively studied in several bacteria. Among them, in the most extensively studied species, *E. coli*, ArgR repressor is a hexamer with molecular weight of 98 kDa and binds to two 18-bp-long palindromic regions called "ARG boxes" in the promoter regions of *argA*, *argD*, *argG*, *argECBH*, *carAB*, *argF*, and *argR* genes [3, 38]. ArgR then represses their expression under conditions of excessive arginine [7]. The consensus sequence of all known ARG boxes and its inverse in *E. coli* is "aNTGAATaattATTCANt," an 18-base-pair-long palindromic region [3]. It also has an "SR" motif, Ser-47 and Arg-48, known as a DNA binging site in its N-terminal region [11]. According to mutational analysis of *E. coli* ArgR, single amino acid substitutions of residues 47 or 48 of the *E. coli* ArgR result in decreased binding of repressor to the DNA [4, 39]. This "SR" motif is well conserved in many bacterial ArgR proteins. In

addition, the eight-amino-acid motif "GTIAGDDTL/I" region located in the C-terminal region is also well conserved in all known arginine repressors. This region is known as the arginine binding domain, and in particular, the "DD" residues are known to be sufficient for oligomerization of ArgR proteins.

In addition to its role as a repressor in arginine biosynthetic gene expression, ArgR protein is involved in many different cellular processes; for example, ArgR plays a vital role in arginine catabolism in *P. aeruginosa* under aerobic conditions, which provides the cell with carbon, nitrogen, and energy [12, 29, 30]. ArgR also activates the *aotJQMOPargR* operon that encodes a system for uptake of arginine and ornithine in *P. aeruginosa* [28]. The regulation protein AhrR, which has ArgR function in *Bacillus subtilis*, activates the *rocABC* and *rocDEF* operons of the arginine deaminase pathway [10]. ArgR also acts as an activator for arginine catabolic genes [10, 17, 28]. Furthermore, ArgR is involved in the initial pairing of the recombination sites [1, 14]. As stated above, ArgR plays multifunctional roles in the cell. These unusual features motivated us to examine the function of ArgR protein in *C. glutamicum*.

In this study, we report characterization of the *C. glutamicum* ArgR repressor and its role in transcriptional regulation of arginine biosynthetic gene expression in *C. glutamicum*.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are described in Table 1. *E. coli* and *C. glutamicum* AS019 strains were grown in luria bertani (LB) as a rich medium at 37°C. As a minimal medium, *E. coli* cells were grown in M9 synthetic medium [27] at 37°C and *C. glutamicum* cells were grown in basal medium for *Corynebacterium* growth (BMCG) [22] or minimal medium XII for *C. glutamicum* (CGXII) [19] at 30°C in the absence or presence of arginine (1 mg/ml). All three of these are defined media based on glucose and ammonia. All plasmids were propagated in *E. coli* DH5α grown in LB medium. When needed, supplements were added to the following final concentrations: ampicillin (50 μg/ml), kanamycin (25 or 50 μg/ml), isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM).

General DNA techniques and transformation

DNA manipulations were conducted using general molecular biological techniques [33]. T4 DNA ligase, Taq polymerase, and restriction endonucleases were purchased from New England BioLabs (NEB) or Boehringer Mannheim Biochemicals (BMB). DNA fragments were separated on 0.8% agarose gels and isolated using a Qiagen extraction kit (Qiagen, Korea). Plasmid DNA was isolated with the Qiagen plasmid purification kit (Qiagen, Korea). *E. coli* and *C. glutamicum* strains were transformed using the $CaCl_2$ or electroporation method. All recombinant plasmids were sequenced at the Solgent sequencing facility (Solgent, Korea). Primers used in this study were synthesized by Bioneer (Bioneer, Korea) and are listed in Table 1.

Computer analysis

Nucleotide and amino acid sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple protein sequence alignments were conducted with the CLUSTAL W programs [37]. Computer analyses of nucleotide and amino acid sequences were done by using the DNAMAN program or Protein Analysis Tools on the ExPASy world wide web molecular biology server of Geneva University Hospital and University of Geneva.

