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Reengineering of an L-Arginine and L-Citrulline Producer of *Corynebacterium glutamicum*

Running title: Arginine/citrulline producer of *C. glutamicum*

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ABSTRACT

Toward the creation of a robust and efficient producer of L-arginine and L-citrulline (arginine/citrulline), we have performed re-engineering of a *Corynebacterium glutamicum* strain using genetic information of three classical producers. Sequence analysis of their *arg* operons identified three point mutations (*argR123*, *argGup92*, *argG45*) in one producer and one point mutation (*argB26* or *argB31*) in each of the other two producers. Reconstitution of the former three mutations or each *argB* mutation on a wild-type genome led to no production. Combined introduction of *argB26* or *argB31* with *argR123* into a wild-type gave rise to arginine/citrulline production. When *argR123* was replaced by *argR*-deleted mutation (*ΔargR*), the production was further increased. The best mutation set, *ΔargR* and *argB26*, was used to screen for the highest productivity in the backgrounds of different wild-type strains of *C. glutamicum*. This yielded a robust producer, RB, but the production was still one-third of that of the best classical producer. Transcriptome analysis revealed that the *arg* operon of the classical producer was much more highly up-regulated than that of strain RB. Introduction of *leuC456*, a mutation derived from a classical L-lysine producer and provoking global induction of the amino acid-biosynthetic genes including the *arg* operon, into strain RB led to increased production, but incurred retarded fermentation. On the other hand, replacement of the chromosomal *argB* by heterologous *Escherichia coli argB*, natively insensitive to arginine, caused three-fold increased production without retardation, revealing that the limitation in strain RB was the activity of the *argB* product. To overcome this, in addition to *argB26*, the *argB31* mutation was introduced into strain RB, which caused higher deregulation of the enzyme and resulted in dramatically increased production, like the strain with *E. coli argB*. This reconstructed strain displayed an enhanced performance, thus allowing significantly higher productivity of arginine/citrulline even at the suboptimal 38ºC.

INTRODUCTION

We have shown reverse engineering of a high-production strain of

Corynebacterium glutamicum using L-lysine fermentation as a model (10, 11, 21). The characteristic that the methodology aims at is robustness of the resulting strain. The classical approach based on random mutation and selection has sacrificed the native robustness of an organism in exchange for enhancing the production abilities to the limits. The high production abilities and delicate constitutions of classical industrial producers are the merits and demerits of the classical approach. Such the inevitable consequence of the classical approach could be understood also from the fact that more than one thousand mutations have been accumulated in the genome of an industrial L-lysine producer of *C. glutamicum* (11). We examined those mutations and identified mutations relevant to L-lysine production. Subsequent assembly of the useful mutations in a robust wild-type strain was shown to substantially improve producer performance (18, 21). In addition, the mechanisms of L-lysine hyperproduction were unraveled through this strain reconstruction (7, 15, 20, 21). As demonstrated in these studies, our work involves re-engineering a more efficient producer using knowledge regarding the mutations that have accumulated over years of industrial strain development. The methodology starts by tracing backwards to an existing classical producer and thus can be called reverse engineering.

With the accumulated knowledge on mutations relevant to production, it becomes possible to combine positive mutations derived from different lines of classical producers in a single wild-type background. Such an advanced approach has recently led to an impressive result in L-arginine and L-citrulline production by *C. glutamicum*. The procedure and impact of this re-engineering methodology are described here.

L-Arginine, a semi-essential amino acid, has lately attracted considerable attention, because the amino acid has been shown to be a precursor to nitric oxide (NO), a key component of endothelial-derived relaxing factor (1). Because of L-arginine's NO-stimulating effect, the amino acid helps, for example, to relax and dilate blood vessels, and thus can be utilized in numerous clinical areas (1). On the other hand, L-citrulline, a precursor of L-arginine biosynthesis (Fig. 1), is also an

important amino acid for our health since it is a source of endogenous L-arginine in the body (5). Since the issue of which amino acid is preferably accumulated is not the subject of this paper, we report here the results for the sum of the two amino acids as arginine/citrulline.

MATERIALS AND METHODS

Bacterial strains and plasmids. *C. glutamicum* arginine producers used for comparative sequence analysis are strains A-27, I-30, and D-77 derived through multiple rounds of mutagenesis from wild-type ATCC 13870, ATCC 13032, and KY10025, respectively (2, 17). Strain ATCC 13870 was previously classified as *Corynebacterium acetoacidophilum*, but by recent molecular taxonomic studies, it is currently re-classified in the original species *C. glutamicum* (13). The wild-type strains used for comparisons of potentials for arginine/citrulline production are *C. glutamicum* ATCC 13870, ATCC13032, and other representative strains listed in our previous report (19). *E. coli* DH5α (27) was used as a donor of the genomic DNA for amplifying the *E. coli argB* gene and also as a host for cloning of the PCR products. Plasmid pESB30, which is non-replicative in *C. glutamicum*, is a vector for gene replacement in *C. glutamicum* (16). Plasmids pCargR123, pCargRG45, pCargB26, pCargB31, pCleuC456, and pCargB2631 that contain the mutated DNAs in vector pESB30 were used to replace the wild-type chromosomal DNAs by the mutated DNAs. Plasmid pCargRd that contained the internally deleted *argR* gene in vector pESB30 was used to replace the wild-type chromosomal gene by the deleted gene. Plasmid pC-EargB that contained the open reading frame (ORF) of *E. coli argB* in vector pESB30 was used to replace the chromosomal *argB* ORF with the heterologous *E. coli argB* ORF.

Media. Complete medium BY (28) and minimal medium MM (28) were used for cultivation of *C. glutamicum*. Solid plates were made by the addition of Bacto-Agar (Difco) to 1.6%. When required, kanamycin was added at the final concentrations of 20 μg/ml for BY plates. RG2 medium used for production in a

300-ml flask consisted of (per liter) 60 g of glucose, 5 g of corn steep liquor, 30 g of $(NH_4)_2SO_4$, 8 g of KCl, 2 g of urea, 0.5 g of KH_2PO_4, 0.5 g of K_2HPO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 1 g of NaCl, 20 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 5H_2O$, 20 mg of nicotinic acid, 20 mg of ß-alanine, 10 mg of thiamin-HCl, 0.2 mg of D-biotin, and 30 g of $CaCO_3$ (pH 7.7). RSG1 medium used for second-seed culture in jar fermentation consisted of (per liter) 60 g of glucose, 22 g of corn steep liquor, 5 g of $(NH_4)_2SO_4$, 5 g of urea, 2 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 5H_2O$, 30 mg of $CaCl_2 \cdot 2H_2O$, 30 mg of $CuSO_4 \cdot 5H_2O$, 1 mg of $ZnSO_4 \cdot 7H_2O$, 1 mg of $NiCl_2 \cdot 6H_2O$, 1 mg of $CoCl_2 \cdot 6H_2O$, 1 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 mg of β-alanine, 10 mg of nicotinic acid, 10 mg of thiamine-HCl, and 0.3 mg of D-biotin (pH 7.2). RPG1 medium used for 5-liter jar fermentors consisted of (per liter) 60 g of glucose, 3 g of corn steep liquor, 30 g of $(NH_4)_2SO_4$, 2.8 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 1 g of NaCl, 20 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 5H_2O$, 40 mg of $CaCl_2 \cdot 2H_2O$, 2 mg of $CuSO_4 \cdot 5H_2O$, 2 mg of $ZnSO_4 \cdot 7H_2O$, 2 mg of $NiCl_2 \cdot 6H_2O$, 2 mg of $CoCl_2 \cdot 6H_2O$, 20 mg of β-alanine, 20 mg of nicotinic acid, 10 mg of thiamine-HCl, and 0.2 mg of D-biotin (pH 6.8). For growth of *E. coli*, Luria-Bertani broth or agar (27) was used.

