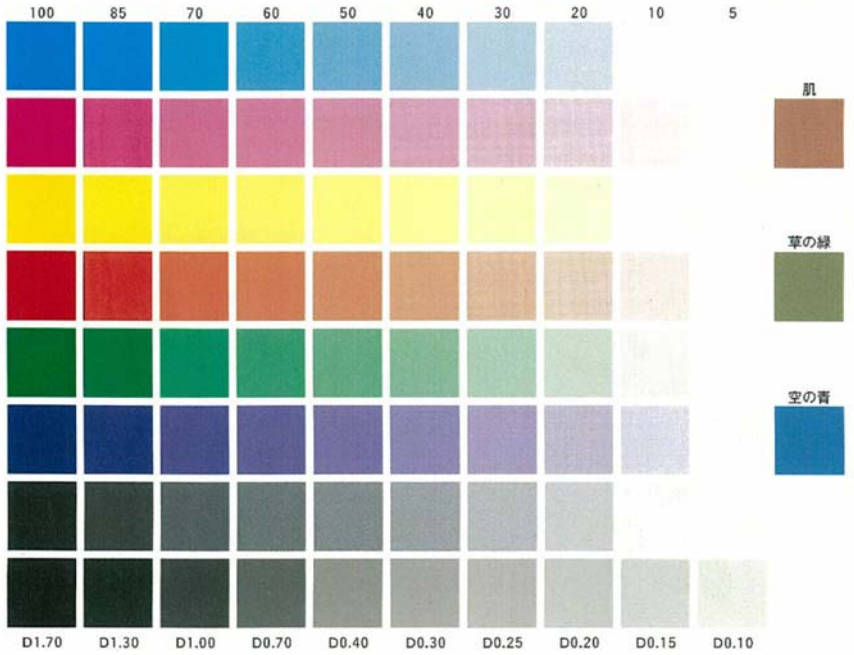

Studies on arginine racemase of *Pseudomonas taetrolens* NBRC 3460 : structure, functions, and application to D-amino acid production

2010

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CONTENTS

PREFACE

D-Amino acids have been considered as unnatural and unessential compounds in nature. But recently, according to the development of analytical technologies, D-amino acids have been found in various living organisms. In bacteria, D-alanine and D-glutamic acid exist as two of the essential components of a cell wall. In animal tissues and foods, various D-amino acids such as D-serine, D-alanine, and D-aspartic acid have been found and some physiological functions of them are extensively studying in detail. Amino acid racemase is one of the representative enzymes concerning D-amino acid metabolisms of various organisms.

The arginine racemase (ArgR) of *Pseudomonas taetrolens* NBRC 3460 catalyzes the racemization of arginine, lysine, and various other amino acids except acidic and aromatic amino acids (94, 95). The broad substrate specificity of the enzyme is unique, and almost all other amino acid racemases such as alanine racemase (62), glutamate racemase (26), and ornithine racemase (12) show a high substrate specificity.

In this thesis, I studied the structure, functions, and application to D-amino acid production of the arginine racemase of *Pseudomonas taetrolens* NBRC 3460. In chapter I section I, I describe the first identification of a pyridoxal 5-phosphate (PLP)-containing amino acid racemase of *P. taetrolens* NBRC 3460 located in a

periplasm. In chapter I section II, I described the physiological function of the arginine racemase of *P. taetrolens* NBRC 3460. In chapter II, I describe the primary structure of the arginine racemase of *P. taetrolens* NBRC 3460 by MALDI-TOF MS, the construction of a C47A/C73A-ArgR by site directed mutagenesis, and the function of these two cysteine residues. In chapter III, I describe D-lysine production by using an L-lysine overproducer of *Corynebacterium glutamicum* ATCC 13032 *lysC* T311I strain harboring an arginine racemase of *P. taetrolens* NBRC 3460, and D-ornithine production by using an L-ornithine overproducer of *C. glutamicum* ATCC 13032::*argF* compared with that of the wild-type strain.

CHAPTER I / SECTION I

A periplasmic, pyridoxal 5'-phosphate-dependent amino acid racemase in *Pseudomonas taetrolens* NBRC 3460

INTRODUCTION

The pyridoxal 5'-phosphate (PLP) dependent amino acid racemases catalyze the racemerization of their substrates by removal of an α-hydrogen bound from a chiral carbon followed by the nonspecific return of a hydrogen to the same carbon. A number of different amino acid racemases of this type has been studied, and the racemases differ remarkably with respect to their substrate specificity. An example for a highly specific amino acid racemase is the alanine racemase, like that of *Bacillus psychrosaccharolyticus* (63) or that of *Corynebacterium glutamicum* ATCC 13032 (62) which is active with alanine only, but not with the structurally related serine, amino butyrate, not to mention aspartate, glutamate, methionine, or lysine. These enzymes, encoded by *alr*, act on L-alanine to provide D-alanine for cell wall synthesis and they are therefore widespread among bacteria to ensure provision of D-alanine as a cell wall building block. In addition to this anabolic alanine racemase, a catabolic alanine racemase is also known. This latter enzyme is less frequently distributed and encoded by *dadX* (but also annotated as *alr*). Thus, *E. coli* K12 has both *alr* and *dadX* (86). Another example of a highly specific amino acid racemase is the catabolic ornithine racemase of *Clostridium sticklandii* DSM 519. The latter is active with ornithine only, but not with L-lysine, L-arginine, L-methionine, or other amino acids (12). In contrast

to these specific racemases, the broad substrate specificity racemases are less frequent. An example for such a racemase with broad substrate specificity is an arginine racemase isolated from *Pseudomonas taetrolens* NBRC 3460 as early as 1971 (94, 95). Its analysis revealed activity with the basic amino acids D-arginine, L-lysine, and L-ornithine, and further amino acids, but almost no activity with hydrophobic, acidic or aromatic amino acids. It was designated arginine racemase, but proteins with similar enzymological features and termed BsRC (for broad specificity racemase) are also present in *Pseudomonas putida* ATCC 17642 (48, 73), or *Aeromonas punctata* subsp. caviae (34), suggesting a close relationship of the proteins. Recently, the gene sequence of *P. putida* ATCC 17642 became available, and the rather weak racemase activity of wild-type BsRC with L-tryptophan as substrate was evolved to increase its activity with this specific substrate about 20-fold (39). Although there is some information on the enzymological features of broad substrate specificity racemases, which is in part due to their relaxed substrate specificity for preparing D-amino acids, there is only limited information on the physiological function of these enzymes. I therefore started to clone the gene encoding the broad substrate specificity racemase of *P. taetrolens* NBRC 3460 but noticed that the open reading frame was apparently larger than the peptide sequence of *P. putida* ATCC 17642 found in the literature (39).

Moreover, I found distracting that in *P. putida* KT2440 (ATCC 47054) which is one of the fully sequenced *P. putida* strains currently available, there are two genes annotated alanine racemases (58). Based on this information, I started to analyze in detail the corresponding genes and enzymes, respectively, of *P. taetrolens* NBRC 3460 leading to the first identification of a PLP-containing amino acid racemase located in a periplasm.

MATERIALS AND METHODS

Materials. The amino acids D- and L-arginine, D- and L-alanine, D-ornithine, and D-lysine were purchased from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). L-Ornithine and L-lysine were purchased from Wako Chemical Co. (Osaka, Japan). DEAE-Toyopearl 650 M and Butyl-Toyopearl 650 M were obtained from Tosoh (Tokyo, Japan). Restriction endonucleases, Ligation mix, and 10 X A attachment mix were purchased from Toyobo (Osaka, Japan) and Takara Bio (Shiga, Japan). All other chemicals were purchased from Kanto Kagaku Co. (Tokyo, Japan), Kishida Chemical Co. (Osaka, Japan), Sigma-Aldrich Co. (MO, USA), or Tokyo Kasei Kogyo (Tokyo, Japan) unless otherwise stated and were of the best commercially available grade.

Strains and growth. The *P. taetrolens* strain used was obtained from the NITE Biological Resource Center, National Institute of Technology and Evaluation, Chiba, Japan as strain NBRC 3460. Aliases of this strain are *P. graveolens* IFO 3460, and *P. taetrolens* ATCC 4683. The further strains and plasmids used are given in Table I-I-1. The medium used for cultivation of *P. taetrolens* NBRC 3460 was usually LB. For carbon source utilization studies, minimal medium No. 154 was used (83). Cells were grown in a 50 ml medium in a 500-ml baffled Erlenmeyer flask at 30°C and 160 rpm on

a rotary shaker. The growth was measured photometrically at 600 nm by UV-VIS photometer V-550 (Jasco Co. Ltd., Tokyo, Japan).

Table I-1 Strains, plasmids, and primers used

	Comments
Strains	
E. coli BL21 (DE3)	Expression host
E. coli GSJ101	Derivative of GSJ100 deleted of Tat apparatus (6)
E. coli GSJ100	Control strain (6)
P. taetrolens NBRC 3460	Wild type
P. taetrolens NBRC 3460::*argR*	Wild type with *argR* disrupted
Plasmids	
pET11b-*argR*	Expression vector, contains full-length *argR*
pET21b-*alr*	Expression vector, contains *C*-terminal His-tagged *alr*
pK18mob-*argR*-int	Non-replicative vector for disruption of *argR*
pBBR1MCS2	Low copy number vector (41)
pBBR1MCS2ArgR-His	Vector with *C*-terminal His-tagged full-length *argR*
pBBR1MCS2ArgR	Vector with full-length *argR*
Primers	
A	GTGCTCAA(A/G)GC(C/G)GA(C/T). First amplification of *argR*
B	GGTGCGGTC(A/G)TAGCC(C/G)AC. First amplification of *argR*
C	GACGCCTA(C/T)GG(A/T/C)CA(C/T)GGT. Second amplification of *argR*
D	GGTGCGGTC(A/G)TAGCC(C/G)AC. Second amplification of *argR*
E	TCAGCATATGCCCTTCTCCCGTAC. Amplification of *argR*
F	GACGGATCCTGGTTCAATATACGG. Amplification of *argR*
G	TATCCATATGCGTCCCGCCCGCGCCC. Amplification of *alr*
H	ATAGGATCCCGTTCGCCGATGTA. Amplification of *alr*

Gene isolation and cloning. Chromosomal DNA was isolated using the FastPure kit of TaKaRa (TaKaRa, Kyoto, Japan). Primers A and B, 0.2 pmol of each, were used together with 1.4 μg chromosomal DNA from *P. taetrolens* NBRC 3460 as a template to generate an amplification product at an annealing temperature at 40°C. This was used as the template in a second PCR at an annealing temperature of 52°C with primers C and D. The product was purified, cloned in pT7 Blue T, and sequenced using the DNA sequencer SQ5500E (Hitachi High-Technologies Corp., Tokyo, Japan). Based on the partial sequence obtained, new primers were designed to perform genome-walking PCR with the LA PCR™ *in vitro* cloning kit supplying HindIII and EcoRI cassettes (TaKaRa, Kyoto, Japan). Based on the 5′- and 3′-ends of *argR* obtained, primers E, F were designed to amplify the entire *argR* gene from genomic DNA of *P. taetrolens* NBRC 3460. The product obtained was digested with NdeI and BamHI, and ligated into NdeI/BamHI digested pET11b to form pET11b-*argR*. Cloning of *alr* was similarly achieved, involving a final amplification step with the primer pair G and H, digestion of the amplification product with NdeI and BamHI, and ligation with the accordingly treated vector to yield pET21b-*alr*. To generate the inactivation vector pK18mob-*argR*-int, an internal HincII fragment of *argR* derived by restriction was prepared from pET11b-*argR*, which was ligated with SmaI-cleaved pK18mob.

Subsequently, pK18mob-*argR*-int was used to transform *P. taetrolens* NBRC 3460 by electroporation to kanamycin resistance. One colony was isolated and PCR analyses used to confirm disruption of *argR*.

Isolation of arginine racemase. Cells of *E. coli* BL21 (DE3) (pET11b-*argR*) were harvested from 1.2 l LB medium and washed twice with cold 0.85% (w/v) NaCl, followed by resuspension in a 74 mM Tris–HCl (pH 8.0) buffer containing 0.5 M sucrose and 1 mM EDTA (buffer A). After centrifugation at 8,000 X *g* for 20 min, the cells were resuspended in ice-chilled water, and after brief incubation centrifuged at 5,500 X *g* for 10 min. The supernatant was collected and used as a cell-free extract. This was dialyzed against a 10 mM potassium phosphate buffer (pH 7.3) containing 0.01% (v/v) 2-mercaptoethanol and 20 μM pyridoxal 5′-phosphate (PLP) (buffer B). The enzyme solution was applied to a column of DEAE-Toyopearl 650 M (2.5 X 30 cm) equilibrated with buffer B, and washed with the same buffer containing 40 mM KCl. Proteins were eluted with a linear gradient of KCl at a concentration of 40 to 80 mM in buffer B, and active fractions combined and dialyzed against buffer B containing 1.2 M $(NH_4)_2SO_4$. The resulting solution was applied to a column of Butyl-Toyopearl 650 M (1.25 X 15 cm) equilibrated with the same buffer, and the column washed with buffer B

containing 0.9M $(NH_4)_2SO_4$. Proteins were eluted with a linear gradient of $(NH_4)_2SO_4$ at a concentration of 0.9 to 0.7 M in buffer B, and active fractions combined and dialyzed against buffer B. Finally, the enzyme solution obtained was applied a second time to DEAE-Toyopearl 650 M chromatography under identical conditions as described to yield pure arginine racemase which was dialyzed and stored frozen in buffer B. Alr was prepared from *E. coli* BL21 (DE3) harboring pET21b-*alr* which was disrupted by sonification. The cell-free extract was dialyzed against a 10 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl and 10 mM imidazole (buffer C), and the protein solution applied to a Ni-NTA agarose column (1.0 X 10 cm) equilibrated with the same buffer. The column was washed with buffer C but containing 50 mM imidazole, and eluted by increasing the imidazole concentration to 250 mM. Alr was finally dialyzed and stored in buffer B.