Construction of *argC* or *argG* promoter–*lacZ* fusion plasmids (pPromC and pPromG) and ArgR expression vector (pET-RR)

The promoter probe vector, pRS415A, was used to estimate the strength of *argC* and *argG* promoters. The *argC* and *argG* promoter regions in pARG11 and pARG25 plasmids were digested and cloned upstream of the promoter-less *lacZ* reporter gene of pRS415 generating pPromC and pPromG, respectively (see Table 1 for more information). Transcriptional activities of *argC* and *argG* promoters were checked by measuring β-galactosidase enzyme activities. To characterize *C. glutamicum* ArgR repressor, the *argR* gene was amplified by polymerase chain reaction (PCR) from the pARG25 plasmid using the argR-F and argR-R primers (Table 1). The PCR products were digested with *Eco*RI and *Hin*dIII restriction enzymes and ligated into the pET28a(+) plasmid, which generates pET-RR plasmid.

Overexpression and purification of ArgR protein

E. coli BL21(DE3)pLysS cells were transformed with pET-RR and grown at 37°C to exponential growth phase in LB medium containing kanamycin (25 μg/ml). ArgR overexpression was induced by addition of 1.0 mM IPTG at $OD_{600} = 0.5–0.6$ for indicated times. The cells were harvested and washed with 50 mM Tris–Cl (pH 8.0). The collected cells were resuspended in 150 μl ice-cold "Arg buffer" [20 mM Tris–Cl (pH 7.5), 10 mM $MgCl_2$, and

Table 1 Bacterial strains, plasmids, and primers used in this study

	Relevant phenotype or genotype[a]	Source or reference
Strain		
C. glutamicum		
CG-RRW	ASO19, Spontaneous rifampicin resistant mutant of ATCC 13059	
CG-RRM	Like CG-RRW except *argR*$^-$ mutant (*argR*::pIRR-1); Km^R	This study
E. coli		
DH5α	*supE44 Δ(lacU)169(ΦlacZΔM15)hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	
BL21(DE3)pLysS		
S17-1	*hsdR pro recA* carrying RP4-2-TC::Mu in the chromosome	[36]
Plasmid[b]		
pMT1	Parental cloning shuttle vector; Ap^R (*E. coli*), Km^R (*C. glutamicum*)	[21]
pARG11	pMT1 with 5.8 kb insert carrying *argCJBDFR*; Ap^R	[43]
pARG25	pMT1 with 6.9 kb insert carrying *argBDFRGH*; Ap^R	[43]
pRS415	Promoter-less *lac* operon fusion vector; Ap^R	[43]
pPromC	pRS415 with 1.6 kb *Hin*dIII-*Kpn*I fragment carrying upstream of *argC*; Ap^R	This study
pPromG	pRS415 with 1.7 kb *Eco*RI-*Bam*HI fragment carrying upstream of *argG*; Ap^R	This study
pET28a(+)	Expression vector; Km^R	Novagen
pET-RR	pET28a(+) with 950 bp *Eco*RI-*Hin*dIII fragment carrying *argR*; Km^R	This study
pSL18	Expression vector; Km^R	Novagen
pIRR-1	pSL18 carrying 277 bp integral *argR* fragment; Km^R	This study
Primer[c]		
argB-F	5′-GCCATGGTGGATGATGATC-3′	This study
argB-R	5′-GACCTGACCAAAGAGCACC-3′	This study
argC-F	5′-TTCAGGATCCACCTGAAGCTTACGCGCCACCTGTAC-3′	This study
argC-R	5′-TGTGGGATCCATTC ACCGAGAATTCTGCCTGCG GTT-3′	This study
argG-F	5′-CTGTGGCAATTCCATACCTG-3′	This study
argG-R	5′-CGTTTGCCTTGATGGTTGGC-3′	This study
argR-F	5′-GAGG***GAATTC***ATGTCCCTTGGCTCAACCCC-3′	This study
argR-R	5′-ACTG***AAGCTT***GTACATGCCGTTTGCCTTGA -3′	This study
argRD-F	5′-CAGC***GGTACC***CAACTGTCTGAATTG-3′	This study
argRD-R	5′-CACC***TCTAGA***ATGAAACTTGCCAGG-3′	This study