Cultivations for arginine/citrulline production. For flask fermentation, a 2.0-ml sample of the seed culture grown to early stationary phase at 30°C in BYG medium (containing 1.0% glucose in medium BY) was inoculated into 20 ml of RG2 medium in a 300-ml flask and cultivated aerobically at 30°C or 38°C until the sugar was consumed.

For jar fermentation, cells grown on a BY plate at 30℃ for 1 day were inoculated into 200 ml of RSG1 medium in a 2-liter flask. After grown to early stationary phase at 30℃ on a rotary shaker, the seed broth was transferred into a 5-liter jar fermentor containing 1,000 ml of RPG1 medium. After the sugar initially added was consumed, a solution containing (per liter) 500 g of glucose, 30 g of $(NH_4)_2SO_4$, 1 g of $NH_4H_2PO_4$, 1.2 g of $MgSO_4 \cdot 7H_2O$, 8 g of KCl, 150 mg of $CaCl_2 \cdot 2H_2O$, 20 mg of β-alanine, 20 mg of nicotinic acid, 20 mg of thiamine-HCl,

and 0.2 mg of D-biotin was continuously fed until the total amount of glucose in the medium reached 572 g. The feeding rate of the solution was controlled to maintain the glucose concentration in the medium at a low concentration (below 0.5%). The culture was basically performed with an agitation speed of 800 rpm, aeration at 2 liter/min, and at 30℃ or 38°C. The pH was maintained at 6.8 with NH_4OH.

Recombinant DNA techniques. Standard protocol (27) was used for the construction, purification and analysis of plasmid DNA, and transformation of *E. coli*. Chromosomal DNA of *C. glutamicum* was extracted from protoplasts by the method of Saito and Miura (25). The protoplasts were prepared by the method of Katsumata et al. (12). Transformation of *C. glutamicum* by electroporation was carried out by the method of van der Rest et al. (24), using Gene pulser and Pulse controller (BioRad, USA). PCR was performed with DNA Thermal Cycler GeneAmp 9600 (Perkin Elmer, USA), using TaKaRa La Taq™ DNA polymerase (Takara Bio, Ohtsu, Japan).

Nucleotide sequence analysis of the *arg* operon. The entire *arg* operon *argCJBDFRGH* (Cgl1394-1401) was PCR amplified using primers arg op-F and arg op-R with each genomic DNA of three arginine producers, A-27, I-30, and D-77, and individual natural ancestors as a template. Primers used in this study are listed in Table 1. Amplified 9.0-kb PCR fragments were purified using GENECLEAN III KIT (Qbiogene, CA, USA). With each PCR fragment as a template, the inner segments of each fragment were amplified by PCR at intervals of approximately 500 bp using primers designed based on the genome sequence of *C. glutamicum* (BA000036) publicly available at http://gib.genes.nig.ac.jp/single/index.php?spid=Cglu_ATCC13032 (10). The nucleotide sequences of the PCR products were then analyzed by an ABI PRISM™ 377 DNA sequencer from Applied Biosystems, with ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer, USA). The subsequent electrophoresis analysis was carried out by Pageset SQC-5ALN 377 (Toyobo, Japan).

Introduction of specific mutations into genome. The mutated *argB* gene regions were PCR amplified using primers argB-F and argB-R with genomic DNAs of arginine producers, I-30 and D-77 as templates. On the other hand, the mutated *argR* gene region was PCR amplified using primers argR-F and argR-R with genomic DNA of arginine producer A-27 as a template. Similarly, the mutated *argRG* gene region was PCR amplified using primers argR-F and argG-R with genomic DNA of arginine producer A-27 as a template. Each PCR fragment was cloned into pESB30 by the TA cloning method (27). The recombinant plasmids constructed by cloning the PCR fragments containing the mutated *argB* gene regions of strains I-30 and D-77 into pESB30 are designated pCargB26 and pCargB31, respectively. The recombinant plasmids constructed by cloning the PCR fragments containing the mutated *argR and argRG* gene regions of strain A-27 into pESB30 are designated pCargR123 and pCargRG45, respectively. The specific mutations on the recombinant plasmids were introduced into *C. glutamicum* strains via two recombination events as described previously (21). For introduction of the *leuC456* mutation into strain RB, plasmid pCleuC456 (7) was used.

Generation of a strain carrying both *argB* mutations. The *argB26* and *arg31* mutations were introduced into the genomic *argB* gene using plasmid pCargB2631, which was constructed as follows. The 5'-region and 3'-region of the *argB* gene were amplified by PCR with pCargB31 as a template using the following primer pairs; argB-F with argB26-R, and argB26-F with argB-R, respectively. As the two primers argB26-R and argB26-F contained regions complementary to each other, fusion PCR was performed using the purified 5'-region and 3'-region fragments as templates and the primers argB-F and argB-R. The resulting 1.0-kb fragment contained the intended *argB* gene region on which both *argB* mutations coexisted. The fragment was cloned into pESB30 by the TA cloning method to yield pCargB2631. This plasmid was used to replace the corresponding chromosomal gene with the double mutated gene.

Chromosomal deletion of *argR*. Plasmid pCargRd containing the internally deleted *argR* gene was constructed as follows, and was used to replace the wild-type chromosomal gene with the deleted gene. The 5'-upstream region of the *argR* gene was amplified by PCR using primers argR-up-F and argR-up-R with ATCC 13032 genomic DNA as a template. Similarly, the 3'-downstream region of the gene was amplified using primers argR-down-F and argR-down-R. Fusion PCR was then performed using the purified 5'-upstream and 3'-downstream fragments as templates and the primers argR-up-F and argR-down-R. The resulting 1.0-kb fragment contained the deleted *argR* gene which was shortened from 513 bp to 132 bp by in-frame deletion of the inner sequence. The fragment was digested by BamHI and then ligated to BamHI-digested pESB30 to yield pCargRd. Defined chromosomal deletion of the *argR* gene was accomplished using the pCargRd, via two recombination events as described previously (21).