Analysis of phosphopyridoxyl peptide. The arginine racemase was reduced with sodium borohydride, digested with trypsin and the peptides were separated by HPLC on a Wakosil-II 5C-18 AR column (Wako, Osaka, Japan). Fluorescence detection was at 330 nm excitation and 390 nm emission. The fluorescent phosphopyridoxyl peptide was isolated and subjected to Edman degradation using an automated protein sequencer

(PPSQ-21, Shimadzu Co., Kyoto, Japan).

Enzyme assays. Amino acid racemase activities with D-arginine or D-lysine as a substrate were in part measured in coupled assays with L-arginase (94, 95) or L-lysine 6-aminotransferase (88). However, generally, amino acid racemase activities were measured via HPLC quantification of the respective enantiomer formed (65). The assay mixtures contained a 0.6 M CHES–NaOH buffer, pH 10.0 (130 µl), 0.1 M L-amino acid (80 µl), 0.1 mM PLP (200 µl), and enzyme. Reactions were carried out at 37°C for different time intervals, stopped by heat treatment at 100°C for 2 min, and subjected to HPLC analysis. Protein was determined with bovine serum albumin as standard, with one unit being defined as the amount of enzyme producing one µmol of enantiomer per min.

Localization studies. Fractionation of *P. taetrolens* NBRC 3460 and *E. coli* BL21 (DE3) harboring pET11b-*argR* was essentially as described (57). Both bacteria were pre-cultured at 30°C in 5 ml LB and 100 µl of this pre-culture transferred into four Sakaguchi flasks (500 ml) containing 100 ml LB and cultivated at 30°C aerobically. Per data point, cells of one flask were harvested by centrifugation at 14,000 X *g* for 20

min at 4°C. The supernatant was collected as the extracellular fraction. The pelleted cells were washed twice with 0.85% (w/v) NaCl. The washed cells were subsequently resuspended in buffer (40 ml per 1 g wet weight) containing 500 mM sucrose and 1 mM EDTA in a 30 mM Tris–HCl (pH 8.0) for disruption by osmotic pressure. After centrifugation at 14,000 X *g* for 20 min at 4°C, the cell debris was resuspended in deionized water and centrifuged again. The supernatant obtained was collected as periplasmic fraction. The residual cell debris was disrupted by ultrasonication and the supernatant was obtained after centrifugation used as cytosolic fraction. Alkaline phosphatase and *β*-galactosidase activities were quantified as marker enzymes present in the periplasm and cytosol, respectively, as described (15, 78).

Translocation studies. Fractionation of *E. coli* GSJ 100 and *E. coli* GSJ 101 into a fraction containing the cytoplasm together with membranes (CP/M) and a periplasmic fraction (PP) was done by using an EDTA-lysozyme spheroplasting method as described earlier (42). Also SDS-PAGE and Western blotting of fractions which correspond to an identical number of cells is described in the reference. For the detection of the His-tagged arginine racemase protein, a penta-His-antibody (Qiagen, Hilden, Germany) was used as the primary antibody in a dilution of 1:1,000. The

detection of transaldolase B protein (TalB) was carried out using an anti-transaldolase B antibody in a dilution of 1:1,000. Further processing was done by use of the ECL Western blotting detection kit (GE Healthcare, Munich, Germany) according to the manufacturer's instructions. The chemiluminescent protein bands were recorded using the Fujifilm LAS-3000 Mini CCD camera and image analyzing system together with the software AIDA 4.15 (raytest Inc., Wilmington, USA).

Accession numbers. The nucleotide sequence for *P. taetrolens* NBRC 3460 *argR* is deposited at DDBJ/EMBL/GenBank as AB096176, that for *alr* as AB296103.

RESULTS

Cloning of *argR* and *alr*. Sequences of the broad specificity amino acid racemases of *Pseudomonas striata* (73) and *Pseudomonas putida* IFO 12996 (39) were used as the basis to enable isolation of *argR* from *P. taetrolens* NBRC 3460. Primers A and B (Table I-I-1) close to the conserved PLP binding site were designed and used to amplify *argR* by PCR applying chromosomal DNA of *P. taetrolens* NBRC 3460 as the template. Two subsequent amplifications were made with increasing annealing temperature, the resulting fragments cloned, and one of them was shown by sequencing to possess the expected PLP motif. This fragment was used for isolation of the entire *argR* by genome-walking which was done with the LA PCR™ *in vitro* cloning kit to result in pET11b-*argR* containing a 3,160 bp genomic fragment of *P. taetrolens* NBRC 3460, with the open reading frame termed *argR*. The arginine racemase gene of *P. taetrolens* NBRC 3460 consists of 1,227 bp and encodes a protein of 408 amino acids. In order to study also the alanine racemase gene of *P. taetrolens* NBRC 3460 for comparison, I used the genome sequence of *P. putida* KT2440 (58) to design primers G and H within the gene annotated *alr*. Using PCR amplification and genome-walking in a procedure similar to that described above the *alr* gene from *P. taetrolens* NBRC 3460 was obtained in pET21b-*alr*. The gene consists of 1,074 bp and encodes a protein of 357 amino

acids.

Isolation of arginine racemase and alanine racemase proteins. Arginine racemase was isolated from *E. coli* BL21 (DE3) harboring pET11b-*argR* enabling expression of the native enzyme without any tag. As shown in Table I-I-2, the isolation procedure based on ion exchange and hydrophobic interaction chromatography yielded 16-fold enriched enzyme with a high specific activity of 1,120 $\mu mol^{-1} min\ mg^{-1}$. After dialysis of the protein against a 10 mM potassium phosphate buffer (pH 7.3) containing 10 mM hydroxylamine for 12 h, the activity was lost. However, the activity was restored to 80% of its initial value upon incubation of the apoenzyme with 10 mM PLP, indicating reconstitution of the protein by PLP addition. The apoenzyme and the PLP-enzyme were reduced with sodium borohydride, and digested with trypsin. Separation of the peptides by reversed-phase liquid column chromatography yielded one strong fluorescent peak in the PLP-enzyme preparation, which was absent in the apoenzyme treated identically. This phosphopyridoxyl peptide was isolated and sequenced, yielding the sequence $SQIX_1AVLX_2ADAYGHGIGL$ corresponding to amino acyl residues 73–83 of the protein as derived from the nucleotide sequence. The X-residues could not be determined. According to the DNA sequence X_1 is a cysteine and X_2 a

lysine, the latter lysine apparently serving to establish the aldimine linkage between its epsilon-amino group and the carbonyl group of PLP. The isolated arginine racemase was furthermore subjected to Edman degradation and the amino-terminal sequence APPLSMTDGV determined. This is at variance with the peptide sequence derived from the *argR* open reading frame used and as present in the *P. taetrolens* NBRC 3460 genome. It corresponds to amino acids 24 to 33 of the deduced peptide sequence, suggesting that native arginine racemase is made as a preprotein processed by cleavage (see below). Alr was isolated from *E. coli* BL21 (DE3) pET21b-*alr* as a carboxy-terminal 6 X His-tagged protein. The homogeneous protein was dialyzed against buffer B and stored at −20°C, as was the arginine racemase protein.

Table I-I-2. Isolation of arginine racemase

Steps	Total activity (U)	Total protein (mg)	Specific activity ($\mu mol\ min^{-1}\ mg^{-1}$)	Yield (%)	Fold enrichment (–)
Cell-free extract	127,000	1,810	70.2	100	1.0
DEAE-Toyopearl	118,000	140	840	92.9	12.0
Butyl-Toyopearl	78,800	72.3	1,090	62.0	15.5
DEAE-Toyopearl	56,000	50.0	1,120	44.0	16.0

Enzyme properties of arginine racemase and alanine racemase proteins. Using gel filtration on a calibrated column a mass of 84 ± 11 kDa was determined for arginine racemase, and one of 79 ± 16 kDa for alanine racemase. Since the calculated monomer mass of mature arginine racemase is 41.5 kDa and of Alr 38.8 kDa, both native enzymes adopt a dimeric structure. The optimum activity of the Alr was at pH 8.0 and this was similar the case for arginine racemase (94). Whereas alanine racemase showed maximal activity at 35°C, this was the case for arginine racemase only at 65°C. The activation energy as calculated from Arrhenius plots was 126 kJ mol^{-1} for Alr with L-Ala and 61.8 kJ mol^{-1} for arginine racemase with L-Arg as a substrate. The specificities of both racemases were determined with L-amino acids as substrates by quantification of the formed enantiomers via HPLC (65). Arginine racemase was most active with L-Lys and L-Arg, whereas with L-Ala only a minor activity was obtained (Table I-I-3), and these values largely agree with that described (94). With Phe as substrate only a trace activity was detectable, and with L-Glu the enzyme was inactive. Interestingly, the BsRC of *P. putida* IFO 12996 exhibits subtle but significant differences in its specificity (39). In contrast to arginine racemase of *P. taetrolens* NBRC 3460 with its broad substrate specificity, the alanine racemase Alr of this organism exhibited activity with L-Ala only and acted neither on L-Arg nor on any other

of the amino acids tested (Table I-I-3).

Table I-I-3. Substrate specificities of arginine racemase and alanine racemase

Substrate	Arginine racemase (%)	Alanine racemase (%)
L-Alanine	12.4	100
L-Lysine	100	0
L-Arginine	95.2	0
L-Ornithine	42.0	n.d.
L-Methionine	12.4	0
L-Ethionine	11.8	n.d.
L-Homoarginine	6.2	n.d.
L-Norvaline	6.5	n.d.
L-Leucine	1.7	0
L-2-Aminobutyrate	1.4	n.d.
L-Phenylalanine	0.02	0

n.d. Not determined

Amino acids were used in a concentration of 40 mM

Localization of arginine racemase in *P. taetrolens* NBRC 3460. Following the observation that arginine racemase was 23 amino acids shorter than expected from the gene sequence, and that SignalP predicted a cleavage site for the full-length protein (4), I studied the localization of the protein in *P. taetrolens* NBRC 3460. Supernatants of cell suspensions yielded the extracellular enzyme fraction, whereas osmotically shocked cells yielded periplasmic and cytoplasmic fractions (57). As shown in Fig. I-I-1, the greatest racemase activities of cultures grown to different A_{600} were always localized in

the periplasm, whereas significantly lower activities were present in the cytosol and the supernatant.

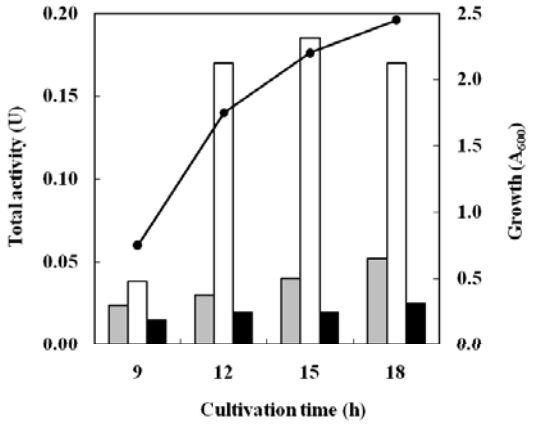


Fig. I-I-1. Localization of arginine racemase activity in *P. taetrolens* NBRC 3460.

Cells were grown on complex medium containing L-arginine with the line showing the growth curve. Samples were removed at the 4 different time points indicated and processed to derive cytoplasmic (gray bar), periplasmic (empty bar), and extracellular fractions (black bar)

Localization of arginine racemase in *E. coli* BL21 (DE3). In addition, localization in *E. coli* BL21 (DE3) harboring pET11b-*argR* used for protein isolation was studied. For this purpose, *E. coli* BL21 (DE3) was grown in 100 ml volumes to four different time points, fractionated, and the arginine racemase activity together with that of alkaline phosphatase and β-galactosidase determined, the latter enzymes serving as lead

enzymes for either periplasmic or cytoplasmic localizations. As shown in Table I-I-4, the largest fractions of arginine racemase activity were present in the periplasmic fraction, and the contribution of the cytoplasmic fraction plus the medium fraction to the total activity was at best 16%. The periplasmic alkaline phosphatase and the cytosolic β-galactosidase quantified as control localized as expected. This confirms that the broad substrate specificity racemase also localizes in *E. coli* BL21 (DE3) in the periplasm and means processing of arginine racemase in the heterologous host.

Table I-I-4. Localization of arginine racemase in *E. coli* BL21 (DE3)

Culture time (h)	Localization	Alkaline phosphatase	β-Galactosidase	Arginine racemase
5	Extracellular	11	0	6
	Periplasm	84	20	92
	Cytoplasm	5	80	2
6.5	Extracellular	3	1	4
	Periplasm	85	7	89
	Cytoplasm	12	92	7
8	Extracellular	1	1	5
	Periplasm	93	6	89
	Cytoplasm	6	93	6
9.5	Extracellular	6	1	8
	Periplasm	88	10	84
	Cytoplasm	6	89	8

Numbers are percentages of the respective enzyme at the respective time point. Total ArgR activity at 5, 6.5, 8, and 9.5 h was 7, 22, 59, and 75 U mg(protein)$^{-1}$, and the respective growth (A_{600}) 0.6, 0.9, 2.4, and 3.3, respectively

Transport of arginine racemase in *E. coli* (GSJ100) and *E. coli*::*tat* (GSJ101). As shown above, enzymatically active arginine racemase can be detected in the periplasm when *argR* is expressed in *E. coli*. The arginine racemase signal peptide lacks a typical Tat-consensus motif and possesses only one arginine at the boundary between the n- and the h-region of the deduced signal peptide, suggesting that arginine racemase might be exported into the *E. coli* periplasm separately from its PLP cofactor via the Sec pathway. However, since it is possible that arginine racemase may be a Tat-substrate with an atypical Tat-consensus motif, the subcellular localization of a *C*-terminal His-tagged version of arginine racemase (ArgR-His) was determined in an *E. coli* wild-type strain and its isogenic Tat-defective mutant derivative, in which all known *tat* genes were deleted (6). *E. coli* (GSJ100) and *E. coli*::*tat* (GSJ101) cells expressing either untagged ArgR or ArgR-His were fractionated into a cytoplasm/membrane (CP/M) fraction and a periplasmic (PP) fraction by EDTA-lysozyme spheroplasting. The fractions were subsequently analyzed by SDS-PAGE and Western blotting, using anti-5 X His antibody for the detection of arginine racemase (Fig. I-I-2a). As a control for the quality of the fractionation experiments, the subcellular distribution of the cytoplasmic enzyme transaldolase B was analyzed in parallel (6). As expected, transaldolase B was found exclusively in the CP/M fraction of all cells examined (Fig.

