

## Molecular Cloning of the Arginine Biosynthetic Genes from *Corynebacterium glutamicum*

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Complementation cloning of the *argC*, *E*, *B*, *D*, *F*, and *G* genes in *Corynebacterium glutamicum* was done by transforming the genomic DNA library into the corresponding arginine auxotrophs of *Escherichia coli*. Recombinant plasmids containing 6.7 kb and 4.8 kb fragments complementing the *E. coli argB* mutant were also able to complement the *E. coli argC*, *E*, *A*, *D*, and *F* mutants, indicating the clustered organization of the arginine biosynthetic genes within the cloned DNA fragments. The insert DNA fragments in the recombinant plasmids, named pRB1 and pRB2, were physically mapped with several restriction enzymes. By further subcloning the entire DNA fragment containing the functions and by complementation analysis, we located the *arg* genes in the order of ACEBDF on the restriction map. We also determined the DNA nucleotide sequence of the fragment and report here the sequence of the *argB* gene. When compared to that with the mutant strain, higher enzyme activity of *N*-acetylglutamate kinase was detected in the extract of the mutant carrying the plasmid containing the putative *argB* gene, indicating that the plasmid contains a functional *argB* gene. Deduced amino acid sequence of the *argB* gene shows 45%, 38%, and 25% identity to that from *Bacillus stearothermophilus*, *Bacillus subtilis*, and *E. coli* respectively. Our long term goal is genetically engineering *C. glutamicum* which produces more arginine than a wild type strain does.

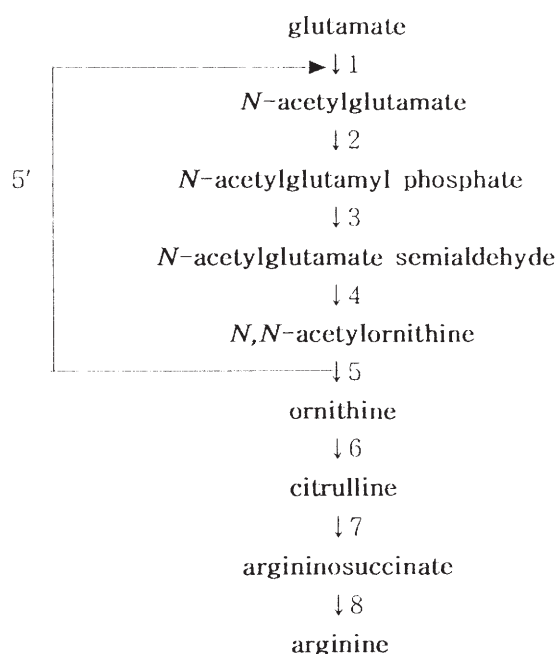
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*Corynebacterium glutamicum*, an aerobic, nonsporeforming, gram-positive organism, is widely used in the industrial production of amino acids and nucleic acids (1, 42, 43). In recent years, advances have been made in the development of cloning vectors and transformation systems for *C. glutamicum*, making it possible to apply recombinant DNA techniques and to study the structure, organization, and regulation of genes and enzymes of this organism. Because of the important role of *C. glutamicum* as an amino acid producer, several genes involved in amino acid biosynthesis, particularly in L-lysine and L-threonine synthesis, have been isolated and characterized, and some of these genes have been used to design engineered strains with improved amino acid production (15, 17, 19 and 41 for reviews). For the same purpose, we have started to study genes for arginine biosynthesis in

*C. glutamicum*.

Arginine biosynthesis in prokaryotes occurs by an eight-step pathway (Fig. 1)(6, 7, 8, 40). The first four steps of the pathway involve *N*-acetylated intermediates, beginning with the acetylornithine. *N*-acetylornithine is converted to ornithine in the fifth step of the pathway, and two separate enzymes have evolved to catalyze this reaction. In members of the family *Enterobacteriaceae*, *N*-acetylornithine is hydrolyzed to ornithine and acetate by the *argE* enzyme *N*-acetylornithinase (22, 38, 39). In other bacteria, including the methanogens, cyanobacteria, pseudomonads, and *Neisseria gonorrhoeae*, the acetyl group of *N*-acetylornithine is transferred to glutamate by the *argJ* enzyme, ornithine acetyltransferase (OATase) (13, 36, 38). The *N*-acetylglutamate produced from this reaction can be cycled back into the arginine biosynthetic pathway (40), bypassing step 1 (Fig. 1). *N*-acetylornithinase has been purified from *Escherichia coli* and ap-