Replacement of chromosomal *argB* with heterologous *E. coli argB*. Plasmid pC-EargB containing the ORF of *E. coli argB* sandwiched between the 3'-region of *C. glutamicum argJ* and the 5'-region of *C. glutamicum argD* was constructed as follows, and was used to replace the endogenous *C. glutamicum argB* ORF with the *E. coli argB* ORF. The 3'-region of *C. glutamicum argJ* was amplified by PCR using two primers argJ-F and argJ-R with ATCC 13032 genomic DNA as a template. On the other hand, the ORF of *E. coli argB* was also amplified with two primers EargB-F and EargB-R with *E. coli* DH5α genomic DNA as a template. In the design of the primer EargB-F, four low-usage codons in the N-terminal coding region of *E. coli argB* were replaced with synonymous high-usage codons in *C. glutamicum* as shown in Table 1 (AAT→AA<u>C</u>, TTA→<u>C</u>T<u>C</u>, ATT→AT<u>C</u>, and AAA→AA<u>G</u>; underlining indicates changed nucleotides). Fusion PCR was performed using the purified *C. glutamicum argJ* and *E. coli argB* gene fragments as templates and the primers argJ-F and EargB-R. The resulting 1.5-kb fragment contained the *E. coli argB* ORF that was preceded by the 3'-region of *C. glutamicum argJ*. The fragment was

digested with BglII and BamHI and then ligated to BamHI-digested pESB30 to yield pESBargB. Similarly, the 5'-region of *C. glutamicum argD* was amplified by PCR using two primers argD-F and argD-R with ATCC 13032 genomic DNA as a template. The resulting 0.7-kb fragment was digested with BamHI and BfrI and then ligated to pESBargB digested by the same restriction enzymes to yield pC-EargB. Replacement of chromosomal *argB* with heterologous *E. coli argB* was conducted using pC-EargB, via two recombination events as described previously (21).

Transcriptome analysis. Total RNAs were prepared from mid-exponential phase cultures of 5-liter jar fermentors as described previously (8). Transcriptome analysis was performed using GeneChip (Affymetrix, USA). Labeling of RNA transcripts, hybridization, and scanning were performed according to the manufacturer's instructions. Gene expression data were analyzed using Microarray Suite 5.0 software (Affymetrix). Changes in expression levels that had a change call of decrease or increase together with a P value of < 0.001 and a signal ratio of more than 1.5-fold were considered significant. The reproducibility of the measurements was confirmed by duplicate, independent cultures and experiments.

Enzyme assay. Crude cell extracts were prepared by sonic disruption of cells from mid-exponential phase cultures of 5-liter jar fermentors as described previously (22). Protein quantity was determined by the method of Bradford (3). The activities of *N*-acetyl-L-glutamate kinase (ArgB) in crude cell extracts were measured colorimetrically at 30℃, essentially as described by Ferández-Murga et al. (6).

Analysis. Cell growth was monitored by measuring OD_{660} of the culture broth with a U-1080 Auto Sipper Photometer (Hitachi, Japan). Glucose concentration was determined using Determinar GL-E (Kyowa Medex Co., Ltd., Japan). L-arginine and L- citrulline titers were determined by HPLC system

(Shimazu, Japan) after derivatization with *o*-phthalaldehyde.

RESULTS

Identification of mutation(s) causing arginine/citrulline production. Our first task was to identify basal mutation(s), namely, mutation(s) that conferred the ability to produce arginine/citrulline on wild-type *C. glutamicum*. For this purpose, three arginine producers, strain A-27, strain I-30, and strain D-77, were used as gene resources. These are classical mutants derived independently from different lines of *C. glutamicum* wild-type strains. From their genomic information, we attempted to identify the basal mutation(s). Generally speaking, such basal mutation(s) are assumed to exist on the *argB* gene encoding the key regulatory enzyme for arginine biosynthesis (Fig. 1), but to our surprise, there was no such mutation in strain A-27, the best arginine producer of the three. Instead, a point mutation was found in each of the *argR* gene, the upstream non-coding region of the *argG* gene, and the *argG* gene (Fig. 2A): a C to T exchange at position 368 in *argR*, leading to an amino acid replacement of Ala-123 by Val (designated mutation *argR123*); a G to A exchange at 92 bp upstream of *argG* (designated mutation *argGup92*); and a G to A exchange at 136 in *argG*, leading to an amino acid replacement of Asp-45 by Asn (designated mutation *argG45*). On the other hand, the *argB* genes of strains I-30 and D-77 were found to have a different point mutation as expected (Fig. 2A): a C to T exchange at position 77 in *argB*, leading to an amino acid replacement of Ala-26 by Val (designated mutation *argB26*); and an A to G exchange at position 91 in *argB*, leading to an amino acid replacement of Met-31 by Val (designated mutation *argB31*).

By using these five specific mutations, we examined their relevance for arginine/citrulline production in a wild-type ATCC 13032 background. First of all, strain A-27-derived three mutations (*argR123*, *argGup92*, and *argG45*) were reconstituted simultaneously on the wild-type genome, but unexpectedly arginine/citrulline production was not observed (Fig. 2B). Separate introduction of the *argB31* or *argB26* mutation into the wild-type resulted in no production as well

(Fig. 2B). However, when the *argB31* or *argB26* mutation was combined with the *argR123* mutation on the wild-type genome, we observed arginine/citrulline production for the first time (Fig. 2B). Based on this finding, the missense mutation, *argR123*, was assumed to impair the repressor function of the ArgR protein. We also examined the effect of in-frame deletion of the *argR*-inner sequence (designated mutation *ΔargR*) on production. As a result, increased production was observed in either combination with *argB31* or *argB26* (Fig. 2B). The level was higher in the combination of *argB26* and *ΔargR* than in the combination of *argB31* and *ΔargR* (Fig. 2B). Taken all together, we specified the basal mutations as *argB26* and *ΔargR*.

The consequences of these mutations on production were more prominent when the culture temperature was shifted from traditional 30ºC to suboptimal 38ºC (Fig. 2B). This indicated that arginine/citrulline fermentation by this organism could be potentially realized even at higher temperatures than is traditionally practiced.

Screening for a wild-type background with best performance. In the re-engineering approach, it is important to start from different wild-type strains to obtain best performance, since a strain finally engineered is supposed to inherit the properties of its original ancestor. For this purpose, the basal mutation set, *argB26* and *ΔargR*, was introduced into six *C. glutamicum* wild-type strains to generate isogenic mutants, which were then screened for the abilities to produce arginine/citrulline at the flask level (data not shown). Among those, we chose two typical producers, the derivatives of wild-type strains, ATCC 13032 and ATCC 13870, and compared their performance in more detail using 5-liter jar fermentors. The evaluation was conducted under suboptimal 38ºC conditions, because fermentation at elevated temperatures (35~40ºC) is industrially advantageous, leading to reduction of cooling costs compared with the traditional 30ºC conditions. Figure 3 shows the profiles of arginine/citrulline production, which revealed that strain ATCC 13032 had a significantly higher potential for arginine/citrulline production at elevated temperatures than the other strain. The selected wild-type ATCC 13032 carrying the basal mutation set, *argB26* and *ΔargR*, on its genome was designated

strain RB and used for subsequent analysis.