I-I-2b). As shown in Fig. I-I-2a, various polypeptides were detected with the anti-5His antibody in the subcellular fractions of the strains expressing the ArgR-His (lanes 1 to 4), but not in the strain expressing the untagged ArgR (lanes 5 and 6). In the CP/M fraction of both the *E. coli* wild-type (lane 1) and the *E. coli*::*tat* strain (lane 3) expressing ArgR-His, a band (p) is detected by the anti-5 X His antibody that corresponds in molecular weight to the unprocessed precursor of ArgR. In addition, a smaller migrating form (*) of ArgR-His is detected in the same fraction of both strains. Most likely, this ArgR-His-derived polypeptide corresponds to a cytosolic degradation product. Importantly, however, in both the *E. coli* wild-type strain (lane 2) and the *E. coli*::*tat* strain (lane 4), a band (m) of identical intensity is detected by the anti-5 X His antibody in the periplasmic fraction. This ArgR-His-derived polypeptide corresponds in size to the correctly processed mature form of ArgR-His. Since no difference in the subcellular distribution of ArgR-His-derived polypeptides (nor in the enzymatic activities of the corresponding subcellular fractions) is detected between the Tat-proficient wild-type strain and the Tat-defective ::*tat* mutant strain, my results clearly demonstrate that membrane translocation of arginine racemase occurs independently of a functional Tat system in *E. coli*.

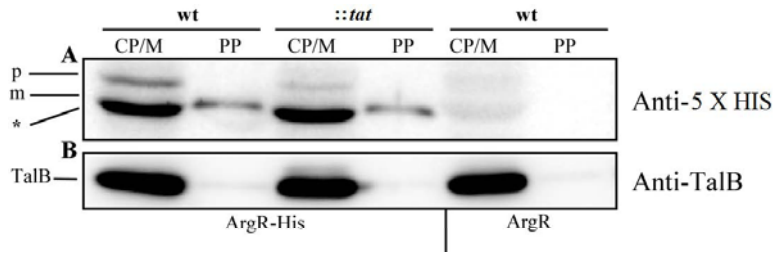


Fig. I-I-2. Subcellular localization of His-Tagged arginine racemase polypeptides.

Cells of the tat wild-type (wt) strain *E. coli* GSJ100 and the tat deletion mutant (::*tat*) GSJ101 containing pBBR1MCS2ArgR-His or pBBR1MCS2ArgR were fractionated into a CP/M fraction and a PP fraction by EDTA lysozyme spheroplasting as described in "MATERIALS AND METHODS" section. Samples were subjected to SDS-PAGE and Western blotting using anti-5xHis antibodies (a) or, as a control, using antibodies directed against the cytoplasmic protein TalB (b). *p* ArgR-His precursor, *m* exported mature form of ArgR-His in the PP fraction, asterisk position of the ArgR-His degradation product in the CP/M fraction.

Utilization of amino acids due to arginine racemase. To permit studies to be made on the physiological function of arginine racemase, the non-replicative vector pK18mob-*argR*-int was constructed to enable disruption of *argR* by homologous recombination (see "MATERIALS AND METHODS" section). The vector was used to transform *P. taetrolens* NBRC 3460 to kanamycin resistance and the correct integration was verified by PCR. The resulting strain was named *P. taetrolens* NBRC 3460::*argR*. An enzyme assay using L-Lys as substrate yielded an activity below 0.01 μmol min^{-1} mg $(protein)^{-1}$, whereas it was 0.43 μmol min^{-1} mg $(protein)^{-1}$ for the wild type, thus confirming absence of racemase activity in the mutant. In minimal medium

(83), I studied the utilization of 10 mM L-, and D-amino acids as a carbon source. Figure I-I-3 shows that the basic amino acids D-Lys, L-Lys, D-Arg, L-Arg, and L-Orn were used to roughly the same extent by *P. taetrolens* NBRC 3460 wild type, whereas D-Orn enabled rather slow growth. Also, the enantiomers of Ala could be utilized as carbon source, whereas this was not the case with Met. In contrast to this, *P. taetrolens* NBRC 3460::*argR* was no longer able to utilize D-Lys, and also the utilization of D-Arg was strongly reduced, showing that arginine racemase plays a key role in the metabolization of these basic D-amino acids. The slightly influenced L-Orn utilization also observed remains unexplained but could indicate, for instance, regulatory interactions of the unknown amino acid degradation pathways. In addition to growth on the synthetic medium, also LB was assayed. It was observed that growth of *P. taetrolens* NBRC 3460::*argR* was slightly retarded to a growth rate of 0.36 h^{-1} as compared to 0.59 h^{-1} for the wild type, which might indicate that arginine racemase is also generally involved in amino acid degradation. The addition of D-Ala did not influence the growth of the wild type or that of the mutant.

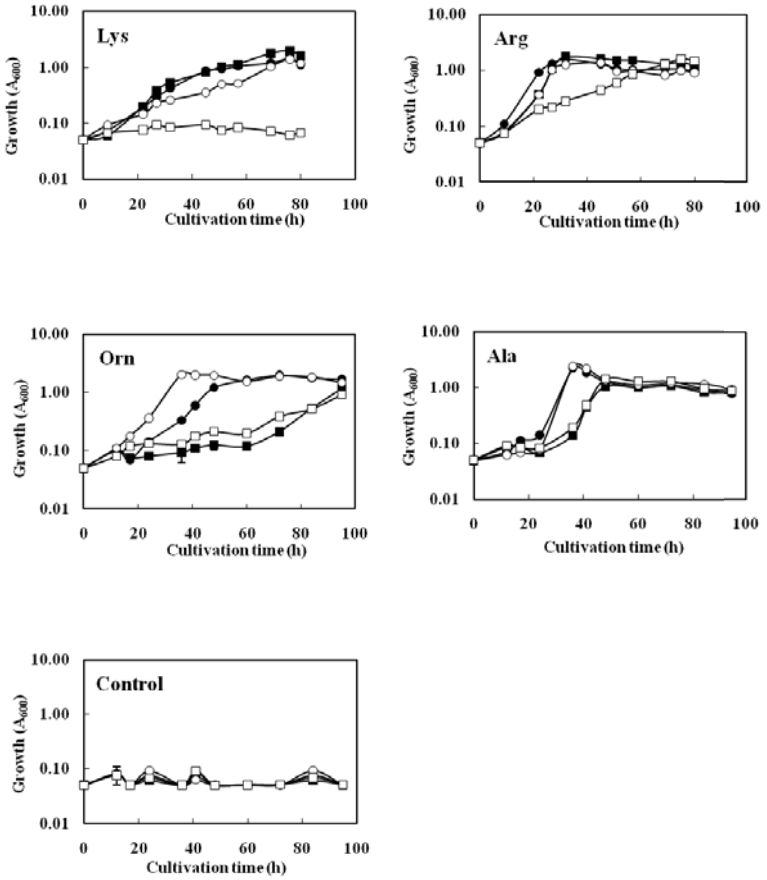


Fig. I-I-3. Utilization of D- and L-amino acids as carbon source by *P. taetrolens* NBRC 3460.

The amino acids used are as indicated. The square indicates D-amino acid, the circle, L-amino acid, wild-type, closed symbols, and mutant with inactivated *argR*, open symbols. Single representative data of two different experiments are shown.

DISCUSSION

The fractionation data obtained with the *P. taetrolens* NBRC 3460 arginine racemase clearly show that arginine racemase is located in the periplasm. Also, in *E. coli* BL21 (DE3) transport of the arginine racemase protein into the periplasm, the processing into its mature form takes place. The signal sequence present shows the typical tripartite structure characteristic of signal peptides, consisting of a positively-charged 'n-region', a central hydrophobic 'h-region', and a polar 'c-region that contains the signal peptidase cleavage site'. However, the sequence itself does not allow translocation to be attributed either via the twin-arginine translocation (Tat) pathway, or the general secretion (Sec) pathway (66). I first suggested that arginine racemase is translocated via the Tat pathway, in particular since it requires pyridoxal 5′-phosphate (PLP) for activity and the Tat pathway translocates its substrates in their fully folded form, including integrated cofactors. One feature of Tat substrates is the eponymous twin-arginine motif at the boundary between the n- and the h-region. However, the arginine racemase signal peptide possesses only one arginine, suggesting that arginine racemase might be exported via the Sec pathway. Indeed, I experimentally confirmed that arginine racemase is translocated into the periplasm of *E. coli* GSJ 100 independent of the Tat pathway and, therefore, must have been

translocated via the Sec route. Generally, this pathway is used to transport proteins devoid of an attached cofactor through the membrane in an unfolded manner, whereas the Tat pathway is mainly used by cofactor-containing proteins. However, it has been demonstrated that some heme-cofactor-containing proteins are in fact translocated into the periplasm via the Sec pathway, followed by further post-translocational modifications, including heme-cofactor insertion. For example, the cytochromes MtrC and OmcA of the Gram-negative bacterium *Shewanella oneidensis* are first translocated across the cytoplasmic membrane using the Sec pathway, and undergo an extensive post-translocational maturation process inside the periplasm to become the heme-containing lipoproteins (79). Due to the Tat-independence of arginine racemase export and the incompatibility of the Sec pathway with transport of folded structures, my findings imply that the PLP cofactor of arginine racemase must have been added to the apoprotein in the periplasm. Otherwise, also the high activity in the periplasm is difficult to explain. Therefore, arginine racemase represents a new class of enzymes utilizing the Sec pathway, where protein and cofactor use different pathways to arrive in the periplasm, but details on these particular events are completely unknown yet. As the growth experiments with *P. taetrolens* NBRC 3460 demonstrate, arginine racemase is a catabolic enzyme. It is necessary for efficient utilization of the two basic D-amino

acids D-Lys and D-Arg, and for D-Lys utilization it is even essential. For D-Arg utilization an additional activity might be present to compensate for the absence of arginine racemase, but this is not Alr. Alr is very specific, just utilizing D, L-Ala as substrates. The localization of arginine racemase within the periplasm of *P. taetrolens* NBRC 3460 is surprising; but this could be related to its catabolic function. It might give the cell an advantage for substrate utilization. Since racemization occurs in the periplasm, the bacterium would then be equipped with fewer specific import systems. However, little is known about the import and catabolism of basic amino acids in *Pseudomonas* (72, 90), and it should also be mentioned that according to genome information *argR* sequences exist which do not have a signal sequence and could be arginine racemases. For instance Q9KSE5 of *Vibrio cholerae* might be a cytosolic arginine racemase (29). In *P. taetrolens* NBRC 3460 the genes adjacent to *argR* exhibit similarities to an *N*-acyl amino acid amidohydrolase and a protease (pfam: PF01546), respectively, which could indicate a relation of the entire gene cluster with catabolic activities. As mentioned, the alanine racemase Alr of *P. taetrolens* NBRC 3460 is highly specific and acts on Ala only, as do all the other alanine racemases studied (84). Interestingly, Alr of *P. taetrolens* NBRC 3460 has slightly greater identity to the catabolic alanine racemase Alr2 of *E. coli* K12, also termed DadX, than

to the biosynthetic alanine racemase Alr1. Due to this fact and the fact that in *P. taetrolens* NBRC 3460 *alr* is located adjacent to *dadA*, the latter gene predicted to encode the small subunit of a D-amino acid dehydrogenase, I initially thought that Alr served only catabolic purposes. However, in an attempt to inactivate *alr* of *P. taetrolens* NBRC 3460 I obtained only very poor growing colonies. The possibility exists that the single alanine racemase in *Pseudomonas* serves both anabolic and catabolic purposes, as I found growth of *P. taetrolens* NBRC 3460 on D-alanine. The interaction of Alr with catabolism and anabolism deserves further study. Due to the apparent similarity of the alanine racemases Alr and DadX and the fact that also arginine racemase sequences are annotated as alanine racemases, I inspected selected genomes of gammaproteobacteria for the occurrence and similarities of such racemases. I found only two genes in *P. putida* KT2440, one corresponding to *alr* of *P. taetrolens* NBRC 3460, and the other to *argR*. A similar situation prevails in the *P. putida* strains GB-1, F1, W619, and further *Pseudomonas* species like *P. aeruginosa* PAO1. From these genomic data and my experimental analysis, it appears that the *Pseudomonas* strains mentioned have the arginine racemase with broad substrate specificity and just one alanine racemase. Whereas, a similar situation exists for *V. cholerae* O1 biovar eltor str. N16961 and *Yersinia pestis* CO92, the situation in *Aeromonas salmonicida*

subsp. *salmonicida* A449 is different. This bacterium has the arginine racemase, together with two alanine racemases. These two alanine racemases have 67% identity, with that encoded by ASA_3286 (gi: 4995230) probably serving anabolic purposes due to its genomic context. Thus, with respect to the two paraloguous alanine racemases, the situation in *A. salmonicida* resembles that in *E. coli* K12 which has the two alanine racemases *dadX* (*alr2*) and *alr* (*alr1*) (90), both specific for alanine, and one for anabolism and one for catabolism, with *alr2* probably expressed on demand. A distinction between the catabolic and anabolic alanine racemases based on sequence identities only is less evident, and this is probably not surprising due to the identical substrate and mechanism they use.