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**Fig. 1.** Arginine biosynthetic pathway. Gene designations are as defined for *E. coli* (40). 1, *N*-acetylglutamate synthetase (*argA*). 2, *N*-acetylglutamate 5-phosphotransferase (*argB*). 3, *N*-acetylglutamate 5-semialdehyde dehydrogenase (*argC*). 4, *N*-acetylornithine 5-amino transferase (*argD*). 5, acetylornithinase (*argE*) or 5', ornithine acetyltransferase (*argJ*). 6, ornithine carbamoyltransferase (*argF*). 7, argininosuccinate synthetase (*argG*). 8, argininosuccinase (*argH*).

pears to function as a monomer with an molecular weight of 62,000 Da (6, 7). In contrast, no OATase from any other source has been characterized, and little is known of the physical properties of this enzyme.

While the biosynthesis of arginine by *E. coli* has been extensively studied as a model of gene regulation, few of the genes encoding the enzymes involved in this pathway in other prokaryotes have been determined for their DNA nucleotide sequences (10, 12, 14, 20). For examples, nucleotide sequence data are available for *E. coli* *argA*, *argF*, *argI*, and *carAB*, for the regulatory region of the *argECBH* operon (2, 4, 5, 16, 26, 28), and for *Pseudomonas aeruginosa* *argF*, *arcB*, and *argA* (21). For *Bacillus subtilis*, *argCEBDF* was also cloned and well characterized (23, 24, 25). Neither *argE* from *E. coli* nor *argJ* from *P. aeruginosa* has been cloned (21, 22). Riley and Glandorff (29) have cloned the 6.3 kb *Bam*HI fragment containing *argD* gene that encodes for acetylornithine- $\delta$ -transaminase. Based on the hybridization with the *argD* gene probe, it has been shown that *argM* gene may have a high similarity to the probe. The *arg* operon in *E. coli* described as above has been studied extensively by many researchers (22, 23, 24, 25, 28, 29, 30).

In some microorganisms the metabolic flow through the acetyl cycle is controlled by arginine-mediated fe-

edback inhibition of the second biosynthetic step, catalyzed by *N*-acetylglutamate kinase which is encoded by *argB* gene (*N*-acetyl-L-glutamate 5' phosphotransferase; EC2.7.2.8: 11, 13, 38). However, in *B. stearothermophilus*, no noticeable inhibition of *N*-acetylglutamate kinase by either ornithine or arginine could be detected. Instead, the target for inhibition was found to be the bifunctional *argJ* (and possibly the *argA*) gene product: Both *N*-acetylglutamate synthase and ornithine acetyltransferase activities were strongly inhibited by ornithine. Arginine, however, did not affect either activity (31, 32, 33). Consequently, in this organism the metabolic intermediate ornithine, rather than the end-product arginine appears to be critical for controlling metabolite conversions in the arginine acetyl cycle.

Here, we describe the cloning of the *argACEBDF* genes which are clustered within a 6.7 kb DNA fragment of the genome in *C. glutamicum*. We also report the cloning of the *argG* gene and sequence analysis of *argB* gene which encodes for *N*-acetylglutamate kinase.

## Materials and Methods

### Bacteria strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Corynebacterium glutamicum* and *Escherichia coli* strains were grown in LB (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose) at 30°C and 37°C, respectively. When needed, ampicillin was added to 50  $\mu$ g/ml for plasmid selection. *E. coli* arginine auxotrophs were used for the complementation and their assays were carried out on minimal medium M9 (5 $\times$ M9 salts, 20% Glucose, 0.1M CaCl<sub>2</sub>, 1M MgCl<sub>2</sub>) (35) supplemented with appropriate growth factors and, when necessary, 0.5% of arginine.

### DNA manipulation

The general molecular biological techniques were used (35). Plasmid isolation from *E. coli* was carried out by the alkaline lysis method. Chromosomal DNA from *C. glutamicum* strains were transformed by electroporation as described (35). Restriction endonucleases and DNA modifying enzymes were purchased from New England BioLabs, Boehringer Mannheim Biochemicals, and Bethesda Research Laboratories (BRL) and used as recommended by the manufacturers. *E. coli* strains were transformed following the CaCl<sub>2</sub> procedure (35) or by electroporation apparatus (Invitrogen, USA).