Transcriptome analysis. Strain RB which carries the *argB26* and *ΔargR* mutations can grow and consume glucose almost as fast as the wild-type strain, and so the fermentation period can be shortened to below half of that traditionally required. In addition, fermentation at high temperatures around 38ºC can be possible. Nevertheless, the final titer of arginine/citrulline production was about one-third of that of classical producer A-27 (Fig. 2B). What is the limitation in strain RB, compared with the classical producer? To get the clue, we here attempted transcriptome analysis of both producers. When total RNAs from mid-exponential phase cultures of 5-liter jar fermentors were used to study differential transcription profiles between each producer and its parental wild-type, an interesting finding that could explain the difference in production levels has emerged. That was the difference in the expression levels of the *arg* operon. In strain RB carrying the *ΔargR* mutation, the expression of the *arg* genes were indeed derepressed by around ten-fold, but to our surprise, an additional up-regulation was observed in classical producer A-27 (Fig. 4). Although strain A-27 carries the *argR123* mutation, such high up-regulations could not be explained by only the mutation. So we hypothesized that a sort of global response, probably the stringent-like response which we observed in classical lysine producer B-6 (9), might occur also in strain A-27, thus leading to further induction of the *arg* operon. In fact, the expression profiles observed for the central metabolic pathways and amino acid-biosynthetic pathways in strain A-27 had similar features to those of the lysine producer (9).

Induction of a global response. If the hypothesis mentioned above were true, introduction of a specific mutation provoking the stringent-like response, the global induction of the amino acid-biosynthetic genes, into strain RB would be expected to result in increased production of arginine/citrulline. Since we have already defined such the mutation, namely *leuC456*, in classical lysine producer B-6 (7), the mutation was introduced into strain RB. As expected, the presence of the mutation

enabled strain RB to accumulate a comparable level of arginine/citrulline to classical producer A-27 in flask cultures (data not shown). However, in 5-liter jar fermentor cultivation at 38ºC, the engineered strain showed retarded fermentation due to decreased growth and sugar consumption (data not shown), thus leaving a problem to be solved for realization of high-speed fermentation.

Verification of the target to be engineered. Plasmid-mediated amplification of the entire *arg* operon could be another strategy for increased expression of the operon. However, this engineering also led to retarded fermentation (data not shown). On the other hand, assays using crude extract of strain RB showed that the mutated ArgB enzyme was inhibited by relatively low concentrations of arginine although less sensitive to arginine than the wild-type enzyme (Fig. 5). This finding reminded us of that if the metabolic flux toward arginine/citrulline were restricted at the regulatory step, the expressional shortage of the *arg* operon in strain RB might be compensated by a further qualitative change of the ArgB enzyme, namely higher desensitization of the enzyme. To verify this, we engineered the genome of strain RB to generate a strain carrying the hybrid *arg* operon where the ORF of the endogenous *argB* gene was replaced with that of *E. coli argB*. This engineering is based on the information that *E. coli* has different control mechanisms for arginine biosynthesis than *C. glutamicum* (4, 14, 23, 26, 29): the feedback control in *E. coli* occurs at the ArgA enzyme, and the ArgB enzyme is natively insensitive to end-product inhibition (Fig. 1). The result was beyond our expectations. In 5-liter jar fermentor cultivation at 38ºC, arginine/citrulline production increased by about three fold during almost the same culture period as that for strain RB (data not shown). From the result, it was verified that the target to be engineered in strain RB was *argB*.

Higher desensitization of the ArgB enzyme. How could we achieve higher desensitization of the ArgB enzyme of strain RB? This became our next objective. To this end, we combined the *argB31* mutation with the *argB26* mutation on the *ΔargR*

background, because the two changed amino acid residues were located very close to each other on the amino acid sequences of the ArgB enzyme and thus were assumed to occur on the same allosteric site of the enzyme. Enzyme assays revealed that the resulting ArgB enzyme was expectedly less sensitive to end product inhibition than the original enzyme carrying either of the two *argB* mutations (Fig. 5). The engineered strain which carried the *argB26*, *argB31*, and *ΔargR* mutations was designated strain RBid (Fig. 6).

Arginine/citrulline production by strain RBid. We examined whether strain RBid displayed considerably increased production, similar to the strain carrying the heterologous *E. coli argB* on its genome. The result obtained from flask cultures met our expectations and arginine/citrulline production by strain RBid reached a level comparable to that of the classical producer, A-27 (Fig. 2B). In 5-liter jar fermentor cultivation, the engineered strain maintained its high production rate even at 38°C, thus leading to completion of fermentation during half the time period required with the traditional strain (Fig. 7).

DISCUSSION

The producer's performance that we demanded in the face of this re-engineering program is robustness which allows high fermentation yields in the shortest possible time even under stressful conditions. The newly engineered strain, RBid, has satisfied the requirements, which is clearly reflected on the difference in the fermentation profiles between the new strain and the classical strain (Fig. 7). The engineered strain actually inherited wild-type levels of tolerances to various stresses including elevated temperatures, high osmolarity, and low oxygen tension, whereas the classical producer showed considerably less sensitivities to such stresses (data not shown). The cellular robustness which is intrinsic to the wild-type is occasionally regarded as a negative characteristic for metabolic engineering because of its counteractive effects on metabolic changes. However, in large-scale industrial fermentation, the ambient conditions vary considerably depending on the

location of cells within fermentor, and thus, the native robustness of an organism would be one of several important characteristics which should be retained in a producer strain working there.

As shown in Figure 6, strain RBid was generated ultimately by assembling the *argB26*, *ΔargR*, and *argB31* mutations derived from three different lines of classical producers, I-30, A-27, and D-77, respectively, on the wild-type genome. This shows that combining relevant mutations from independently developed classical producers could have a prominent effect on amino acid production. Furthermore, the combination of such mutations and a specific host is also an important consideration in this re-engineering approach. The host strain where the three mutations were incorporated is ATCC 13032 which was selected from among various *C. glutamicum* wild-type strains as a strain with high potential for industrial arginine/citrulline production. If another wild-type strain had been used as a host, such a clear result as presented here may not have been obtained.

The three specific mutations introduced into the wild-type are all relevant to the terminal pathway for arginine biosynthesis, and no mutation relevant to other metabolic pathways and regulations are included in the final strain. Nevertheless, the re-engineered strain achieved a high level of production above the classical producer with a long breeding history of more than 10 years. This result seems to suggest that carbon will direct toward a desired end-product from central metabolism if appropriate deregulation of a relevant terminal pathway is attained. However, it is also true that a comparable level of production as with the classical producer was attained by the introduction of the *leuC456* mutation into strain RB although fermentation was retarded in that case. Furthermore, it is certain that classical producer A-27 has no mutation in *argB*. These findings suggest alternative mechanisms of production as follows: a specific mutation such as the *leuC456* mutation might give rise to the stringent-like response leading to the global induction of the amino acid biosynthetic genes. This induction, coupled with the derepression of the *arg* operon by the *argR* defect, likely contributes to production through tremendous overexpression of the operon which we observed in the

transcriptome of the classical producer.