SUMMARY

The pyridoxal 5′-phosphate (PLP)-dependent amino acid racemases occur in almost every bacterium but may differ considerably with respect to substrate specificity. I here isolated the cloned broad substrate specificity racemase arginine racemase of *Pseudomonas taetrolens* NBRC 3460 from *Escherichia coli* BL21 (DE3) by classical procedures. The racemase was biochemically characterized and amongst other aspects it was confirmed that it is mostly active with lysine, arginine and ornithine, but merely weakly active with alanine, whereas the alanine racemase of the same organism studied in comparison acts on alanine only. Unexpectedly, sequencing the amino-terminal end of arginine racemase revealed processing of the protein, with a signal peptide cleaved off. Subsequent localization studies demonstrated that in both *P. taetrolens* NBRC 3460 and *E. coli* BL21 (DE3) arginine racemase activity was almost exclusively present in the periplasm, a feature so far unknown for any amino acid racemase. An ArgR-derivative carrying a carboxy-terminal His-tag was made and this was demonstrated to localize even in an *E. coli* mutant devoid of the twin-arginine translocation (Tat) pathway in the periplasm. These data indicate that arginine racemase is synthesized as a prepeptide and translocated in a Tat-independent manner. I therefore propose that arginine racemase translocation depends on the Sec system and a post-translocational insertion of PLP occurs. As further experiments showed, arginine racemase is necessary for the catabolism of D-arginine and D-lysine by *P. taetrolens* NBRC 3460.

CHAPTER I / SECTION II

Physiological function of an arginine racemase in *Pseudomonas taetrolens* NBRC 3460

INTRODUCTION

Various amino acid racemases have been found in bacteria, but most of their physiological functions are not clarified until now. Alanine racemase (EC 5.1.1.1) (62) and glutamate racemase (EC 5.1.1.3) (13) are known to play an important role to synthesize two of essential components of a peptideglycan, and ornithine racemase (EC 5.1.1.12) (12) relates to D-ornithine catabolism. I clarified the physiological function of the arginine racemase of *Pseudomonas taetrolens* NBRC 3460 by using *P. taetrolens* NBRC 3460::*argR* in Chapter I Section I. One of the important physiological functions of the arginine racemase is to catabolize D-lysine as a carbon source.

A signal peptide exists in *N*-terminal of the arginine racemase and the arginine racemase is transported into a periplasm by the function of this signal peptide. Therefore, it suggests that D-amino acids produced by the arginine racemase may function outside of a cytoplasm. In this study, I prepared a peptideglycan function of *P. taetrolens* NBRC 3460 to investigate a possibility of existence of D-amino acids in a peptideglycan fraction of this microorganism.

MATERIALS AND METHODS

Materials. L- and D-Met were purchased from Wako Chemical Co. (Osaka, Japan). All other chemicals were purchased from Kanto Kagaku Co. (Tokyo, Japan), Kishida Chemical Co. (Osaka, Japan), Sigma-Aldrich Co. (Missouri, USA), Tokyo Kasei Kogyo (Tokyo, Japan), or Watanabe Chemical Industries Ltd. (Hiroshima, Japan) unless otherwise stated and were of the best commercially available grade.

Determination of D-amino acid in medium. *P. taetrolens* NBRC 3460 and *P. taetrolens* NBRC 3460::*argR* were pre-cultivated on a LB agar medium, and then a single colony on the medium was inoculated into a LB medium in a 500-ml of Erlenmeyer flask with a baffle, and cultivated at 30°C for 24 h with shaking (150 rpm) until a growth of microorganism reaches to a stationary phase. After centrifugation at 5,500 X *g* for 15 min at 4°C, the collected cells were washed with a 0.85% (w/v) NaCl aqueous solution, and inoculated into a 50 ml of 154 medium (83) with final absorption 1.0 at 600 nm. After 24 h, the culture broth was taken from the flask and centrifuged at 16,100 X *g* for 15 min at 4°C. The supernatant was diluted 1,000 times with a 0.1 M sodium acetate buffer (pH 7.2) and reacted with *o*-phthalaldehyde (OPA) and *N*-acetyl-L-cysteine (NAC) according to the method of Aswad D. W. (2) after filtration

with a GL chromatodisc (0.2 μm, GL Science, Tokyo, Japan). An aliquot of the reaction mixture was subjected to HPLC analysis.

Preparation of peptideglycan fraction. *P. taetrolens* NBRC 3460 and *P. taetrolens* NBRC 3460::*argR* cells were grown and harvested by the method described above. The cells obtained were suspended in a 50 ml of an ice-chilled NaCl solution, and centrifuged at 16,100 X *g* for 15 min at 4°C. The cells were resuspended in 15 ml of a 10 mM potassium phosphate buffer (pH 7.0) plus 0.9% (w/v) NaCl aqueous solution, and dropped into 15 ml of a boiling 10% (w/v) SDS solution with vigorously stirring for 4 h. The mixture was stirred overnight at 37°C. An insoluble fraction containing a peptideglycan was collected by ultracentrifugation at 27,500 X *g* for 30 min at 30°C (P70AT Rotor; Himac CP70MX Preparative Ultracentrifuge, Hitachi Koki Co., Ltd., Tokyo, Japan), and incubated at 100°C for 2 h in a 1.0% (w/v) SDS solution. After ultracentrifugation again, the insoluble fraction obtained was washed four times with deionised water, and used as a peptideglycan fraction.

Hydrolyzation of peptideglycan. The peptideglycan fraction was hydrolyzed with hydrogen chloride by using a Pico-tag Work station (Waters, Tokyo, Japan) at 110°C for

20 h according to the protocol available from the manufacturer. The hydrolysed samples were dried under reduced pressure and resuspened in a mixture containing (95%, v/v) 5 mM disodium phosphate in deionized water adjusted with acetic acid to pH 7.4 and (5%, v/v) acetonitrile. The sample was adjusted to pH 2.3 with 0.1 M HCl and slowly passed through a Pasteur pipette packed with a glass wool plug in its bottom and Dowex 50W X 8 resin (bed volume: 0.5 X 3.5 cm). The adsorbed sample was eluted with a 4 M NH_3 solution and derivatized to *N* (O)-pentafluoropropionyl 2-propyl ester according to the method described previously (17, 21, 76).

Analytical methods. HPLC analyses of D- and L-amino acids were carried out under the same conditions used previously (65). GC-MS analysis was carried out by a gas chromatography GC-2010 system equipped with a Model QP2010 mass spectrometer (GC-MS, Shimadzu, Kyoto, Japan). A fused silica capillary column Chirasil®-L-Val (Varian Inc., Darmstadt, Germany) was used to separate and quantify D- and L-amino acids according to the method previously reported (1, 70, 71).

Effects of osmotic pressure. *P. taetrolens* NBRC 3460 and *P. taetrolens* NBRC 3460::*argR* were grown at 30°C for 20 h until their growths reach stationary phase.

The cells were then collected by a low speed centrifugation at 4°C, and washed with a 150 mM NaCl aqueous solution once before resuspending in 150, 50, 5, or 0 mM NaCl. The cells were incubated in 150, 50, 5, or 0 mM NaCl for 10 min at room temperature, and their viabilities were estimated by inoculating them on LB agar plates and cultivating at 30°C for 16 h.

Other methods. The growth of microorganism in a culture broth was measured photometrically at 600 nm with a UV-VIS photometer V-550 (Jasco Co. Ltd., Tokyo, Japan).

RESULTS

Detection of D-amino acids extracellularly produced by *P. taetrolens* NBRC 3460 and *P. taetrolens* NBRC 3460::*argR*. After *P. taetrolens* NBRC 3460 or *P. taetrolens* NBRC 3460::*argR* was grown in a 154 medium at 30°C for 24 h, D-amino acids produced in the medium was measured with HPLC. In the culture broth of *P. taetrolens* NBRC 3460, D-alanine (0.02 mM), D-leucine (0.01 mM), and D-methionine (0.03 mM) were detected. However, in the culture broth of *P. taetrolens* NBRC 3460::*argR*, D-alanine (0.02 mM) was detected, and D-leucine and D-methionine were not detected.

Detection of D-amino acids in a peptideglycan fraction. The peptideglycan fractions of *P. taetrolens* NBRC 3460 and *P. taetrolens* NBRC 3460::*argR* were prepared by the boiling-SDS method, and their D-amino acid content was measured with GC-MS (Fig. I-II-1). D-Alanine, D-methionine, and D-glutamic acid were detected in the peptideglycan fraction of *P. taetrolens* NBRC 3460, but only D-alanine and D-glutamate were detected in that of *P. taetrolens* NBRC 3460::*argR*. The ratio of D-methionine and D-glutamic acid to D-alanine were calculated to be 0.60 and 2.74, respectively. These results suggest that the arginine racemase of *P. taetrolens* NBRC 3460 plays an

important role to produce D-methionine in the peptideglycan fraction of this microorganism.

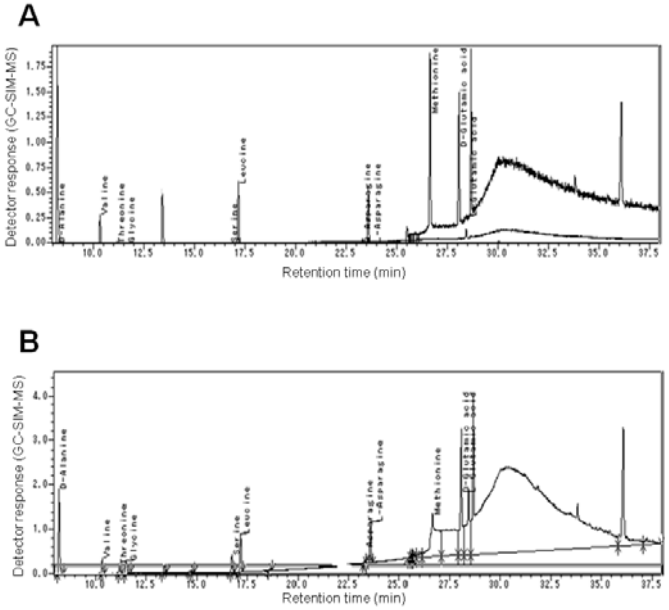


Fig. I-II-1. GC-MS analysis of D- and L-amino acids in peptideglycan fractions of *P. taetrolens* NBRC 3460 and *P. taetrolens* NBRC 3460::*argR*

A: *P. taetrolens* NBRC 3460; B: *P. taetrolens* NBRC 3460::*argR*

Effects of osmotic pressure. After treatment with a NaCl solution of different concentrations, the *P. taetrolens* NBRC 3460 and *P. taetrolens* NBRC 3460::*argR* cells were cultivated on LB agar plates, and then the colonies appeared on agar plates were

counted. In case of *P. taetrolens* NBRC 3460, the average numbers of colonies were 214, 196, 120, and 96 and in case of *P. taetrolens* NBRC 3460, 172, 60, 11, and 9 when the cells were treated with a 150, 50, 5, or 0 mM NaCl solution, respectively (Fig. I-II-2).

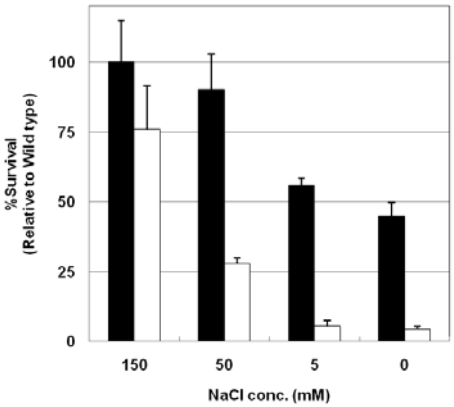


Fig. I-II-2. Effects of osmotic pressure on *P. taetrolens* NBRC 3460 and *P. taetrolens* NBRC 3460::*argR* cells

■: *P. taetrolens* NBRC 3460; □: *P. taetrolens* NBRC 3460::*argR*

DISCUSSION

D-Amino acids have been found recently in a much broader range of living organisms with development of improved analytical and detection techniques (21, 76). For example, D-serine was demonstrated to act as a neuromodulator in human (17), and D-alanine is most likely to serve as an osmoregulator since the level of D-alanine as well as that of L-alanine increased with increasing environmental salinity in the tissues of the brackish-water bivalve *Corbicula japonica* (59) and the hard clam *Meretrix lusoria* (64). In bacteria, studies of D-amino acids and their metabolism have focused intensively on microbial amino acid racemases and D-amino acid transaminases, since D-alanine and D-glutamate are known to be two essential components of the peptideglycan in the microbial cell wall (18, 51). In addition, D-ornithine was found in *Bacillus subtilus* and plant pathogenic *Corynebacterium* species (67), and D-methionine was found in *Vibrio cholerae* (45).

As compared with the molecular diversity of the peptideglycan in gram positive bacteria, gram negative bacteria apparently follow more uniform rules in building up their peptideglycan (28). Much difference was not observed for gram negative bacteria between the results of amino acid analyses of the hydrolyzed peptideglycans so far. Some defferences from the compositions of "standard" *Escherichia coli*

peptideglycan might be due to uncertainties of the analyses or insufficient purification of the peptideglycan (28). In this study, I found that D-methionine is contained in a peptideglycan fraction of *Pseudomonas taetrolens* NBRC 3460. *V. cholerae* also contains D-methionine in its peptideglycan, and two putative amino acid racemase genes (*vc1312, bsrV*) are encoded in its genome (45). It suggests that D-methionine produced by these racemases is incorporated into a peptideglycan. The primary structure similarity between the putative amino acid racemases of *V. cholerae* and the *P. taetrolens* NBRC 3460 arginine racemase is about 50%, and the function of the arginine racemase is probably similar to that of the putative amino acid racemases of *V. cholerae*. The osmotic stress strongly affected on the survival rate of *P. taetrolens* NBRC 3460::*argR* like *V. cholerae*::*bsrV*. Accordingly, D-methionine in a peptideglycan may be a common essential component for bacteria to adapt the outer environment of the cells.