Additional information is given in "Materials and methods"

[a] Superscript "R" indicates resistance to the following antibiotics: *Ap* ampicillin, *Km* kanamycin

[b] All plasmids have been sequenced

[c] Restriction enzyme sites are represented as italic and bold characters. Abbreviation "F" and "R" represents forward and reverse, respectively

10 mM β-mercaptoethanol]. One hundred fifty microliters of 2× sodium dodecyl sulfate (SDS) gel-loading buffer [100 mM Tris–Cl (pH 6.8), 200 mM dithiothreitol (DTT), 3% SDS, 1.2% bromophenol blue, and 20% glycerol] was then added to the samples. Cells were disrupted by vigorous vortexing for 3 min on ice. Protein samples from all stages were denatured by heating (100°C, 5 min) and then centrifuged (10,000×*g*, 10 min) at room temperature. The supernatants were transfered to clean tubes. The overexpressed ArgR protein was checked by SDS–polyacrylamide gel electrophoresis (PAGE) using a 10–15% running gel, 5% stacking gel that was stained with Coomassie brilliant blue after electrophoresis. The overexpressed recombinant ArgR repressor was purified using Ni^{2+}-affinity chromatography (Novagen, Korea). *E. coli* BL21(DE)pLysS cells carrying the pET-RR plasmid were cultured and induced with 1 mM IPTG at OD_{600} = 0.5–0.6 followed by incubation for 3 h. Cells were centrifuged at 5,000×*g* for 5 min, resuspended in 4 ml ice-cold 1× binding buffer [5.0 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl (pH 7.9)] and disrupted in a FISHER sonic dismembrator model 300 for 20 min on ice. The extracts were centrifuged at 39,000×*g* for 30 min to remove debris. The supernatant was applied to a Novagen His-Bind resin column, and the column was washed first with 25 ml 1× binding buffer, and then with 15 ml 1× washing buffer [60 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl (pH 7.9)]. The bound ArgR proteins

were eluted with 15 ml elution buffer [100 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl (pH 7.9)]. The protein concentration was measured by a Bradford protein assay.

Gel filtration assay

The molecular mass of native ArgR was determined using a gel filtration assay on a Superose-12 prep grade column. Proteins of known molecular mass were used as molecular markers: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), phosphorylase b (97.4 kDa), and bovine serum albumin (67 kDa). All steps were carried out at a cold room (4°C). Approximately 2–3 μg pure proteins in 200 μl Arg buffer was loaded on a Superose column. The ArgR proteins were eluted with Arg buffer containing 0.15 M NaCl and monitored by SDS–PAGE.

In vitro gel retardation

DNA–protein complexes were examined by gel retardation assays on 1.5% agarose gels. A 400-bp fragment corresponding to the upstream of the *argC* gene was prepared by PCR amplification from pARG11 using argC-F and argC-R primers (Table 1). One hundred nanograms of 400-bp DNA fragment was incubated with 7 μg crude proteins for 30 min at 37°C in 20 μl binding buffer [50 mM Tris–Cl (pH 8.0), 250 mM NaCl, 5.0 mM $MgCl_2$, 2.5 mM DTT, 2.5 mM ethylenediamine tetraacetic acid (EDTA), and 20% glycerol] with arginine (10 mM or 30 mM). Electrophoresis was carried out at 50 V with 0.5× tris-acetate-EDTA (TAE) buffer.

Construction of *C. glutamicum argR* mutant (CG-RRM) and transformation of *C. glutamicum*

To make a CG-RRM strain, a pIRR-1 plasmid was constructed by inserting a 277-bp internal *argR* fragment (115–392 residues) into pSL18 (Table 1). The internal *argR* fragment was amplified by PCR from the pET-RR plasmid using argRD-F and argRD-R primers (Table 1). The pIRR-1 plasmid was then introduced into a mobilizing strain of *E. coli* S17-1 by electroporation. *E. coli* S17-1 carries an RP4 derivative integrated into the chromosome, which provides the necessary transfer functions for mobilization. The plasmid mobilization from *E. coli* to *C. glutamicum* was performed by conjugation [34], and transconjugants were selected on a LB agar plate containing kanamycin (50 μg/ml) and nalidixic acid (30 μg/ml). pIRR-1 was inserted into the chromosomal DNA of *C. glutamicum* by single crossover recombination, resulting in CG-RRM [35]. The recombination was confirmed by performing Southern hybridization and PCR.