C. glutamicum has a long history of classical breeding, which results in a huge variety of industrially useful mutants. Their beneficial mutations have mostly lain idle within individual mutants, but their exploitation is now progressing in our laboratories. In near future, most of them will be regenerated for useful knowledge that is widely available for the amino acid industry. Thereby, the conventional style of selecting improved strains by phenotypes will undoubtedly be replaced by the new style of re-engineering strains by assembling only desired genotypes. This work as well as our previous studies on lysine fermentation (11, 21) will be a paradigm for future strain development in fermentation industries.

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FIGURE LEGENDS

FIG. 1. L-Arginine-biosynthetic pathways and the relevant genes in *C. glutamicum*. and *E. coli*. The broken arrows indicate the reactions specific to *E. coli* and the corresponding *E. coli* genes are shown in parentheses. The separate reactions specified by the *argA* and *argE* genes are coupled in case of *C. glutamicum* as a single reaction, which is specified by the *argJ* gene. Other reactions are common to both microorganisms.

FIG. 2. Reconstitutions of defined mutations on a wild-type genome and their effects on arginine/citrulline production. (A) The five specific mutations identified on the *arg* operons of three classical producers A-27, I-30, and D-77 are indicated under the corresponding *arg* genes. (B) Production abilities of wild-type ATCC 13032 carrying each mutation(s) were evaluated in shake flasks at 30ºC (black bars) and 38ºC (gray

bars) with classical producer A-27 and the wild-type as controls. Production is shown as the sum of arginine and citrulline. For reference, the molar ratio of arginine to citrulline is shown inside each bar as arginine:citrulline. Data represent mean values from three independent cultures. The standard deviations from the means are indicated as error bars.

FIG. 3. Comparison of the abilities to produce arginine/citrulline between two isogenic mutants, strain ATCC 13032 carrying the *argB26* and *ΔargR* mutations (●) and strain ATCC 13870 carrying the same two mutations (■). The molar ratios of arginine to citrulline on the final titers of the former and the latter strains were 82:18 and 81:19, respectively. Fermentation was carried out at 38°C using 5-liter jar fermentors. Values are means of replicated cultures, which showed <5% differences between each other.

FIG. 4. Ratio of mRNA levels of the *arg* genes in strain RB (white bars) and classical producer A-27 (gray bars) to those in their parental wild-type strains, ATCC 13032 and ATCC 13870, respectively. Total RNAs from mid-exponential phase cultures of 5-liter jar fermentors were used to study differential transcription profiles. Transcriptome analysis and the relevant experimental approaches were performed as described in Materials and Methods. Data are the mean values of two data sets for each gene, which showed <20% differences between each other.

FIG. 5. Arginine-sensitivities of various ArgB enzymes. Symbols: ■, ArgB activity of wild-type strain ATCC 13032; ●, ArgB activity of strain ATCC 13032 carrying the *argB26* and *ΔargR* mutations; ▲, ArgB activity of strain ATCC 13032 carrying the *argB31* and *ΔargR* mutations; ◆, ArgB activity of strain ATCC 13032 carrying the *argB26*, *argB31*, and *ΔargR* mutations; □, ArgB activity of strain ATCC 13032 whose endogenous *argB* was replaced by *E. coli argB*. Values are means of replicated assays, which showed <5% differences between each other.

FIG. 6. Schematic diagram of the creation of new strain RBid. Useful mutations identified in classical producers are indicated together with unnecessary mutations (×).

FIG. 7. Fermentation kinetics of the newly developed strain RBid at 38ºC in 5-liter jar fermentor cultivation. For comparison, the profiles of classical producer A-27 which was cultured under its optimal 30 ºC conditions are shown as controls. Symbols: ○, arginine/citrulline of strain RBid; ●, growth of strain RBid; □, arginine/citrulline of strain A-27; ■, growth of strain A-27. The molar ratios of arginine to citrulline on the final titers of strains RBid and A-27 were 66:34 and 90:10, respectively. Values are means of replicated cultures, which showed <5% differences between each other.

TABLE 1. Oligonucleotide primers

Primer[a]	Sequence (5'→3')[b]
arg op-F	CGCATCACTGGTCGCTTGTG
arg op-R	GTTTCCAACATGTCGGTGATGG
argB-F	AATGCGGCTCGCACTGTTGC
argB-R	ATCGGTGTACAGACCTTCCAC
argR-F	TCCTGCCTACCGTGGCAAAG
argR-R	TTGCATCAACAACGATGGACTC
argG-R	AGGGCGTCGACAAGCTCGG
argB26-R	TCGGCAGCAAAAACAGCCTTGA
argB26-F	ATCTCAAGGCTGTTTTTGCTGCC
argR-up-F (BamHI)	**CCTGA***GGATCC*CCCAGCAGGCCTTAAGGGTA
argR-up-R	CCCATCCACTAAACTTAAACAGCCAAGGGACATGTCTTACCT
argR-down-F	TGTTTAAGTTTAGTGGATGGGGATAGGGTGGGGCTGAAAGAA
argR-down-R (BamHI)	**AGGCA***GGATCC*CTCAGCGAACTCATCCTTTG
argJ-F (BglII)	**CGGCCG***AGATCT*TTGACACCCTGGATATTGATGG
argJ-R	AGCTTGATGATGAGTGGGTTCATCATCCATGGCAACGCCTCAGCG
EargB-F	ATGATGAAcCCAcTcATcATCAAgCTGGGCGGCGTACTGCTG
EargB-R (BamHI, BfrI)	**CGATAT***GGATCC***TGCTCAT***CTTAAG*CTAAAATCCGCGTACCC
argD-F (BfrI)	**TTTTAG***CTTAAG*AAGGCACCGTTTTTAGAAAAGA
argD-R (BamHI)	**CATCGG***GGATCC*TCAAGATGCCGTACTCATCGC

[a] Restriction endonuclease sites existing in primer sequences are indicated in parentheses.

[b] Restriction endonuclease sites are italicized, and linker bases are in bold type. The codons corresponding to the *argB26* mutation (A26V) in primers argB26-R and argB26-F are boxed. Lowercase letters in primer EargB-F indicate changed nucleotides to synonymous codons for codon optimization of the N-terminal coding region of *E. coli argB* in *C. glutamicum*. The complementary sequences in primer pairs are underlined.