### Cloning of arginine biosynthetic genes by complementation

A *C. glutamicum* genomic DNA library was made of 4-

**Table 1.** Bacterial strains and plasmids used in this study

| Strains or plasmids  | Relevant genotypes or phenotypes <sup>a</sup>  | Sources or reference |
|----------------------|--|----------------------|
| <i>C. glutamicum</i> |  |                      |
| ASO19                | Spontaneous rifampicin resistant mutant of ATCC 13059  | 29                   |
| <i>E. coli</i>       | F <sup>-</sup> $\Phi$ 80dlacZDM15 $\Delta$ ( <i>lacZYA-argF</i> )U169                        |                      |
| DH5 $\alpha$         | <i>deoR endA1 hsdR17 supE44 thi-1 recA1</i><br><i>gyrA96 relA1</i> $\lambda$ <sup>-1</sup>   |                      |
| CGSC6176             | F <sup>-</sup> $\lambda$ <i>argA81::Tn10 IN(rrnD-rrnE)1</i>                                  | CGSC                 |
| CGSC5421             | Hfr <i>lacZ43(Fs) <math>\lambda</math> relA1 argB62 thi-1</i>                                | CGSC                 |
| CGSC1184             | F <sup>-</sup> <i>galT23 <math>\lambda</math> IN(rrnD-rrnE)1 argC24</i>                      | CGSC                 |
| CGSC4538             | Hfr <i>thr-1 leuB6 proA30 lacZ4 glnV44(AS) <math>\lambda</math> rpsL8 argD37 thi-I mut+</i>  | CGSC                 |
| CGSC6760             | Hfr <i>ara-41 lacY1 or lacY40 <math>\lambda</math><sup>md</sup> xylA7 mtlA2 argE86::Tn10</i> | CGSC                 |
| CGSC4896             | Hfr <i>araF58 relA1 spoT1 metB1</i>  | CGSC                 |
| CGSC5961             | F <sup>-</sup> <i>argG78 rpsL257</i>   | CGSC                 |
| Plasmids             |  |                      |
| pMT1                 | Shuttle vector; Ap <sup>r</sup> ( <i>E. coli</i> ), Km <sup>r</sup> ( <i>C. glutamicum</i> ) | 18                   |
| pBluescriptII KS(+)  | Ap <sup>r</sup> lacZ   | Stratagene           |
| pRB1                 | pMT1 with 4.8 kb insert carrying <i>argB</i> ; Ap <sup>r</sup>                               | This work            |
| pRB2                 | pMT1 with 6.7 kb insert carrying <i>argB</i> ; Ap <sup>r</sup>                               | This work            |
| pRC1                 | pMT1 with 3.5 kb insert carrying <i>argC</i> ; Ap <sup>r</sup>                               | This work            |
| pRE1                 | pMT1 with 3.9 kb insert carrying <i>argE</i> ; Ap <sup>r</sup>                               | This work            |
| pRE4                 | pMT1 with 3.4 kb insert carrying <i>argE</i> ; Ap <sup>r</sup>                               | This work            |
| pRB211               | pBluescript II KS (+) with 1.7 kb <i>HindIII-HindIII</i> fragment; Ap <sup>r</sup>           | This work            |
| pRB213               | pBluescript II KS (+) with 1.8 kb <i>HindIII-HindIII</i> fragment; Ap <sup>r</sup>           | This work            |
| pRB214               | pBluescript II KS (+) with 2.0 kb <i>ClaI-ClaI</i> fragment; Ap <sup>r</sup>                 | This work            |
| pRB216               | pBluescript II KS (+) with 1.0 kb <i>HindIII-HindIII</i> fragment; Ap <sup>r</sup>           | This work            |
| pRB217               | pBluescript II KS (+) with 1.5 kb <i>ClaI-XbaI</i> fragment; Ap <sup>r</sup>                 | This work            |
| pRB221               | pBluescript II KS (+) with 3.0 kb <i>KpnI-EcoRI</i> fragment; Ap <sup>r</sup>                | This work            |
| pRB224               | pBluescript II KS (+) with 2.5 kb <i>HinIII-XbaI</i> fragment; Ap <sup>r</sup>               | This work            |

<sup>a</sup>Superscripts indicate resistance. Ap: ampicillin, Km: kanamycin.

<sup>b</sup>CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn, USA

to 13-kb *MboI* fragments cloned into the *E. coli-Corynebacterium* shuttle vector pMT1 (18). *C. glutamicum* ASO 19 chromosomal DNA partially digested with restriction enzyme *MboI* was size-fractionated by 10 to 40% sucrose gradient centrifugation, ligated to the *BamHI*-digested vector, and transformed into *E. coli* DH5 $\alpha$ . *E. coli* arginine auxotrophic mutant cells as shown in Table 1 were transformed with the plasmid DNA library and plated onto the M9 minimal medium containing ampicillin, and appropriate supplements. The plates were incubated at 37°C. Transformed colonies were isolated and screened for the plasmid content. The purified plasmids were retransformed into the *E. coli* mutants and the plasmids from transformants were analyzed.