RT-PCR analysis

Total RNA was purified using a MasterPure™ RNA purification kit (Epicentre, Madison, WI). Equal quantities of total RNA (6 μg) were reverse-transcribed into complementary DNA (cDNA) by using a reverse-transcription (RT)-PCR kit (Bioneer, Korea) and Superscript II reverse transcriptase (Invitrogen, Korea). Transcribed cDNAs were used as a template for the PCR amplification reaction using primer pairs argB-F/-R and argG-F/-R (Table 1) with 23 cycles of amplification. RNA pol was used as an internal control to monitor the relative level of each transcript in CG-RRW and CG-RRM.

Measurement of arginine biosynthesis in *C. glutamicum*

For analysis of arginine biosynthesis, *C. glutamicum* was grown in an amino acid fermentation medium, CGXII containing 2% glucose at 30°C for 16 h. Overnight culture of *C. glutamicum* was harvested and washed with 0.9% NaCl, and cells were transferred to CGXII medium to give 1.0 initial optical density at 600 nm. Cells were grown at 30°C and 250 rpm. A high-performance liquid chromatography system (LC 1090; Hewlett-Packard, Avondale, CA) was used for quantitative determination of arginine biosynthesis.

Results and discussion

Nucleotide sequence analysis of the *argC* and *argG* promoter regions

We previously showed that the arginine biosynthesis genes of *C. glutamicum* are clustered on the chromosomal DNA in the order *argCJBDFRGH* [5, 43]. However, *argCJBDFR* and *argGH* are independently transcribed from two promoters in front of the *argC* and *argG* genes, respectively [43] (Fig. 1b). Figure 1c shows the upstream and downstream regions of *argC* and *argG* translational start sites (ATG). Although no experimental transcriptional studies have been carried out, our bioinformatic analysis of the nucleotide sequence revealed the putative -10 and -35 regions, Shine Dalgarno (SD) sequences, and ATG start codons of the *argC* and *argG* genes (Fig. 1c). The transcription start sites (TS) of the *argCJBDFR* and *argGH* transcripts, "A" and "T" residues, are indicated with gray-colored characters [43]. The "A" and "T" residues are located 30 nt and 44 nt upstream of the *argC* and *argG* translation start sites, respectively. When the transcriptional element search software (TESS, http://www.cbil.upenn.edu/tess/techreports/1997/CB) program was applied to the

upstream sequences of the *argC*, *argG*, and *argR* genes to examine the presence of transcriptional regulator binding motif(s), a single ARG box was detected in the *argC* and *argG* promoter regions but not in the *argR* promoter. The predicted ARG box of the *argC* gene is "TATGCATG AATAATtgcATGATCATGCAATA," corresponding to nucleotides 48 through 17 nucleotides upstream of the *argC* translational start site (Fig. 1c). It displayed a characteristic AT-rich sequence, as is typically the case for the consensus sequences of various ARG boxes. The predicted ARG box of the *argC* gene resembles those of arginine-regulated genes in *E. coli*, *S. clavuligerus*, and *B. stearothermophilus*. In particular, a relatively higher similarity was found with the sequence of the *S. clavuligerus argC* ARG box (Fig. 2). It was revealed that the *argG* gene also contains a single putative ARG box ("GCTTGTTAATGCA") (Fig. 1c). However, the ARG box in the *argG* promoter appears to have very weak similarity with other conserved ARG box motifs (Fig. 2). In contrast to the situation in *E. coli*, *C. glutamicum argR* is not preceded by an ARG box, suggesting that expression of *argR* may not be autoregulated. These results correspond to our finding that two transcripts of *argCJBDFR* and *argGH* exist in arginine biosynthesis of *C. glutamicum*.