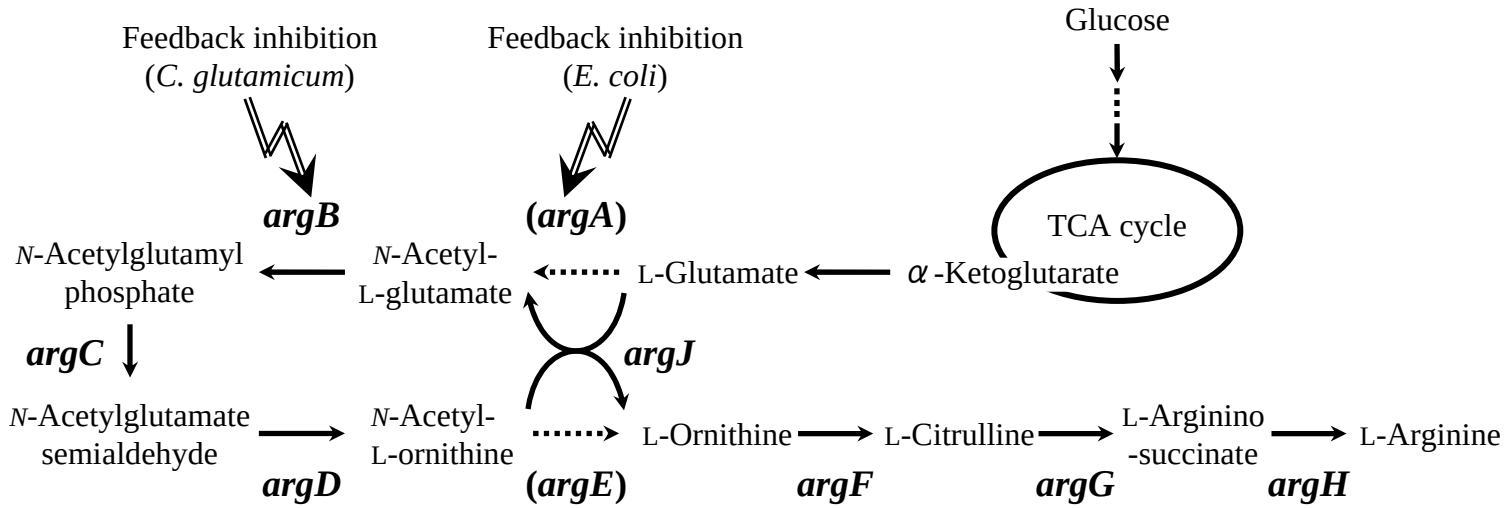


Fig. 1. Ikeda et al.

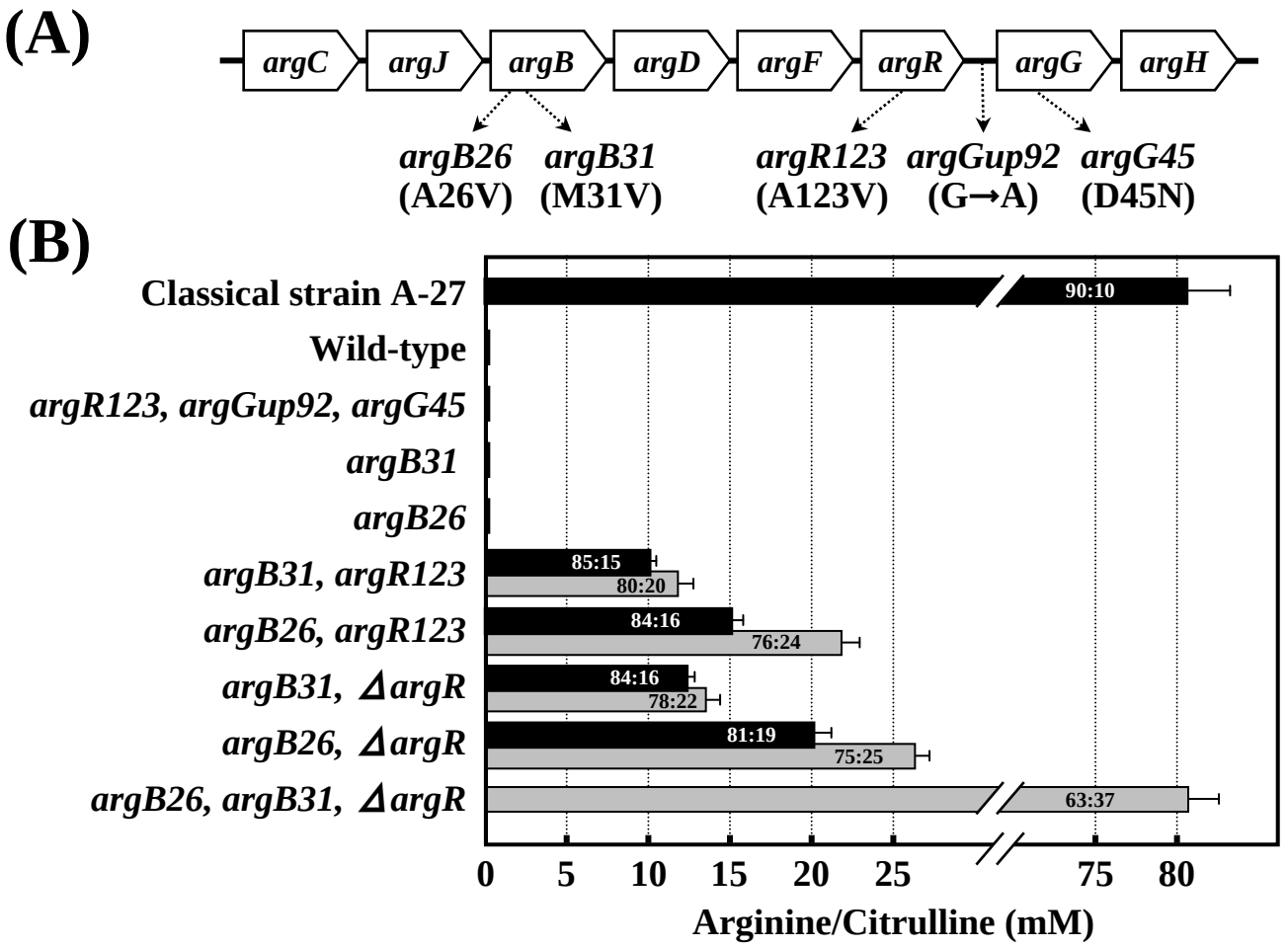


Fig. 2. Ikeda et al.