### Restriction enzyme mapping and subcloning of the insert DNA

For further determination of location of each gene for arginine biosynthesis, the recombinant plasmids pRB1 and pRB2 were digested by several restriction enzymes including *ClaI*, *EcoRI* and *HindIII*. Partial pertinent res-

triction enzyme map for inserts of plasmids pRB1 and pRB2 is shown in Figure 2. Based on the resulting restriction pattern, several smaller fragments were isolated by gel extraction and religated into linearized pBluescript plasmid (stratagen) as the manufacture's suggestion. These subclones, termed pRB211, pRB221, pRB213 and pRB214 (see Table 1), in addition to the original clones, named as pRB1, pRB2, pRC1, pRE1 and pRE4, were transformed for complementation analysis into each of *E. coli* arginine auxotrophic mutants.

### DNA nucleotide sequence determination

Overlapping clones of double-stranded DNA cloned in the Bluescript-II SK(+) were sequenced in an Applied Biosystems model 373A DNA sequencer, using the standard PRISM DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) protocol. The sequencing primers used in this study were T3, T7, PR1 (5'-TTGTCGCGG-AAAGTGCTGCAACCAT-3'), PR2 (5'-GCTCATGTCATT-GACGGCCGCATCG-3'), and PR3 (5'-TCATCGCACAG-CTCGCGCACTGCTT-3'). Sequence patches were assem-

bled and interpreted by using the DNA Strider version 1.2, AssemblyLIGN 1.0.5./MacVector 4.0 (International Biotechnologies Inc.), and Genetics Computer Group software packages (9). Analysis of nucleotide and amino acid sequences were done by DNAMAN computer program (Lynnon Biosoft, USA).

### Preparation of cell extracts and enzyme assays

*C. glutamicum* cells from exponential phase cultures were harvested by centrifugation (10 min, 7000×g) and washed in 0.9% (w/v) NaCl. *E. coli* mutants were harvested in the late exponential phase (about  $2 \times 10^8$  cells ml<sup>-1</sup>) and washed in their respective mineral media. Cells were suspended in 50 mM-Tris/HCl buffer (pH 8.0) and disrupted by sonication for 5 min in a Heat Systems ultrasonic oscillator (100W, 20 kHz). After sonication, the resulting suspension was centrifuged (15 min, 20000×g). All these operations were done below 10°C. The supernatant was used for enzyme assays.