Amino acid sequence analysis of *C. glutamicum* ArgR protein

According to the computer analysis, the ORF of *argR* consists of 516 nucleotides with total G + C content of 57.9%. The ArgR protein contains 171 amino acids with molecular mass of 18.5 kDa. The amino acid sequence of *C. glutamicum* ArgR was aligned with those from four other bacteria. *C. glutamicum* ArgR showed relatively high similarity of 51% to ArgR protein from *M. tuberculosis*, 50% to *S. clavuligerus*, 35% to *B. stearothermophilus*, and 30% to *E. coli* (Fig. 3). According to the amino acid sequence, the *C. glutamicum* ArgR monomer appears to have two highly conserved motifs: the "SR" motif for DNA binding (residues 57–58) in the N-terminal region and the "GTIAG**DD**TV" motif for arginine binding (residues 141–149) and oligomerization (residues 146–147) in the C-terminal region (Fig. 3).

Characterization of *C. glutamicum* ArgR protein

ArgR protein was overexpressed in *E. coli* BL21(DE3)-pLysS and identified on SDS–PAGE. The synthesized his-tagged ArgR protein has molecular weight of about 23 kDa, which includes 4.0 kDa for the pET28a vector

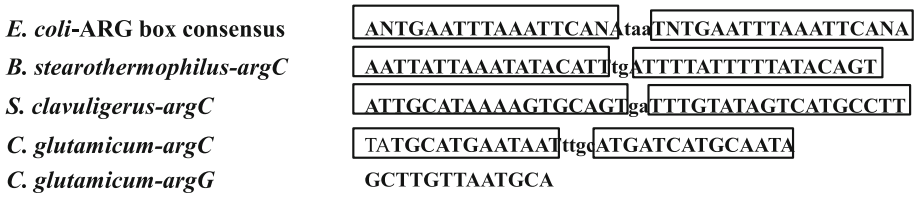

Fig. 2 Alignment of arginine repressor binding sites. *E. coli* consensus [3], the *B. stearothermophilus argC* ARG box [15], and the *S. clavuligerus argC* ARG box [31] are shown. *Rectangles* indicate putative or actual ArgR repressor binding regions

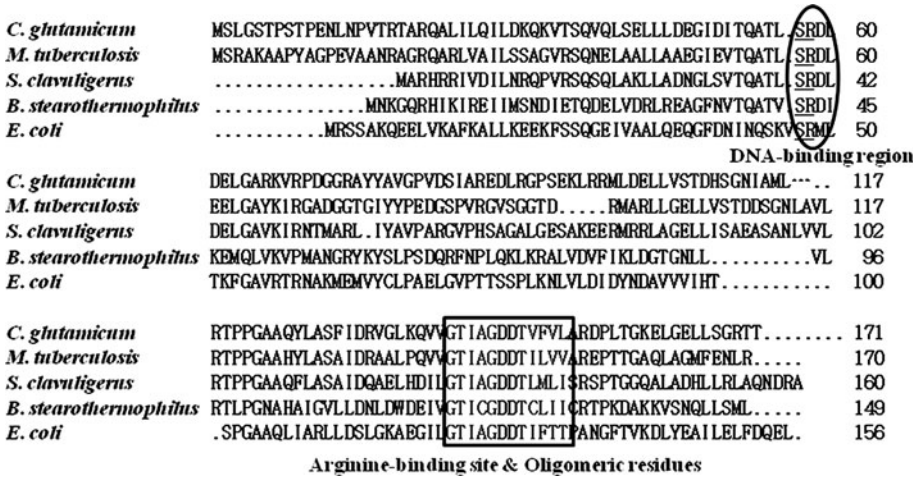

Fig. 3 Amino acid sequence alignment between the ArgR protein from *C. glutamicum* and those from other bacteria. The consensus amino acids for the DNA-binding region, "SR" motifs, are indicated within a *circle*. The box indicates the consensus amino acids for the arginine binding site, including the "DD" residues needed for its oligomerization