SUMMARY

I analyzed the peptideglycan fraction of *P. taetrolens* NBRC 3460 and *P. taetrolens* NBRC 3460::*argR* and found that D-methionine was detected only in the peptideglycan fraction of *P. taetrolens* NBRC 3460. Accordingly the arginine racemase of *P. taetrolens* NBRC 3460 probably synthesizes D-methionine in the peptideglycan fraction. The osmotic stress strongly affects the survival rate of *P. taetrolens* NBRC 3460::*argR*, and it suggests that D-methionine is one of the essential components of a peptideglycan to protect the cells against osmotic stress. Accordingly, the arginine racemase of *P. taetrolens* NBRC 3460 has two functions of both the anabolism of D-methionine and the catabolism of D-lysine.

CHAPTER II

Detection and function of the intramolecular disulfide bond in an arginine racemase: an enzyme with broad substrate specificity

INTRODUCTION

The arginine racemase (ArgR) from *Pseudomonas taetrolens* NBRC 3460 catalyzes the racemization of arginine, lysine, and various other amino acids, except acidic and aromatic amino acids (94, 95). The broad substrate specificity of this enzyme is unique, and almost all other amino acid racemases, such as alanine racemase (62), glutamate racemase (26), and ornithine racemase (12), show high substrate specificities. The arginine racemase of *P. taetrolens* NBRC 3460 belongs to the fold-type III group of pyridoxal 5'-phosphate (PLP)-dependent enzymes with a homodimeric structure and a subunit molecular mass of 42 kDa. The signal peptide is located at the *N*-terminus of the enzyme, which functions by exporting the enzyme to the periplasm (53). Recently, I clarified the physiological function of the arginine racemase by using an arginine racemase integrated mutant, *P. taetrolens* NBRC 3460::*argR*. I found that arginine racemase, catalyzing the catabolism of D-lysine as a carbon source, is an essential, catabolic enzyme for *P. taetrolens* NBRC 3460. Arginine racemase is one of the few examples of an amino acid racemase with an identified function. The homology search revealed that there are two cysteine residues (C47 and C73) in the *N*-terminal region of the primary structure of arginine racemase that are not found in the primary structures of alanine racemases and other amino acid

racemases. In addition, when the region of the *argR* gene corresponding to the signal sequence was truncated, arginine racemase was expressed in the cytoplasm of *Escherichia coli* BL21 (DE3), and the expression level decreased. Based on these findings, I hypothesized that C47 and C73 of arginine racemase would form a disulfide bond that is important for arginine racemase to mature. An intramolecular disulfide bond in a PLP-dependent amino acid racemase has never been reported so far. In this study, I analyzed the primary structure of arginine racemase by MALDI-TOF-MS, constructed a C47A/C73A-ArgR by site directed mutagenesis (cysteine to alanine), and examined the function of these two cysteine residues.

MATERIALS AND METHODS

Materials. The amino acids D- and L-arginine, D- and L-alanine, D-ornithine, and D-lysine were purchased from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). L-Ornithine and L-lysine were purchased from Wako Chemical Co. (Osaka, Japan). DEAE-Toyopearl 650 M and Butyl-Toyopearl 650 M were obtained from Tosoh (Tokyo, Japan). Restriction endonucleases, Ligation mix, and 10 X A attachment mix were purchased from Toyobo (Osaka, Japan) and Takara Bio (Shiga, Japan). All other chemicals were purchased from Kanto Kagaku Co. (Tokyo, Japan), Kishida Chemical Co. (Osaka, Japan), Sigma-Aldrich Co. (MO, USA), or Tokyo Kasei Kogyo (Tokyo, Japan) unless otherwise stated and were of the best commercially available grade.

Bacterial strains, plasmids, and growth conditions. The culture strain of *Pseudomonas taetrolens* NBRC 3460 was obtained from the NITE Biological Resource Center, National Institute of Technology and Evaluation, Chiba, Japan, and was used for the preparation of the genomic DNA. The strain was grown aerobically at 30°C in Luria–Bertani (LB) medium for 14 h. *Escherichia coli* BL21 (DE3) was obtained from Merck KGaA (Darmstadt, Germany) and was grown aerobically at 37°C in LB medium supplemented with ampicillin (100 μg/ml). Further details on the bacterial

strains, primers, and plasmids used are compiled in Table II-1.

Expression and purification of arginine racemase. A DNA fragment containing the full-length coding region of *argR* was amplified by PCR (polymerase chain reaction) using *P. taetrolens* NBRC 3460 genomic DNA as a template. The primers P1 and P3 (Table II-1) were used for the amplification of *argR* with the signal peptide. The primers P2 and P3 (Table II-1) were used for the amplification of *argR* without the signal peptide. Each PCR product obtained was ligated into an NdeI-BamHI or NcoI-BamHI digested pET11b vector to form an *argR*-pET11b or *argR*::*signal*-pET11b vector, respectively. Each plasmid constructed was transformed into *E. coli* BL21 (DE3), and the protein expressed in the *E. coli* clone cells was purified by the same methods as previously described (53).

Table II-1. Strains, plasmids and primers used

Name	Sequence*/Features	Source/Reference
Bacteria		
P. taetrolens NBRC 3460	Wild type	Culture collection
E. coli BL2. (DE3)	Expression host	Culture collection
Primers		
P1	5'-TCA **GCA TAT G**CC CTT CTC CCG TAC-3'	*argR* sense primer
P2	5'-**GCC ATG G**CG CCA CCC CTG TCG ATG ACC G-3'	*argR* without signal peptide sense primer
P3	5'-GCA **GGA TCC** TCG TTC AAT ATA CGG-3'	*argR* antisense primer for pET11b
P4	5'-GTC GCA GAT **CGC C**GC CGT ACT CAA GG-3'	Site-directed mutagenesis of cysteine 47
P5	5'-CCT TGA GTA **CGG C**GG CGA TCT GCG AC-3'	Site-directed mutagenesis of cysteine 47
P6	5'-GGT GTT CCC **GCT** GTC GGT GT-3'	Site-directed mutagenesis of cysteine 73
P7	5'-ACA CCG ACA **GC**G GGA ACA CC-3'	Site-directed mutagenesis of cysteine 73
P8	5'-ATA **GGA TCC** CGC TGA TCT TTC AGG-3'	*argR* antisense primer for pET21b
Plasmids		
pET11b-*argR*	Expression vector, contains full-length *argR*	Chapter I, Section I
pET11b-*argR* without SP	Expression vector, contains *argR* without *N*-terminal signal peptide	This work
pET21b-*argR*	Expression vector, contains *C*-terminal His-tagged *argR*	This work
pET11b-*C47A/C73A-argR*	Expression vector, contains *C47A/C73A-argR*	This work
pET21b-*C47A/C73A-argR*	Expression vector, contains *C*-terminal His-tagged *C47A/C73A-argR*	This work

*Sequences shown in boldface type are as follows: CATATG, NdeI site (P1); CCATGG, NcoI site (P2); GGATCC, BamHI site (P3, P8); GCC/GGC, the site of site-directed mutagenesis (P4/P5); GCT/AGC, the site of site-directed mutagenesis (P6/P7)

Detection of a disulfide bond in arginine racemase by MALDI-TOF-MS. The purified arginine racemase (10 ng) was denatured with an 8 M urea solution (10 μl) and then exposed to one of the following three independent treatments: i) reduction with dithiothreitol (DTT) and *S*-alkylation with iodoacetamide, ii) *S*-alkylation with iodoacetamide, or iii) no treatment. Each mixture was diluted with 50 mM NH_4HCO_3 solution (80 μl), reacted with trypsin (350 ng, Promega, Madison, WI, USA), and incubated at 37°C overnight. The tryptic digested samples were desalted and concentrated to approximately 8 μl by a ZipTipC18 (Millipore, Billerica, MA, USA). The concentrated sample (0.5 ml) was mixed with 0.5 ml of a CHCA solution (5 mg/ml in 50% (v/v) CAN containing 0.1% (v/v) TFA) on a MALDI target plate and analyzed after air-drying. The mass spectra were collected by both reflectron positive ion mode and linear positive ion mode with an AXIMACFRTM-plus MALDI-TOF MS instrument (Shimadzu/Kratos, Manchester, UK), according to the method of Yamaguchi *et al.* (89).

Construction of *C47A/C73A-argR* by site-directed mutagenesis and expression of the recombinant enzyme. I used an overlapped extension PCR method to construct *C47A/C73A-argR*. The first PCR was done to introduce C47 with primers P1 and P5

amplifying the genomic DNA of *P. taetrolens* NBRC 3460 in a Gene Amp 9700 PCR system (PE Applied Biosystems Japan, Tokyo). The program used consisted of three sequential steps of 98°C for 10 s, 55°C for 30 s, and 68°C for 60 s, and it ran for 25 cycles. The second PCR was performed under the same conditions as used for the first PCR, except that primers P3 and P4 were used instead of P1 and P5. The PCR products obtained by the first and second PCR were mixed and used as megaprimers M1 and M2 in the third PCR. The program used for the third PCR consisted of four sequential steps of one cycle of 94°C for 5 min, 25 cycles of 98°C for 10 s, 55°C for 30 s, 68°C for 60 s, and one cycle of 68°C for 7 min. The PCR product obtained was used for the fourth and fifth PCR as a template DNA. The fourth and fifth PCR were performed to introduce C73 with two pairs of primers, P1 and P7, and P3 and P6, respectively. The program used for the fourth and fifth PCR consisted of four sequential steps of one cycle of 94°C for 5 min, 25 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 60 s, and one cycle of 68°C for 7 min. The PCR products obtained by the fourth and fifth PCR (approximately 100 ng) were mixed and used as megaprimers M3 and M4 in the sixth PCR. The program used for the sixth PCR was the same as that for the third PCR. The *C47A/C73A-argR* obtained was ligated into an NdeI-BamHI digested pET11b vector to form a pET11b-*C47A/C73A-argR* vector. The

constructed plasmid was transformed into *E. coli* BL21 (DE3), and the protein expressed in the *E. coli* clone cells was purified by the same methods as described in (53).

Comparison of the protein expression efficiency of C47A/C73A-arginine racemase with that of arginine racemase. To compare the protein expression efficiency of C47A/C73A-ArgR with that of arginine racemase, I constructed pET21b-*argR* or pET21b-*C47A/C73A-argR*. A PCR was performed with primer P1 and primer P8 against pET11b-*argR* or pET11b-*C47A/C73A-argR* as a template, and the PCR product obtained was digested with NdeI and BamHI and ligated into an NdeI-BamHI digested pET21b vector to form a pET21b-*argR* or pET21b-*C47A/C73A-argR*. The constructed plasmid was transformed into *E. coli* BL21 (DE3), and the protein expression efficiency was analyzed by the gel documentation system Chemidoc XRS (Bio-Rad, California, USA) after the recombinant protein was stained with an Invision His-tag In-gel Stain kit (Invitrogen, California, USA).

Enzyme assays. Arginine and lysine racemase activities were measured in a coupled assay system with L-arginase from bovine liver (Sigma, MO, USA) (94). Arginine,

lysine, alanine, methionine, and ornithine racemase activities were also assayed with the HPLC system LC-10A (Shimadzu Co., Kyoto, Japan) equipped with a Crownpak CR(+) column (0.4 X 15 cm; Daicel Chem. Ind. Ltd., Osaka, Japan) by measuring the amino acid enantiomer produced from each substrate amino acids. The mobile phase was H_2O containing $HClO_4$ (pH 1.5), and the flow rate was 0.4 ml/min. The eluted amino acids were detected with a UV detector SPD-10A at 215 nm. The mixtures in a 1.5-ml microtube contained a 0.6 M CHES-NaOH buffer (pH 10.0, 130 μl), 0.1 M D- or L-amino acid (80 μl), 0.1 mM PLP (200 μl), and an enzyme solution. The enzyme reaction was carried out at 37°C in a heating block TAL-1G (TAITEC, Saitama, Japan), stopped after 0, 0.5, 1, 2, 5, or 10 min by heat treatment at 100°C for 2 min, and then 10 ml of the mixture was subjected to HPLC (87). The enzyme reaction under anaerobic and reduced conditions was carried out in a Vacuum Globe Box SGV-65V (AS ONE Co., Osaka, Japan) in the presence of 20 mM DTT.

Fluorescence-quenching analysis of tryptophan residues in C47A/C73A-ArgR and ArgR by acrylamide. The fluorescence-quenching analysis of the tryptophan residues in C47A/C73A-ArgR and ArgR was done by addition to the enzyme solution (0.5 mg/ml, 0.1 ml) of various concentrations (0, 0.05, 0.10, 0.15, 0.20, and 0.25 mM)

of acrylamide dissolved in 0.25 ml of a 20 mM potassium phosphate buffer pH 7.3. The fluorescence intensity was measured with a Hitachi 650-60 fluorescence spectrophotometer (Hitachi, Ibaragi, Japan) equipped with a data processor at 25°C; slit width, 5 nm. The sample was exited at 295 nm, and the fluorescence observed was measured at 330 nm. The data obtained were analyzed with the modified Stern–Volmer equation (25, 46):

$$F_0/(F_0-F_Q) = 1/f_a + 1/K_{sv}\,[Q]$$

where F_0 and F_Q are the fluorescence emission intensities in the absence and presence of acrylamide at the concentration [Q], respectively. K_{sv} is a linear Stern–Volmer quenching constant showing the accessibility of the fluorophore to the quencher, and f_a is the fraction of fluorescence from the residues accessible to the quencher.

Effect of temperature and pH. The effect of the temperature on the activity of arginine racemase was examined at 15, 20, 25, 30, 35, 40, 45, and 50°C. The effect of the pH was examined at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, and 11.5.

Kinetic analysis. Kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) for arginine racemase were

determined with Lineweaver–Burk plots. Arginine, lysine, alanine, and ornithine (D- and L-enantiomers) were used as substrates.