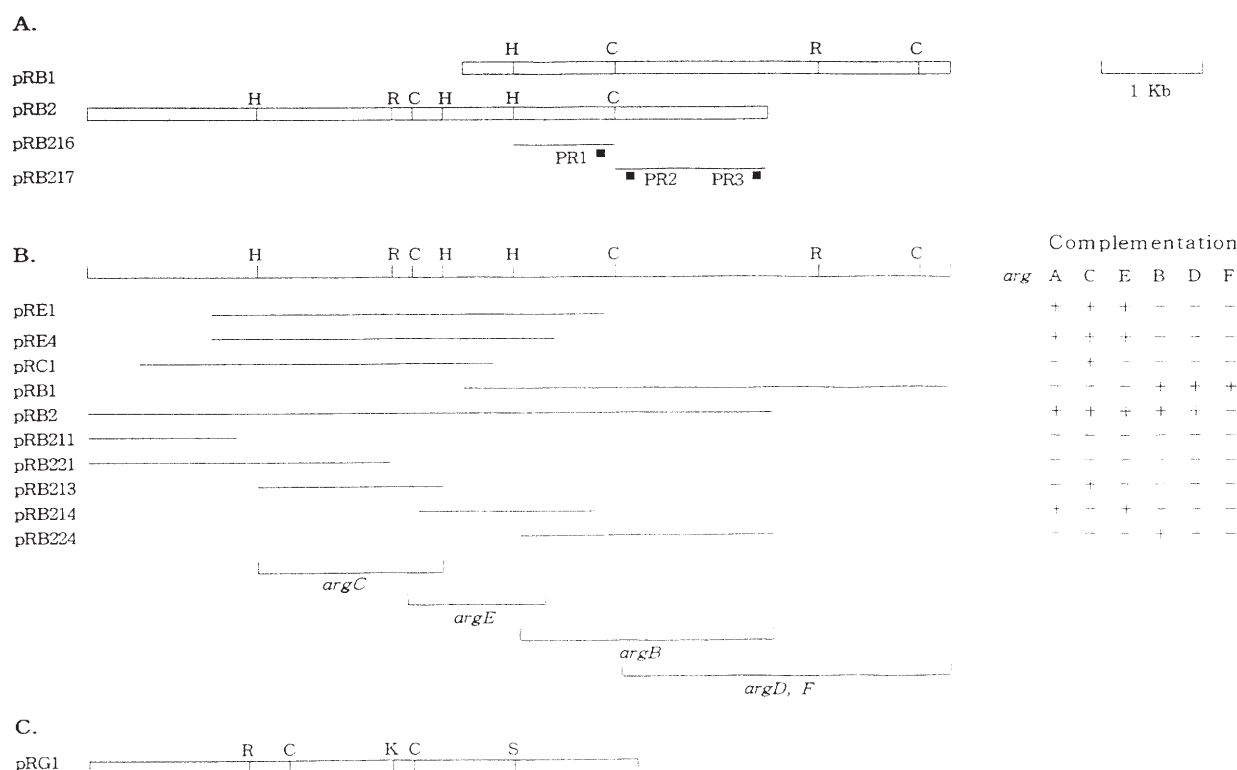
*N*-Acetylglutamate 5-phosphotransferase activity was measured by the ferric chloride method (38, 39). The incubation mixture consisted of: 200 mM Tris/HCl buffer (pH 8.0), 40 mM *n*-acetylglutamate, 40 mM MgCl<sub>2</sub>, 40

mM ATP, 400 mM NH<sub>4</sub>OH (pH 8.0) and extract (0.1~0.5 mg protein) in a final volume of 0.5 ml. The reaction was started by the addition of 1.0 ml of 1.0 M HCl containing 5% (W/V) FeCl<sub>3</sub>·6H<sub>2</sub>O and 4% (W/V) trichloroacetic acid. The absorbance of the hydroxamate-Fe<sup>3+</sup> complex was measured at 500 nm. Formation *N*-acetylglutamyl 5-hydroxamate was a linear function of time up to 120 min incubation. Protein concentration was determined by Bradford method (3) with bovin serum albumin as a standard.

## Results and Discussion

### Cloning of arginine biosynthetic genes from *C. glutamicum* by complementation

Since there have been numerous reports that *C. glutamicum* genes can be cloned by heterologous complementation of *E. coli* mutants, we started this study by transforming genomic DNA library of *C. glutamicum* into *E. coli* arginine auxotrophs. The *arg* genes encoding arginine biosynthetic enzymes from *C. glutamicum* were screened by complementation of several *E. coli* mutants (see Table 1) by transforming a *C. glutamicum* genomic



**Fig. 2.** Restriction map of the pRB1, pRB2, and pRG1 inserts, and deletion analysis of the insert DNA fragments. (A) Restriction map of the pRB1 and pRB2. Abbreviations: H (*Hind*III), R (*Eco*RI), and C (*Cla*I). The restriction sites in the outline show the overlap between the two clones. Physical map of inserts in pRB216 and pRB217 and the location of primers PR 1, 2, and 3 used for sequencing analysis are indicated. (B) Complementation of *E. coli* arginine auxotrophs by specific restriction-generated fragments subcloned in the plasmid, pBluescript II KS(+). (C) Restriction map of the pRG1 containing *argG* gene. Abbreviation: R (*Eco*RI), C (*Cla*I), S (*Sal*I).



library cloned in the vector pMT1 (18). A *C. glutamicum* ASO19 genomic library previously constructed in *Corynebacterium-E. coli* shuttle vector pMT1 was screened for the complementation of an *E. coli* *argB* and other arginine auxotrophic mutants that bear mutations or deletion in one or more *arg* biosynthesis genes. Ampicillin resistant candidate colonies on LB plates were selected and screened for complementation of the arginine auxotrophy of *E. coli* on plates with minimal medium. Several candidates which grew in the absence of arginine were isolated, and their plasmids were characterized by several restriction analysis. All transformants showed the *arg*<sup>+</sup> phenotype, i.e. they grew fluently on minimal medium when transformed again into the same *E. coli* arginine auxotrophs. The insert DNA also had all common restriction fragments, suggesting that these inserts contain overlapped regions.