region (Fig. 4). This is consistent with the predicted size, 18.5 kDa, of the ArgR repressor. The overexpressed ArgR protein was purified using a Novagen His-Bind resin column, and the purified protein was used for a gel filtration assay to estimate the molecular mass of the native protein. Our gel filtration assay showed that the molecular mass of the native ArgR protein was 110 kDa. This result is consistent with a hexamer of identical 18.5-kDa subunits. This configuration is similar to hexameric structures found in several bacteria; for example, active ArgR repressors are a hexamer of 98 kDa in *E. coli* K12 [23], 100.2 kDa in *B. subtilis* [26], 100 kDa in *B. licheniformis* [24], and 103 kDa in *S. clavuligerus*. These hexameric ArgR proteins are distinguished from those of most other prokaryotes, which are typically dimers, and occasionally trimers and tetramers; for example, active ArgR repressors of *P. aeruginosa* PAO1 and *B. stearothermophilus* behave as dimers (74 kDa) and trimers (48 kDa) of the same subunits, respectively [8, 29].

Negative effect of ArgR protein on *argC* promoter activity

In many microorganisms, the arginine biosynthesis pathway is regulated by arginine and ArgR at the transcriptional level. Therefore, the effect of ArgR on the promoter activities of the *argC* and *argG* genes, the first genes of the *argCJBDFR* and *argGH* transcripts, were analyzed using an ArgR-overexpressing vector (pET-RR) and *argC* or *argG* promoter–*lacZ* fusion plasmids (pPromC and pPromG). Under conditions of limited arginine (e.g., arginine-free M9 media), *E. coli* cells with pPromC vector only showed relatively high levels of *β*-galactosidase activity, while ArgR-overexpressing *E. coli* strains bearing both pPromC and pET-RR plasmids showed 85% decrease in *β*-galactosidase activity (142.8 to 22.8 *μ*/mg protein) (Fig. 5). The addition of 50 mM arginine to the medium caused an additional 34% decrease in the *β*-galactosidase level in *E. coli* strains containing the pPromC and pET-RR plasmids (22.9 to 15.2 *μ*/mg protein) (Fig. 5). Taken together, our results suggest that *argCJBDFR* gene expression is strongly dependent on the ArgR repressor. In *E. coli* cells bearing pPromG, the *β*-galactosidase level was hardly affected by ArgR protein and/or arginine (Fig. 5). This indicates that ArgR exerts a negative effect only on the *argC* promoter but not on the *argG* promoter in *C. glutamicum*.

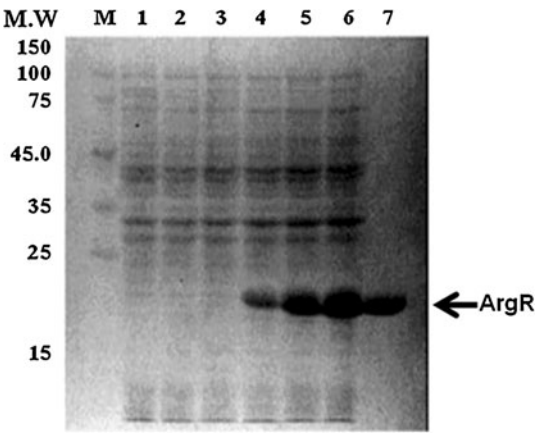

Fig. 4 Overexpression and purification of the ArgR protein. *lane M* size marker, *lane 1* uninduced cells of pET28a(+), *lane 2* uninduced cells of pET-RR, *lane 3* 1-h-induced cells of pET28a(+) with IPTG, *lane 4* 1-h-induced cells of pET-RR with IPTG, *lane 5* 2-h-induced cells of pET-RR with IPTG, *lane 6* 3-h-induced cells of pET-RR with IPTG, *lane 7* ArgR protein purified by His-Bind resin column