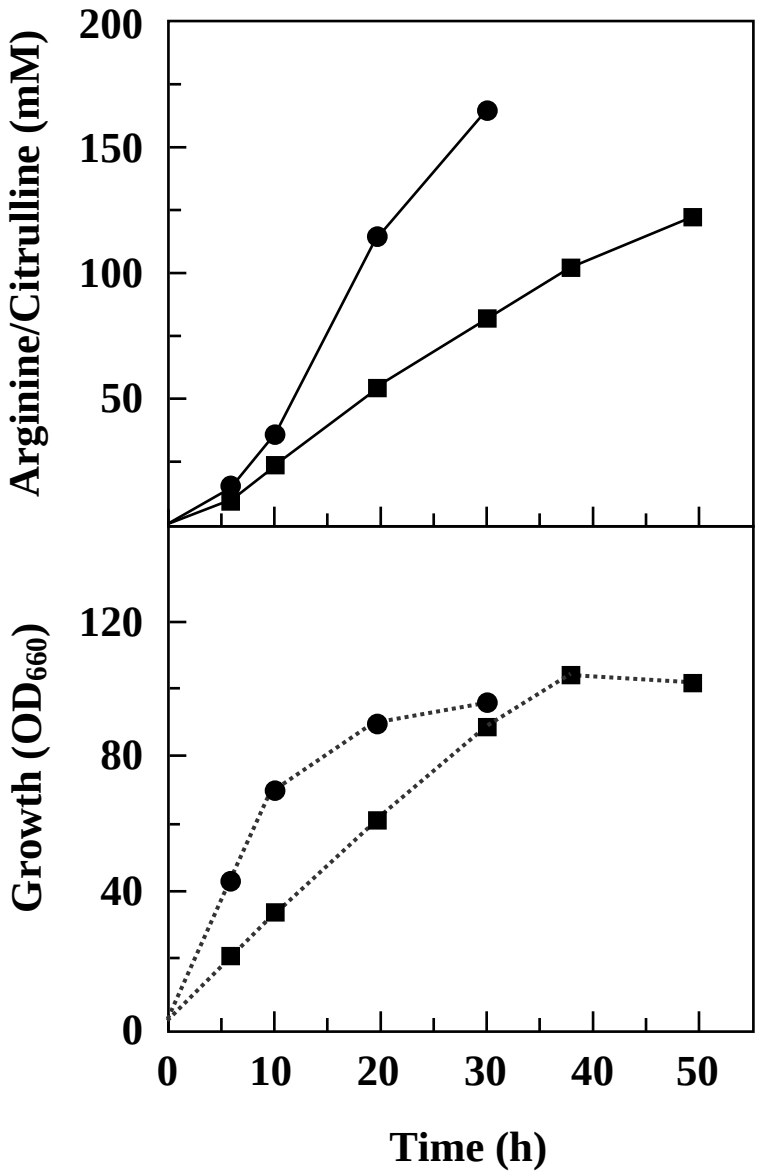
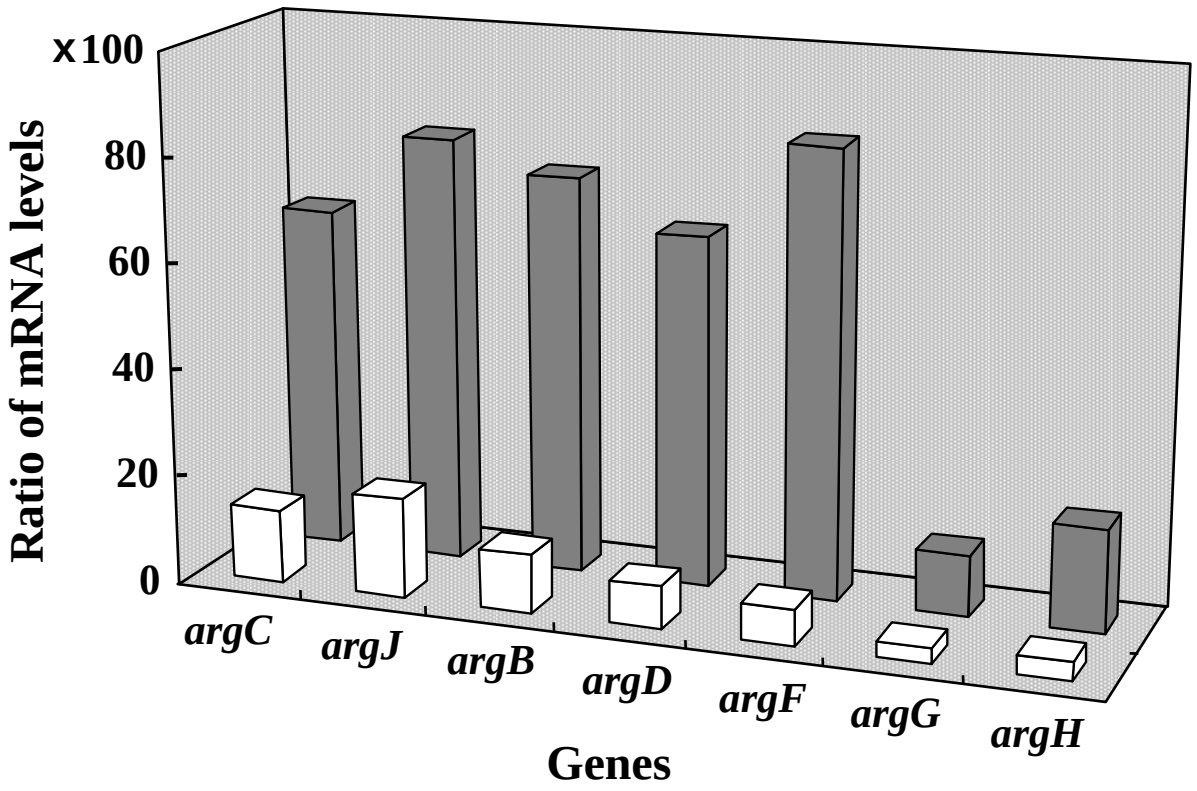


Fig. 3. Ikeda et al.



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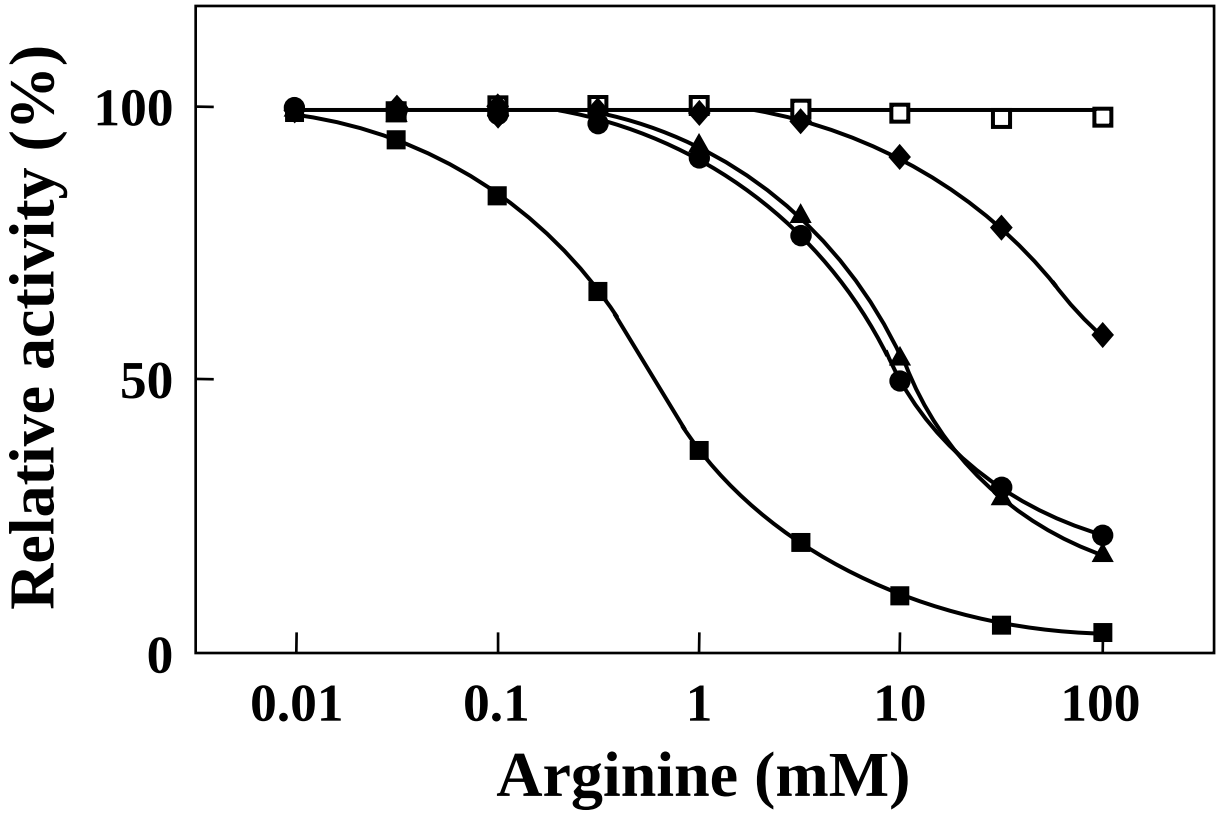