Among the positive clones, plasmid pRB2 (Fig. 2) carried the insert DNA which was 6.7 kb in size, and this plasmid was analyzed further. Plasmid pRB1 (Fig. 2) carried a 4.8 kb insert DNA. Recombinant DNA pRB2 containing 6.7 kb insert was able to complement other *E. coli* arginine auxotrophs, *argC*, *argE*, *argB*, *argD*, *argF* and *argA*. This suggests the clustered organization of the *argACEBDF* genes on the *C. glutamicum*. In addition, We also cloned the *argA*, *argC*, *argE*, *argD*, and *argF* genes through the same complementation technique by transforming the DNA library into the corresponding *E. coli* mutant. By restriction analysis, we found that all inserts were partially overlapped with the inserts of pRB1 and pRB2 (see Fig. 2A and B). This confirms the clustered organization of each gene in *Corynebacterium*. We also cloned the *argG* gene by complementation of the *E. coli* *argG* auxotroph, which is a newly cloned gene except *E. coli*. Among the several candidates plasmids we have screened, the clone pRG1 contains 5.4 kb DNA fragment which maps to other region of *Corynebacterium* genome (data not shown).

### Complementation of each *E. coli* *arg* mutants by the cloned fragments

To determine the location and the organization of each arginine biosynthetic genes, a series of *E. coli* auxotrophic strains bearing mutations or deletions in one or more of the arginine biosynthesis genes (Table 1) were transformed with plasmids pRB1, pRB2, pRC1, pRE1 and pRE4, which were originally isolated clones by complementation. Several transformants for each *E. coli* auxotroph were examined for the ability to grow on minimal medium (M9) lacking L-arginine. The plasmid pRB2 contained the *argACBD* genes of *C. glutamicum* for example (Fig. 2). Others are also indicated as in Fig. 2. It appears

that the organization of the *arg* genes of *C. glutamicum* is very similar to that of *E. coli* and identical to that of *B. stearothermophilus* and *Bacillus subtilis* (25, 28, 31). In addition, Sakabyan *et al.* have also reported the cloning of the *argJBD* cluster in *C. glutamicum* (34). Taken together, we concluded that plasmids pRB1 and pRB2 complemented mutations in *E. coli* genes (*argA*, *C*, *E*, *B*, *D* and *F*), indicating that the 6.7 and 4.8 kb fragments of *C. glutamicum* contain these genes.

### Clustered organization of the arginine biosynthetic genes

To further determine the order and location of each gene within the fragment, we performed subcloning and deletion analysis of the insert DNA. First, for restriction mapping, the insert DNA fragments were digested with several enzymes as shown in Fig. 2A. Then, based on this map, the inserts were subcloned into several smaller fragments. The recombinant plasmids, designated pRB1 and pRB2, were separately digested with pertinent restriction enzymes, and fragments were purified, ligated into the multiple cloning sites of the plasmid pBluescript II KS(+) and transformed into *E. coli* mutants (See Table 1). Transformants were selected on LB medium containing ampicillin and screened on minimal media for complementation of *arg* marker. As shown in Fig. 2B, the plasmids pRB211, pRB221, pRB213 and pRB214 deletion mutants were negative for *N*-acetylglutamate kinase activity and were unable to complement *E. coli* *argB* mutant, indicating that at least *argB* gene is located within the 2.5 kb *Hind*III-*Xba*I fragment termed pRB224 (Fig. 2). Recombinant plasmids pRB211 and pRB221 were negative for production of all *arg* biosynthesis genes. pRB213 was able to complement *E. coli* *argC*, and pRB214 was able to complement *E. coli* *argA* and *argE*. Based on the complementation analysis, we located the *arg* genes in the order of ACEBDF on the restriction map (Fig. 2B).

### Nucleotide and predicted amino acid sequences of the *argB* gene

The strategy for sequencing 2.5 kb of the *C. glutamicum* DNA fragment present in pRB2 is indicated in Fig. 2A. We sequenced and analyzed the entire 2.5 kb DNA fragment. Among these the nucleotide sequence of a 1,395-bp fragment which contains the putative *argB* gene and the amino acid sequence are shown in Fig. 3. An open reading frame (ORF) of 951 nucleotides (nt), extending from nt 445 to nt 1395, was found, with a total G+C content of 59.3%. The ArgB protein contained 317 amino acid, with a molecular weight of 33,552 Da. Computer analysis of amino acid sequence homology revealed a high similarity to that of the ArgB from *Bacillus* (31, 32,



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