Other methods. Spectrophotometric measurements were done with a spectrophotometer U-3210 (Hitachi High-Technologies Co., Ibaragi, Japan). The absorption spectra (250–500 nm) of each enzyme (protein concentration: 0.5 mg/ml) were measured with a spectrophotometer V-550 (Jasco Co. Ltd., Tokyo, Japan) at room temperature; path length, 1 cm. Protein concentrations were determined at room temperature by a standard Bradford assay (7) using a spectrophotometer V-550 (Jasco Co. Ltd., Tokyo, Japan). The molecular mass of ArgR and C47A/C73A-ArgR were estimated by gel filtration on a HiLoad 16/60 Superdex 200 column (1.6 X 70 cm; GE Healthcare UK Ltd., Amersham Place, UK) and with thyroglobulin (669 kDa), catalase (232 kDa), albumin (69 kDa), and chymotrypsinogen A (25 kDa) as standard proteins. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (44). The gels were strained with Coomassie Blue R-250.

RESULTS

Detection of the disulfide bond in arginine racemase. The tryptic peptides derived from arginine racemase were analyzed by MALDI-TOF MS. The MALDI-TOF mass spectra of arginine racemase recorded under the three conditions described in the "MATERIALS AND METHODS" are shown in Fig. II-1. The specific peptide peaks were observed at m/z 861.20 and 3,184.15, when arginine racemase was reduced with dithiothreitol (DTT) and subsequently *S*-alkylated with iodoacetamide (Fig. II-1, a and b). These two peaks corresponded to the tryptic fragments containing carbamidomethyl cysteine of the *S*-alkylated arginine racemase. These values fit well with the theoretical m/z values (m/z 861.48 and 3,184.55) calculated from the amino acid sequence of arginine racemase (Table II-1). In contrast, the peak at m/z 4,048.01 was specifically observed for untreated or iodoacetamide treated arginine racemase without reduction with DTT (Fig. II-1, c) and fits well with the theoretical value (m/z 4,043.04) calculated from the tryptic peptide fragment containing the arginine racemase disulfide linkage (Table II-2). These results show that the single intramolecular disulfide bond between C47 and C73 exists in the primary structure of arginine racemase (Fig. II-2).

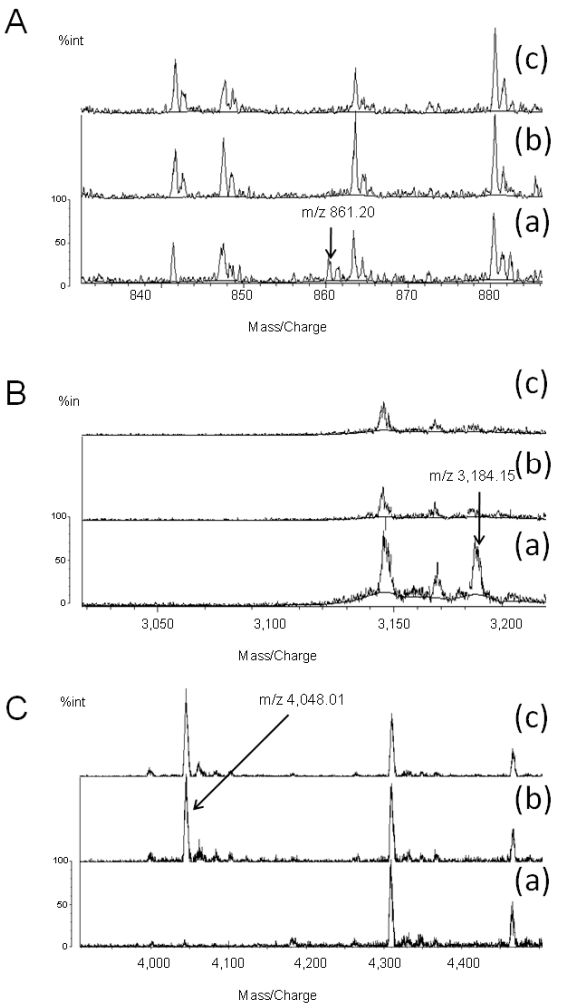


Fig. II-1. MALDI-TOF MS spectra of arginine racemase

The acquired profiles were focused on mass ranges from 830 to 885 (A), 3,025 to 3,225 (B), and 3,900 to 4,500 (C). The purified arginine racemase was treated independently with the three following conditions: (a) reduction with dithiothreitol (DTT) and *S*-alkylation with iodoacetamide; (b) *S*-alkylation with iodoacetamide; and (c) no treatment. Arrows and numerical values indicate specific peptide peaks and observed masses, respectively.

Table II-2. Theoretical and observed monoisotopic mass values for tryptic digestes of ArgR

Mass value	Position	MC	Observed mass value (a) (b) (c)	(a)	(b) (c)	Peptide sequence
4,308.1393	215-254	0	4,309.21			ITLHAANSFATLEVPESHLD MVRPGGALFGDTVPSHTEYK
3,184.5587	52-83	0		3,184.15	N.D.	ADAYGHGIGLLMPSVIAMGV PCVGVASNEEAR
3,145.5687	353-381	0	3,144.56			AEITQAEIEDINGALLADLY TVWGNSNPK
2,908.4648	99-125	0	2,909.21			TAALSELEAALPYNMEELVG NLDFAVK
2,785.3461	1-26	0	2,784.60			APPLSMTDGVAQVNTQDSNA WVEINK
2,616.3674	126-150	0	2,616.97			ASLIAEDHGRPLVVHLGLNS SGMSR
2,033.0021	196-212	0	2,033.71			AFNQQAQWLMNVAQLDR
1,834.8925	323-339	0	N.D.			VSMNTLMVDVTDAPDVK
1,645.8002	177-191	0	1,646.38			AIMTHFAVEDAADVR
1,428.7117	289-301	0	1,429.23			LANITVGYSDGYR
1,372.6855	340-352	0	1,373.14			SGDEVVLFGHQGK
1,188.6007	261-271	0	1,188.94			SHVASVNSYPK
1,149.5317	151-161	0	1,149.64			NGVDMTTAQGR
978.5843	308-316	0	978.64			GIVLINGHR
957.4901	27-34	0	957.70			AAFEHNIR
902.5305	35-43	0	902.67			TLQTALAGK
861.4862	44-51	0		861.20	N.D.	SQICAVLK
826.4781	170-176	0	826.59			VPNLEVR
823.4057	272-279	0	N.D.			GNTVGYGR
717.4141	163-169	0	N.D.			DAVAITK
710.3831	280-285	0	710.33			TYTLGR
652.3487	256-260	0	652.29			VMQFK
598.3922	317-322	0	N.D.			VPVVGK
586.3671	92-96	0	598.00			GQLIR
580.3089	303-307	0	586.32			AFTNK
567.2773	87-91	0	N.D.			ESGFK

MC, number of missed cleavages;
(a) (b) (c) stand for the sample conditions in Detection of a disulfide bond in ArgR by MALDI-TOF MS'.
ND, not detected.

Table II-3. Theoretical and observed mass values for disulfide-linked peptides

Mass value	Position	MC	Modification	Modified mass value	Observed mass value	Peptide sequence
3,184.56	52-83	0				ADAYGHGIGLLMPSVIAMGVPCVGVASNEEAR
			Disulfide bond: 47-73	4,043.04	4,048.01	
861.49	44-51	0				SQICAVLK

MC, number of missed cleavages;
Disulfide linkages are indicated by lines

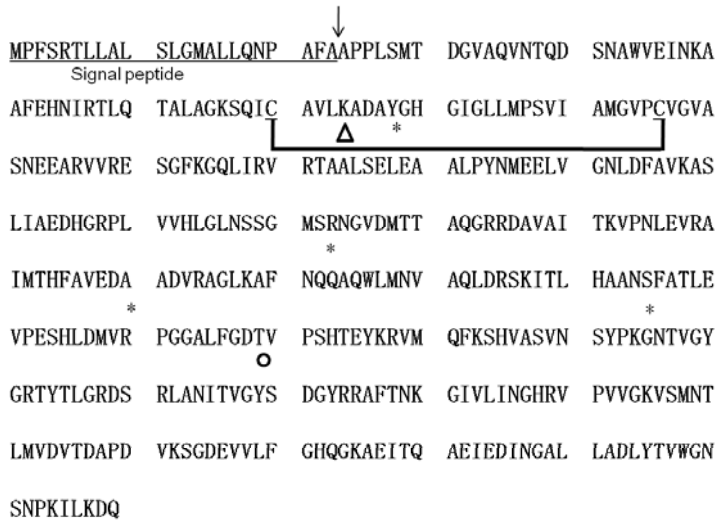


Fig. II-2. Complete primary structure of arginine racemase

The triangle and circle point to the PLP-binding lysyl residue and tyrosyl residue abstracting α-hydrogen of L-amino acid. Asterisks indicate amino acid residues involved in PLP binding. An arrow indicates the cleavage site by a signal peptidase.

Comparison of arginine racemase expression in the cytoplasm or the periplasm.

The total arginine racemase activity detected was 13,900 ± 110 U when arginine racemase was expressed in the periplasm. The total activity decreased characteristically to 500 ± 16 U when arginine racemase was expressed in the cytoplasm. Thus, an about 30 times higher activity of arginine racemase was detected in the periplasm compared to that in the cytoplasm.

Expression and characterization of C47A/C73A-ArgR. C47A/C73A-ArgR was expressed in the soluble fraction of *E. coli* BL21 (DE3) by transformation of the plasmid pET21b-*C47A/C73A-argR*. The specific activity of the arginine racemase in the cell-free extract was 70.2 ± 0.5 U/mg, while that of C47A/C73A-ArgR was 10.5 ± 0.1 U/mg. I analyzed the expression level of both proteins by using an Invision His-tag In-gel Stain kit (Fig. II-3). The area of the protein bands of C47A/C73A-ArgR and ArgR was determined to be 420 and 5,000, respectively. Hence, an approximately ten times higher value was observed for the protein band of arginine racemase. The molecular mass of the subunit and non-denatured protein of C47A/C73A-ArgR were estimated to be 41.5 and 84.0 kDa, respectively. These values corresponded to those of ArgR, and the mutations of C47 and C73 to A47 and A73 in arginine racemase did not affect the quaternary structure of C47A/C73A-ArgR. The absorption spectrum of C47A/C73A-ArgR showed a peak at 420 nm, and this characteristic absorption disappeared and shifted to 310 nm, when the protein was reduced with $NaBH_4$. These results suggest that, like arginine racemase, C47A/C73AArgR contains PLP as a cofactor.

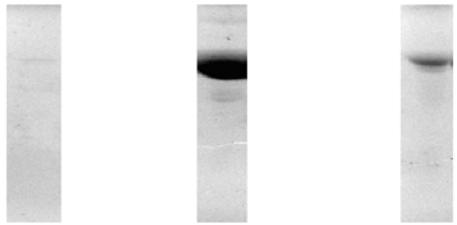
	Blank	ArgR	C47A/C73A-ArgR
Arginine racemase activity (U/mg)	0.031	70.2	10.5
His-tag in-gel stain			
Area	100	5,000	420

Fig. II-3. Comparison of expression level of C47A/C73A-ArgR with that of ArgR

Thermal and pH profiles of C47A/C73A-ArgR. The optimum temperature and pH of C47A/C73A-ArgR were determined to be 70°C and pH 10. C47A/C73A-ArgR was relatively unstable with heat treatment as the half-life ($t_{1/2}$) at 30°C was determined to be 21 min. C47A/C73A-ArgR was stable in the pH range of 8.0 to 11.0. The thermal and pH profiles observed for C47A/C73A-ArgR corresponded well to those of arginine racemase and the mutations of C47 and C73 to A47 and A73 in arginine racemase did not affect the thermal and pH profiles of C47A/C73A-ArgR.

Fluorescence quenching analysis of the tryptophan residues in C47A/C73A-ArgR and ArgR. The Stern–Volmer plot for C47A/C73A-ArgR did not coincide with that

for arginine racemase (Fig. II-4). The linear Stern–Volmer quenching constants (K_{sv}) calculated for C47A/C73A-ArgR and ArgR were 4.44 and 5.16, respectively. The accessibility of acrylamide to the tryptophane (W) residues was increased by the mutations of C47 and C73 to A47 and A73 in arginine racemase, which may have caused the conformational change. The K_{sv} values for arginine racemase determined in the presence or absence of DTT were similar, and the disulfide bond between C47 and C73 did not affect the overall structure of arginine racemase after the enzyme was matured.

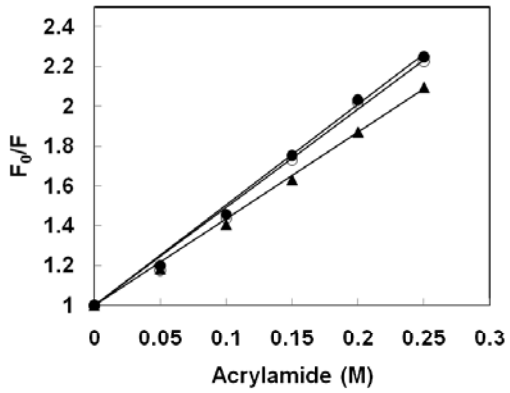


Fig. II-4. Stern-Volmer plot for C47A/C73A-ArgR and ArgR

●, ArgR; ○, ArgR under reduced conditions; ▲, C47A/C73A-ArgR

Comparison of substrate specificities and kinetic parameters between ArgR and C47A/C73A-ArgR. The substrate specificity of C47A/C73A-ArgR was characteristically different from that of arginine racemase (Table II-4). The relative activities of C47A/C73A-ArgR for L-arginine, L-lysine, L-ornithine, D-arginine, D-lysine, and D-ornithine corresponded well to those of arginine racemase, but, interestingly, the relative activities for L-alanine and D-alanine increased to 25 and 26%, respectively. The relative activities of arginine racemase for all the amino acids tested in the presence or absence of DTT were similar, and the disulfide bond between C47 and C73 did not affect the substrate specificity of arginine racemase, once the enzyme was matured (Table II-5). The kinetic constants (k_{cat}, K_m, and k_{cat}/K_m) of the amino acid racemase activity of arginine racemase and C47A/C73A-ArgR for arginine, lysine, ornithine, and alanine are shown in Table II-4. The catalytic efficiency (k_{cat}/K_m) of C47A/C73A-ArgR for arginine, lysine, and ornithine was very similar to that of arginine racemase. However, k_{cat}/K_m of C47A/C73A-ArgR for both L-alanine and D-alanine increased approximately five times as compared to those of arginine racemase.