In vitro binding of ArgR protein to the *argC* promoter

To investigate the specific interaction of the ArgR repressor with the *argC* promoter in *C. glutamicum*, a gel retardation experiment was performed with protein extract from cells containing the ArgR expression vector (pETRR) and a 400-bp PCR product carrying a putative ARG box and the initial part of the *C. glutamicum argC* gene. The 400-bp DNA probe and protein extract were incubated together and subjected to electrophoresis on a 1.5% agarose gel followed by ethidium bromide (EtBr) staining. No shift was observed upon analysis of a binding reaction mixture containing the 400-bp DNA probe and protein extract from cells with empty vector (pET28a was used as a negative control) (lane 1, Fig. 6). In contrast, the migration of the 400-bp DNA probe was significantly decreased in the reaction mixture with cell extract from cells containing the ArgR expression vector, pETRR, in the presence of arginine (10 and 30 mM) (lanes 2 and 3, Fig. 6). This result suggests that the *argC* promoter region is shifted by the ArgR protein and that it forms a single DNA/ArgR

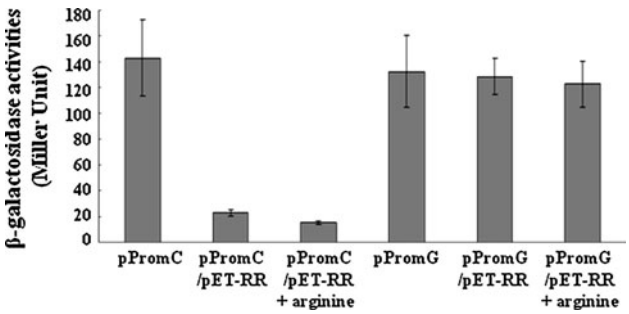

Fig. 5 *β*-Galactosidase activities in *E. coli* cells carrying *argC* or *argG* promoter–*lacZ* fusion plasmids (pPromC or pPromG) and/or ArgR-expressing vector (pET-RR). Cells were grown in M9 medium supplemented with or without 0.5% L-arginine. One unit of activity was defined as the amount of enzyme that hydrolyzes 1 mmole *ortho*-nitrophenyl-*β*-galactoside (ONPG) in 1 min at 30°C. Values are means ± standard deviation of three independent enzyme assays

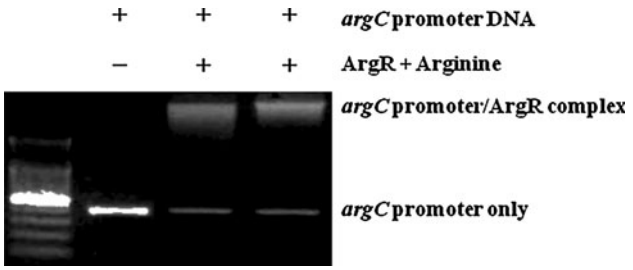

Fig. 6 Gel shift assay to examine binding of crude ArgR to *argC* and *argG* promoter region DNA. Crude extract was prepared from BL21/pET28a and BL21/pETRR. *Lane M* DNA size marker, *lane 1 ArgC* promoter DNA and cell extract from cells carrying the empty vector, pET28a with 30 mM arginine, *lane 2 ArgC* promoter DNA and cell extract from cells carrying pETRR, with 10 mM arginine, *lane 3 ArgC* promoter DNA and cell extract from cells carrying pETRR, with 30 mM arginine

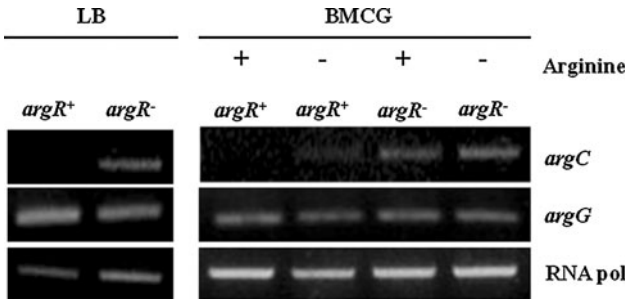

Fig. 7 Comparison of *argC* and *argG* gene expression in *C. glutamicum argR* wild-type (*argR*$^+$, CG-RRW) and mutant (*arg*$^-$, CG-RRM) cells using RT-PCR analysis with 23 cycles of amplification. Cells were grown in LB at 37°C or in BMCG medium supplemented with or without 0.5% L-arginine. RNA pol was used as an internal control

complex. However, a complete shift of all of the probe was not observed with 7 μg total crude protein. This may indicate a lack of sufficient ArgR protein in the crude extract. Replacing 7 μg total crude protein with 3 μg purified ArgR protein resulted in a complete shift of the DNA probe band (data not shown). Using purified ArgR protein, some band shift was also found in absence of arginine, but the amount of shifted DNA was very small compared with that of shifted DNA in the presence of arginine (data not shown). This is consistent with the results of our in vivo β-galactosidase expression assays, which showed that *argC* promoter activity was repressed by ArgR even under conditions of limited arginine.