Fig. 5. Ikeda et al.

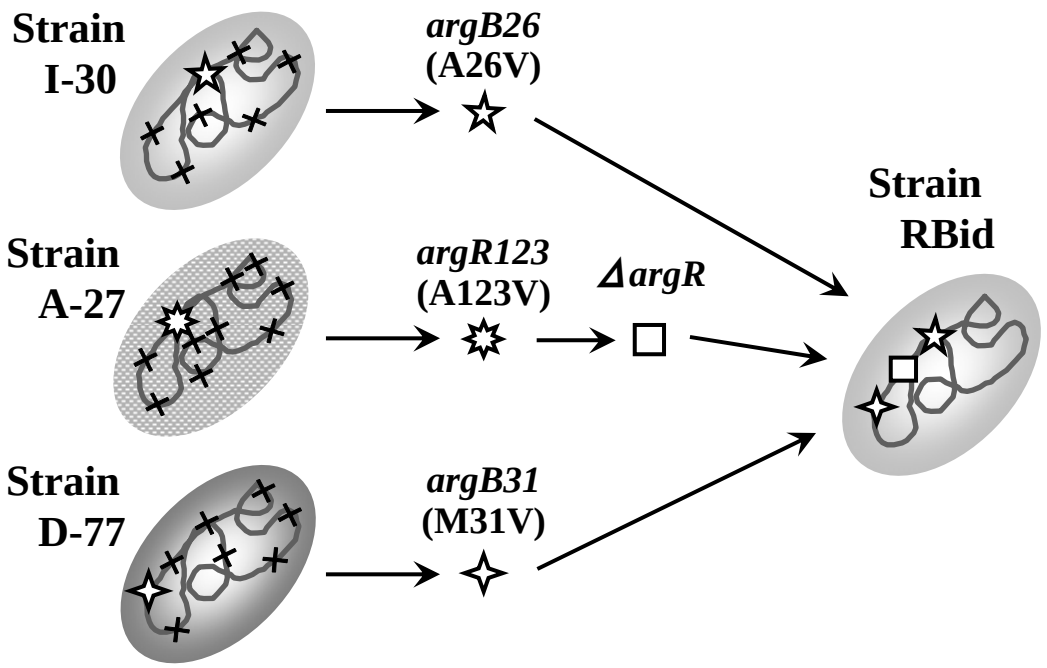

Fig. 6. Ikeda et al.

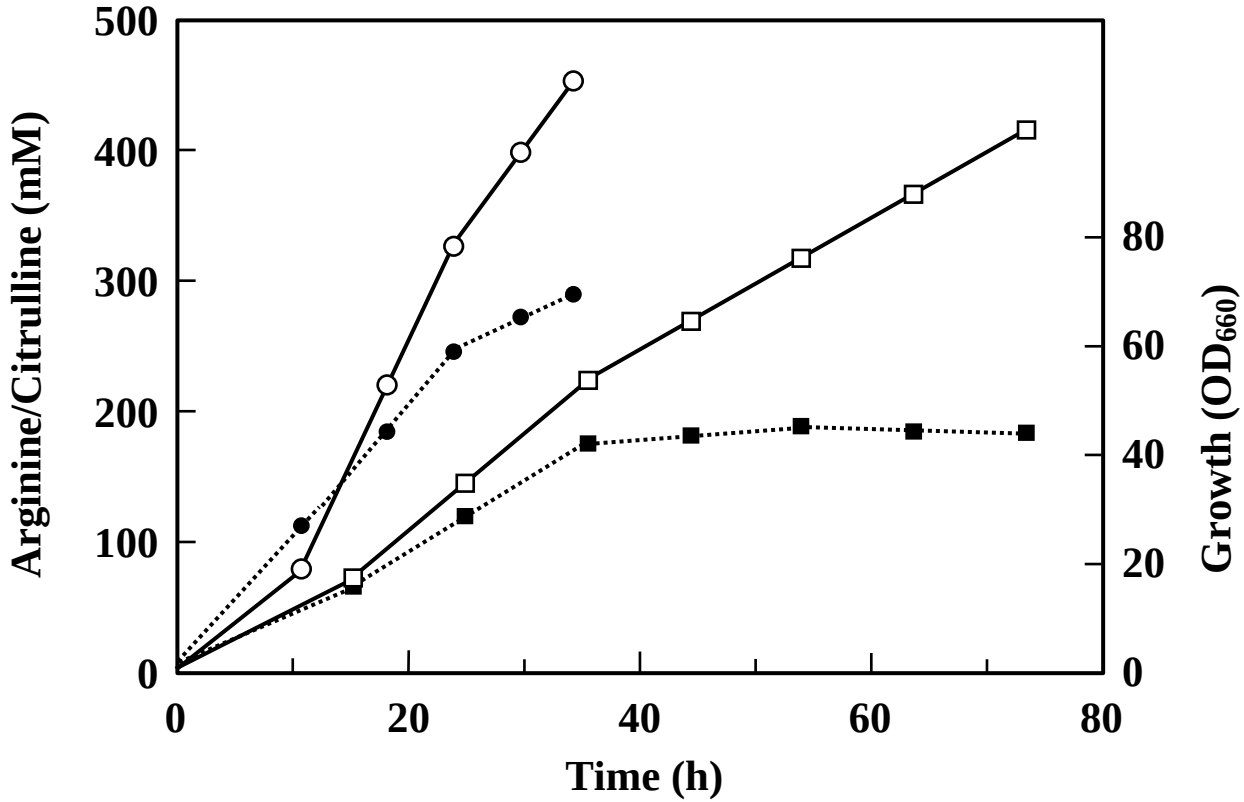

Fig. 7. Ikeda et al.