Table II-4. Substrate specificities

Substrate	oxidized ArgR		reduced ArgR		C47A/C73A-ArgR	
	S.A. (U/mg)	R.A. (%)	S.A. (U/mg)	R.A. (%)	S.A. (U/mg)	R.A. (%)
L-Arginine	1,510 ± 10.4	100	1,510 ± 5.4	100	1,140 ± 13.2	100
L-Lysine	1,570 ± 9.8	104	1,560 ± 10.3	103	1,170 ± 11.1	103
L-Ornithine	660 ± 3.2	44	641 ± 8.3	42	501 ± 8.3	44
L-Alanine	177 ± 6.2	12	161 ± 4.2	11	280 ± 3.1	25
D-Arginine	1,490 ± 18.4	100	1,500 ± 12.4	100	1,100 ± 20.4	100
D-Lysine	1,540 ± 21.5	103	1,550 ± 13.2	103	1,150 ± 18.3	105
D-Ornithine	626 ± 6.8	42	611 ± 5.3	41	490 ± 13.2	45
D-Alanine	168 ± 5.2	11	160 ± 4.0	11	291 ± 5.4	26

Reported values are means ± SD (n=3)
S.A., Specific activity; R.A., Relative activity

Table II-5. Kinetics analysis

Substrate		ArgR			C47A/C73A-ArgR		
		k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Lysine	L-form	109 ± 5.1	2.36 ± 0.42	46.2 ± 8.1	84.9 ± 2.1	1.79 ± 0.01	47.4 ± 0.3
	D-form	104 ± 4.3	2.38 ± 0.52	43.7 ± 5.2	81.7 ± 1.3	1.81 ± 0.02	45.1 ± 1.1
Arginine	L-form	119 ± 3.8	1.17 ± 0.01	101 ± 3.0	93.5 ± 1.9	1.01 ± 0.02	92.6 ± 1.0
	D-form	119 ± 0.2	1.24 ± 0.4	96.0 ± 0.5	94.6 ± 2.1	1.00 ± 0.1	94.6 ± 0.91
Ornithine	L-form	87.7 ± 0.5	3.74 ± 0.8	23.4 ± 4.1	68.8 ± 0.02	3.01 ± 0.04	22.9 ± 0.04
	D-form	83.3 ± 0.3	3.62 ± 1.2	23.0 ± 1.7	67.5 ± 0.03	3.06 ± 0.03	22.1 ± 0.02
Alanine	L-form	2.41 ± 0.01	15.7 ± 0.2	0.15 ± 0.05	6.29 ± 0.3	8.45 ± 0.01	0.74 ± 0.3
	D-form	2.62 ± 0.04	15.0 ± 0.1	0.17 ± 0.01	6.81 ± 0.4	8.82 ± 0.2	0.77 ± 0.2

Reported values are means ± SD (n=3)

DISCUSSION

Disulfide bonds have been detected in the PLP-independent amino acid racemases such as *Pyrococcus horikoshi* OT3 aspartate racemase (AspR) (49) and *Haemophilus influenzae* diaminopimelate epimerase (DapF) (14). In *P. horikoshi* OT3 AspR, the inter-subunit disulfide linkage is formed between C73 and C73'. This disulfide bond may play an important role in increasing the thermostability of the enzyme, because the highly thermostable enzyme was derived from a hyperthermophilic archaeum. It is likely that this disulfide bond is dispensable for dimerization, because other dimeric AspRs from *Desulfurococcus* strain SY. (92) and *Streptococcus thermophilus* (93) have no corresponding cysteine residues. In the *H. influenzae* DapF the intramolecular disulfide bond has been shown, by X-ray crystallographic analysis, to be formed between C73 and C217 (14). However, DapF shows the maximum activity in the presence of a thiol reductant. It is important to note that this disulfide bond might be an artifact formed during the crystallization and unnecessary *in vivo*. In the case of the PLP-dependent amino acid racemases, there is no previous report of a protein with a primary structure containing a disulfide bond. I found that the single intramolecular disulfide bond between C47 and C73 exists in the primary structure of arginine racemase, and arginine racemase is the first example of a PLP-dependent

amino acid racemase that contains a disulfide bond. The amino acid racemase activities were also detected when the disulfide bond of arginine racemase was disrupted by site-directed mutagenesis or reduced with DTT (Table II-3). The thermal and pH profiles and the quaternary structure of arginine racemase did not change when the disulfide bond of arginine racemase was disrupted by site-directed mutagenesis. The substrate specificity and the overall structure did not change when the disulfide bond of arginine racemase was reduced with DTT after the protein was matured, but these properties changed when the disulfide bond of arginine racemase was disrupted by site-directed mutagenesis before the protein was matured (Fig. II-4 and Table II-3). The total activity of arginine racemase decreased when the disulfide bond of arginine racemase was disrupted by site-directed mutagenesis before the protein was matured (Table II-4) or when arginine racemase was expressed in the cytoplasm. Based on these results, I can conclude that the disulfide bond of arginine racemase is essential for arginine racemase to fold and mature as an amino acid racemase with broad substrate specificity. Similar functions of the disulfide bond in protein folding and in stabilizing the folded protein were found in various enzymes, such as an alkaline phosphatase (8, 82), a lysozyme (35, 36), a prochymosin (96), and a beta-propeller phytase (11).

Two cysteine residues (C47 and C73) of arginine racemase were found to be

located around the PLP-binding lysine K51. The homology search revealed that these two cysteine residues are conserved only in the primary structure of the amino acid racemases with broad substrate specificity (BsRC; EC 5.1.1.10) (39) of *Pseudomonas putida* IFO 12996 and not in other amino acid racemases previously reported (Fig. II-5). The signal peptide of arginine racemase is located at the *N*-terminus, and its homologous sequence also exists in the *N*-terminus of BsRC (53). I reported that arginine racemase was exported into the periplasm by the Sec system (53). The reducing environment of the cytoplasm was maintained by thioredoxin reductase and glutathione reductase, which prevent the formation of a disulfide bond (32). Arginine racemase was exported into the periplasm, where it likely interacts with the disulfide bond factor (Dsb) (56) to form the disulfide bond within the oxidative environment of the periplasm (Fig. II-6). Accordingly, the two cysteine residues together with the signal peptide are essential for arginine racemase, and probably also for BsRC, for the maturation as an amino acid racemase with broad substrate specificity.

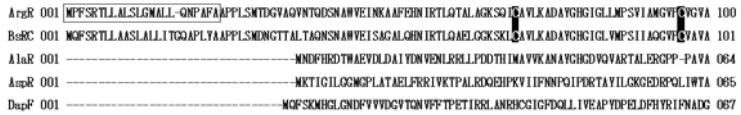


Fig. II-5. *N*-terminal sequence alignments of PLP-dependent and independent amino acid racemases.

The *N*-terminal sequences are from the following proteins: ArgR, arginine racemase of *Pseudomonas taetrolens* NBRC 3460 (accession no. AB096176); BsRC, amino acid racemases with broad substrate specificity of *Pseudomonas putida* IFO 12996 (accession no. BD373122); AlaR, alanine racemase of *Bacillus stearothermophilus* (accession no. M19142); AspR, aspartate racemase of *Pyrococcus horikoshi* OT3 (accession no. BA000001-693); and DapF, diaminopimelate epimerase of *Haemophilus influenzae* (accession no. L42023-730). The signal sequence is shown in the box. The cysteine residues that form a disulfide bond are shown in a white letter on a black background.

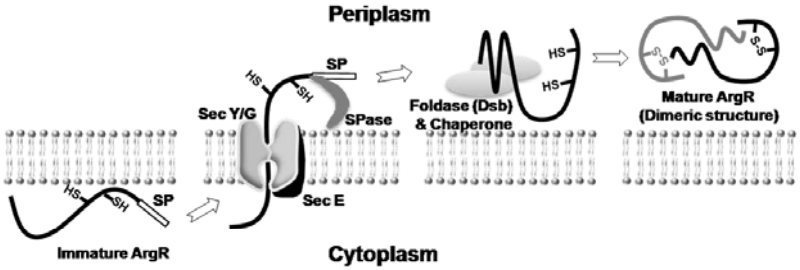


Fig. II-6. Proposed export and folding mechanism of arginine racemase.

After the export from the cytoplasm to the periplasm, the signal peptide (SP) of arginine racemase is removed by a signal peptidase (SPase).

SUMMARY

I found that a single intramolecular disulfide bond between the cysteines C47 and C73 exists in the primary structure of arginine racemase (ArgR) from *Pseudomonas taetrolens* NBRC 3460, and this is the first example of a pyridoxal 5'-phosphate (PLP)-dependent amino acid racemase that contains a disulfide bond. The amino acid racemase activity was still detected, when the disulfide bond of arginine racemase was disrupted by site-directed mutagenesis or reduced with dithiothreitol (DTT). The thermal and pH profiles and the quaternary structure of arginine racemase did not change when the disulfide bond of arginine racemase was disrupted by site-directed mutagenesis. The substrate specificity and the overall structure did not change when the disulfide bond of arginine racemase was reduced with DTT after the protein was matured. However, these properties changed when the disulfide bond of arginine racemase was disrupted by site-directed mutagenesis before protein maturation. The total activity of arginine racemase decreased when the disulfide bond of arginine racemase was disrupted by site-directed mutagenesis before the protein was matured or when arginine racemase was expressed in the cytoplasm. Based on these results, I can conclude that the disulfide bond of arginine racemase is essential for arginine racemase to fold and mature as an amino acid racemase with broad substrate specificity.

CHAPTER III / SECTION I

D-Lysine production by *Corynebacterium glutamicum* ATCC 13032 cells harboring an arginine racemase gene of *Pseudomonas taetrolens* NBRC 3460

INTRODUCTION

D-Amino acids have been considered as biologically inactive substances, although L-amino acids are components of proteins and play important roles in various metabolic pathways. However, almost all bacteria synthesize and utilize D-amino acids, such as D-alanine, D-glutamate, D-valine, and D-phenylalanine as a component of their cell membrane (10, 77) or antibiotics (51, 52, 75). Moreover, D-aspartic acid was found in mammalian cells, and it is suggests that the formation of D-aspartic acid relates to aging (22, 24, 50, 55). D-Lysine is a useful compound as an analog of lutenizing-hormone-releasing hormone (3) and as a drug carrier in the form of polylysine (68). It has been biochemically prepared by asymmetric hydrolysis of carbobenzoxylysine with an L-amino acid oxidase, by microbiological asymmetric hydrolysis of ε-acyllysine or 5-substituted hydantoin, and by microbial resolution of α-amino-ε-caprolactam. However, none of these methods seems to be not applicable to commercial production of D-lysine, since the raw materials and the activity of the enzyme and selectivity are not sufficient to synthesize D-lysine.

Corynebacterium glutamicum ATCC 13032 has been used for industrial production of L-lysine for about 50 years. During the recent years, the classical way to prepare the L-amino acid overproduced strain by random mutagenesis and selection has

been changed to novel approaches based on a rational engineering of the cell. For example, genome breeding, based on the introduction of beneficial mutations identified by comparative sequence analysis between wild type and classical producer strains has generated efficient L-lysine producers (60, 61).

In this study, I constructed the L-lysine overproducer of *C. glutamicum* ATCC 13032, and examined the D-amino acid production by using this strain harboring the arginine racemase gene of *Pseudomonas taetrolens* NBRC 3460.

MATERIALS AND METHODS

Materials. D-lysine, Boc-D-Lys(Boc)-OH, Boc-L-Lys(Boc)-OH, H-L-Ala-OtBu·HCl, and WSCI·HCl were purchased from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). L-Lysine and dichloromethane were purchased from Wako Chemical Co. (Osaka, Japan). Restriction endonucleases were purchased from Toyobo (Osaka, Japan). Ligation mix and 10 X A attachment mix were purchased from Takara Bio (Shiga, Japan). All other chemicals were purchased from Kanto Kagaku Co (Tokyo, Japan), Kishida Chemical Co. (Osaka, Japan), Sigma-Aldrich Co. (Missouri, USA), or Tokyo Kasei Kogyo (Tokyo, Japan) unless otherwise stated and were of the best commercially available grade.

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table III-I-1. Luria-Bertani (LB) medium was used as a complex medium for *Escherichia coli* JM 110, and also for *Corynebacterium glutamicum* ATCC 13032 to isolate their genomic DNA. *C. glutamicum* ATCC 13032 and its mutants were cultivated on either a brain-heart infusion (BHI) agar medium (Difco, BD, France) or a salt agar medium CGXII (38). Kanamycin (25 μg ml^{-1}), spectinomycin (25 μg ml^{-1}), or sucrose (10%, w/v) was added to the medium, if

necessary. The cultivation temperatures of *E. coli* JM 110 and *C. glutamicum* ATCC 13032 were 37°C and 30°C, respectively.

pK18mobsacB, which is a nonreplicative plasmid in *C. glutamicum* ATCC 13032, was used for a gene replacement in a genomic DNA of *C. glutamicum* ATCC 13032. A plasmids pClysC311, which consists of a *lysC*T311I gene of *C. glutamicum* ATCC 13032 ligated into a vector pK18mobsacB, was used to replace a *lysC* gene in a chromosomal DNA of *C. glutamicum* ATCC 13032. A plasmid, pEKEx3 was used to express the arginine racemase gene of *P. taetrolens* NBRC 3460 in *C. glutamicum* ATCC 13032 cells. Plasmids pPargR and pPSPargR, which consist of the arginine racemase and the arginine racemase with the signal peptide of *Pseudomonas taetrolens* NBRC 3460, respectively ligated into a vector pEKEx3, were used to express these genes into *C. glutamicum* ATCC 13032. The *lysE* deletion mutant strain of *C. glutamicum* ATCC 13032 *C. glutamicum* ATCC 13032::*lysE* (LysE type translocator) was obtained from Institut für Biotechnologie, Forschungszentrum Jülich, Germany.