Considering these results, we therefore suggest that the ArgR repressor directly binds to the *argC* promoter, in which arginine increases its binding as a co-repressor.

Transcription from the *argC* and *argG* promoters in *C. glutamicum argR* wild-type and mutant strains

We have shown a negative effect of *C. glutamicum* ArgR on *argC* promoter activity in *E. coli*. This was further confirmed by using *C. glutamicum argR* wild-type (CG-RRW) and mutant (CG-RRM) strains. CG-RRM was constructed by disrupting the arginine binding site and oligomerization region of the chromosomal *argR* gene in *C. glutamicum*. The transcription levels from *argC* and *argG* promoters were analyzed using RT-PCR in CG-RRW and CG-RRM cells grown in LB or BMCG medium supplemented with or without arginine. In CG-RRW cells, *argC* gene expression was strongly repressed in the LB and BMCG media with excess arginine (1 mg/ml). However, it was also considerably repressed even in the BMCG medium without arginine relative to the CG-RRM strain (Fig. 7). In the case of CG-RRM cells, the *argC* gene expression was not decreased in any of the tested media (Fig. 7). Again, *argGH* transcription was unresponsive to the presence of the ArgR repressor and/or arginine (Fig. 7). This result is consistent with previous experiments involving cotransformation of pER-RR and pRomG into *E. coli* DH5α.

Taken together, our results suggest that ArgR represses expression of *argCJBDFR* genes but not of *argGH* genes at the transcriptional level in *C. glutamicum*.

Increased arginine productivity in CG-RRM

We have shown that ArgR exerts a negative effect on the *argC* promoter in *C. glutamicum*. We further investigated the effect of ArgR on arginine productivity in *C. glutamicum*. For this purpose, the levels of arginine productivity in CG-RRW and CG-RRM strains were compared after these strains were grown in an amino-acid fermentation medium, CGXII. CG-RRM cells showed slower growth than that of CG-RRW cells but produced more arginine, with a maximum of 0.96 g/l arginine. CG-RRW cells produced almost no arginine under the same conditions (Fig. 8). Our data suggest that this approach may be applied to other arginine-producing bacteria to improve arginine productivity. We showed that disruption of the *argR* gene caused overexpression of the first transcript *argCJBDFR* but not of the other transcript of *argGH* in *C. glutamicum*. However, it is expected that coordinated amplification of the entire group of enzymes involved in the arginine biosynthetic pathway will be necessary to improve amino acid production levels markedly.

In summary, our study elucidates the transcriptional regulation of the arginine biosynthesis genes in *C. glutamicum*, in which ArgR repressor strongly inhibits *argC* promoter activity in an arginine-dependent manner but does not affect the *argG* promoter.

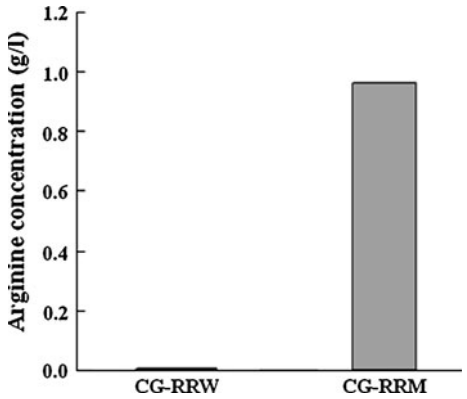

Fig. 8 Measurement of arginine yield from *C. glutamicum argR* wild-type (CG-RRW) and *argR* mutant (CG-RRM) cells. Cells were grown in CGXII containing 10% glucose

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