Table III-1-1. Strains, plasmids, and oligonucleotides used

Strains, plasmid, or oligonucleotides	Relevant characteristics or sequence	Source, reference, or purpose
Strains		
Escherichia coli JM 110	*rpsL (Str^r) thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44 d(lac-proAB) [F' traD36 proAB lacI^q ZΔM15]*	The National Institute of Genetics
Corynebacterium glutamicum ATCC13032	WT strain ATCC 13032, biotin-auxotrophic	Institut für Biotechnologie, Forschungszentrum, Jülich, Germany
C. glutamicum ATCC 13032 *lysC*T311I	Exchange T311I in the *lysC* gene	This study
C. glutamicum ATCC 13032: *lysE*	WT deleted of the fragment of *lysE* gene	Institut für Biotechnologie, Forschungszentrum, Jülich, Germany
Plasmid		
pET11b-*argR*	Expression vector, contains full-length arginine racemase gene	Chapter I, Section I
pK18mobsacB	pUC18 derivative, *lacZ*α Km^r, *mob* site, *sacB* gene	Institut für Biotechnologie, Forschungszentrum, Jülich, Germany
pClys*C*311	pK18mobsacB carrying *lysC*T311I gene	This study
pEKEx3	P*tac*, *lacI^q*, Spc^r	Institut für Biotechnologie, Forschungszentrum, Jülich, Germany
pPargR	pEKEx3 carrying arginine racemase gene without the signal peptide of *Pseudomonas taetrolens* NBRC 3460	This study
pPSPargR	pEKEx3 carrying arginine racemase gene with the signal peptide of *Pseudomonas taetrolens* NBRC 3460	This study
Oligonucleotides		
P1	5'-AA**G AATTC**T TGG CAG CTG CTT TGA-3'	Primer for allelic replacement of *lysC*
P2	5'-GCA GGT GAA GAT GAT GTC GGT G-3'	Primer for allelic replacement of *lysC*
P3	5'-CAC CGA CAT CAT CTT CAC CTG C-3'	Primer for allelic replacement of *lysC*
P4	5'-AA**G GATCC**A AAC GAA CAG TGT CAG-3'	Primer for allelic replacement of *lysC*
P5	5'-TTT **CTG CAG** AAG GAG ATA TAG ATA TGG CGC CAC CCC-3'	Primer for expression of arginine racemase gene without the signal peptide
P6	5'-TTC **TGC AG**A AGG AGA TAT AGA TAT GCC CTT CTC-3'	Primer for expression of arginine racemase gene with the signal peptide
P7	5'-GCA **GGATCC** TCG TTC AAT ATA CGG-3'	Primer for expression of arginine racemase gene

Sequences shown in boldface type are as follows: GAATTC, EcoRI (P1); GGATTC, BamHI (P4/P7); CTGCAG, PstI (P5/P6). Underlined sequences show ribosome binding sites.

Construction of pClysC311. Oligonucleotide sequences used for this study are given in Table III-1. I used an overlapped extension PCR method to construct a *lysC* (aspartate kinase gene, 1,266 bp, YP_224551) T311I. The first PCR was done to replace a gene encoding T311 with a gene encoding I311 to introduce a T311I point mutation into a *lysC* with primer P1 and primer P2 against a genomic DNA of *C. glutamicum* ATCC 13032 by using a Gene Amp PCR system 9700 (PE Applied Biosystems Japan, Tokyo, Japan). The program used consisted of 3 sequential steps of 98°C for 10sec, 55°C for 30 sec, and 68°C for 60 sec, and was run repeatedly for 25 cycles. The second PCR was performed under the conditions used for first PCR with an exception that primer P3 and primer P4 were used instead of primer P1 and primer P2, respectively. The PCR products obtained in the first and second PCR were mixed together and used as megaprimer 1 and megaprimer 2 in third PCR. The program used for third PCR consisted of 4 sequential steps of one cycle of 94°C for 5 min, 25 cycles of 98°C for 10 sec, 55°C for 30 sec, 68°C for 60 sec, and one cycle of 68°C for 7 min. The PCR product obtained was used for fourth PCR as a template DNA. A *lysC*-T311I was obtained and ligated into an EcoRI-BamHI digested pK18mobsacB vector to form a plasmid pClysC311 vector.

Allelic replacement of *lysC*. The allelic replacement of *lysC* in a genomic DNA of *C. glutamicum* ATCC 13032 was done with a pClysC311 by using a Gene Pulser Xcell^{TM} (Bio-Rad, California, USA). The electroporation was done at 25 μF, 200 Ω, and 2,500 V. Since a plasmid pClys311 cannot replicate in *C. glutamicum* ATCC 13032 cells and causes an allelic replacement with *lysC* in a genomic DNA of *C. glutamicum* ATCC 13032, the *C. glutamicum* ATCC 13032 cells transformed with the plasmid can be easily selected with a kanamycin resistance. The allelic replacement occurred by an integration of the plasmid *lysC* via a single-crossover homologous recombination. The kanamycin-resistant integrant obtained was grown on an LB agar medium without kanamycin for 1 day to develop a second recombination by inoculation of an appropriate dilution (10^4-10^5 cells) on a BHI agar medium containing 10% (w/v) sucrose. The genomic DNA was prepared from randomly chosen sucrose-resistant colonies, and the presence of the *lysC* mutant was examined by using a DNA sequencer SQ5500E (Hitachi High-Technologies Co., Tokyo, Japan).

Preparation of pEKEx3-*argR* and pEKEx3-*SP-argR*. A DNA fragment containing the full-length coding region of the arginine racemase gene was amplified by PCR using pET11b-*argR* as a template (53). The primers P5 and P7 were used to amplify the

argR without a signal peptide. The primers P6 and P7 were used to amplify the *argR* with a signal peptide. The primers P5 and P6 contain a ribosome binding site (RBS) between PstI site and *argR* sequence, and a start codon was added just before *argR* without signal peptide. Each PCR product obtained was ligated into a PstI-BamHI digested pEKEx3 vector to form a pPargR and a pPSPargR vector, respectively. Each plasmid constructed was transformed into *C. glutamicum* ATCC 13032 and *C. glutamicum lysC* T311I by Gene Pulser Xcell Electroporation System (Bio-Rad, California, USA).

Course of D- and L-lysine production by *C. glutamicum* ATCC 13032 and *C. glutamicum* ATCC 13032 *lysC* T311I transformed with pEKEx3, pPargR, or pPSPargR. *C. glutamicum* ATCC 13032 and *C. glutamicum lysC* T311I transformed with a pEKEx3, a pPargR or a pPSPargR vector, respectively, was pre-cultivated on a brain-heart infusion (BHI) agar medium (Difco, BD, France), and then a single colony on the medium was inoculated into a 50 ml of a BHI medium in a 500 ml of Erlenmeyer flask with baffles. The flask was placed on a rotary shaker and cultivated at 30°C for 24 h with shaking (150 rpm) until the growth of microorganism reaches a stationary phase. After centrifugation at 5,500 X *g* for 15 min at 4°C, the collected cells were

washed with an ice-chilled CGXII medium (38), and inoculated into a 50 ml of the same medium at a final A_{600} of 1.0. After 0, 3, 6, 9, 11.25, 22.5, 25.25, 28.25, 31, 33.5, 47, or 56 h, 0.5 ml of the culture broth was taken from the flask and centrifuged at 16,100 X *g* for 15 min at 4°C. The supernatant obtained was diluted with a 0.1 M sodium acetate buffer (pH 7.2) 1 or 10 times, and delivertized with *o*-phthalaldehyde after filtration with a GL chromatodisc (0.2 μm, GL Science, Tokyo, Japan). The sample was subjected to HPLC analysis under the same conditions previously reported (21). The course of D- and L-ornithine production by *C. glutamicum* ATCC 13032 were also studied by the same method described above.

Synthesis of L-Lys-L-Ala and D-Lys-L-Ala. L-Lys-L-Ala and D-Lys-L-Ala were chemically synthesized by a solid-phase method. Boc was used to protect an N^{α} terminus and the side-chain amino group of Ala and Lys, and tBu was used to protect an α-carboxyl group of Ala. Boc-L-Lys(Boc)-OH (1.00 g, 2.89 mmol) or Boc-D-Lys(Boc)-OH (1.00 g, 2.89 mmol), and HCl·H-Ala-OtBu (0.525 g, 2.89 mmol) were reacted in a mixture containing CH_2Cl_2 (50.0 ml), HONB (0.518 g, 2.89 mmol), WSCI·HCl (3.18 mmol, 0.610 g), and Et_3N (885 μl, 6.36 mmol). After addition of chloroform (20 ml), the peptide produced was extracted with a saturated sodium

hydrogen carbonate solution (20 ml), and subsequently with a saturated NaCl solution (20 ml). The solution obtained was evaporated, and the protecting groups of the peptide were removed with trifluoroacetic acid (TFA, 5.00 ml) containing 5.00 ml of CH_2Cl_2 and 5.00% (v/v) phenol. The deprotected peptide solution was evaporated, and the peptide was crystallized with 5.00 ml of diethylether. The final preparation of the synthesized peptide was analyzed with an FT NMR (JEOL, Tokyo, Japan), and an LC-MS API3000 (Applied Biosystems, CA, USA).

Assay of D-lysine export. *C. glutamicum* ATCC 13032 and its mutant strains constructed were cultivated under the same conditions described in "Course of D- and L-lysine production by *C. glutamicum* ATCC 13032 and *C. glutamicum* ATCC 13032 *lysC* T311I with pEKEx3, pPargR, and pPSPargR". To determinate the export rates in short-term experiments, the *C. glutamicum* ATCC 13032 cells were inoculated into a pre-warmed CGXII medium containing 2 mM L-Lys-L-Ala or D-Lys-L-Ala. The growth of each strain in the culture broth was measured spectrophotometrically at 600 nm, and L- or D-lysine extracellularly produced was measured with an HPLC.

To examine a possibility of D-lysine export by LysE, *C. glutamicum* ATCC 13032::*lysE* was transformed with a pPargR. The pre-culture was done under the same

conditions described above, then amino acid secretion was examined by resuspending the cells in a pre-warmed CGXII medium containing 2 mM L-Lys-L-Ala plus spectinomycin. *C. glutamicum* ATCC 13032::*lysE* transformed with a pPargR or a pEKEx3, *C. glutamicum* ATCC 13032 transformed with a pPargR in CGXII with 2 mM L-Lys-L-Ala and spectinomycin or in CGXII with spectinomycin were used as references.

Analytical method. HPLC analysis was carried out with a high-performance liquid chromatography LC-10AD system (Shimadzu, Kyoto, Japan). A Hypersil ODS column (particle size: 5 μm, ϕ4 X 250 mm; Agilent, California, USA) was used to separate D- and L-ornithine according to the modified method of Erbe T. *et al* (18). The column temperature was kept at 40°C, and the adsorbed sample was eluted with a linear gradient of a 0.1 M acetate buffer, pH 7.2 (solvent A) and methanol (solvent B). The column was equilibrated with a solvent A containing 50% of solvent B at a flow-rate of 0.8 ml min^{-1}, and then the concentration of solvent B was increased to 70% from 0 to 13 min. After elution of D- and L-ornithine, the concentration of solvent B was increased to 85% from 13 to 17 min. Before analysis of a next sample, the column was re-equilibrated with a solvent A containing 50% of solvent B for 5.5 min.

The sample for HPLC analysis was delivertized with *o*-phthalaldehyde and *N-tert*-butyloxycarbonyl-L-cysteine (BocC) by the modified method of Buck R. H. *et al* (9). A sample (10 μl) was mixed with methanol (0.1 ml) containing 7.5 mg of *o*-phthalaldehyde and 7.5 mg *N-tert*-butyloxycarbonyl-L-cysteine (BocC), and then 5.0 ml of a 0.4 M sodium borate buffer (pH 10.4) containing 15 μl of Brij 30 was added to the mixture.

Other methods. The growth of microorganism in a culture broth was measured spectrophotometricaly at 600 nm (A_{600}) with a photometer V-550 (Jasco Co. Ltd., Tokyo, Japan). Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (44). Gels were strained with Coomassie Blue R-250. The protein band of the arginine racemase in SDS-PAGE was subjected to an in-gel digestion with trypsin as described previously (31). The samples were analyzed with a MALDI-TOF MS (the Biflex III spectrometer, Bruker Daltonics, Bremen, Germany).

RESULTS

Preparation of *Corynebacterium glutamicum* ATCC 13032 *lysC* T311I and its L-lysine production. A non-replicative vector, pK18mobsacB-*lysC* T311I was constructed to replace a gene encoding Thr311 with a gene encoding Ile311 of *lysC* by an allelic replacement: a C-to-T exchange at position 932 in *lysC*. The vector was transformed into the *C. glutamicum* ATCC 13032 cells and the desired clone was selected with kanamycin resistance and sucrose sensitivity. The vector was excluded by itself from the cells after an allelic replacement occurs (Fig. III-I-1). The mutation was confirmed by using a DNA sequencer. The mutant obtained was named *C. glutamicum* ATCC 13032 *lysC T311I*. The *lysC* mutant produced about 19 mM of L-lysine when it was cultivated in a glucose-containing CGXII medium at 30°C for 36 h. The growth rate of *C. glutamicum* ATCC 13032 *lysC* T311I was similar to that of its wild-type strain.

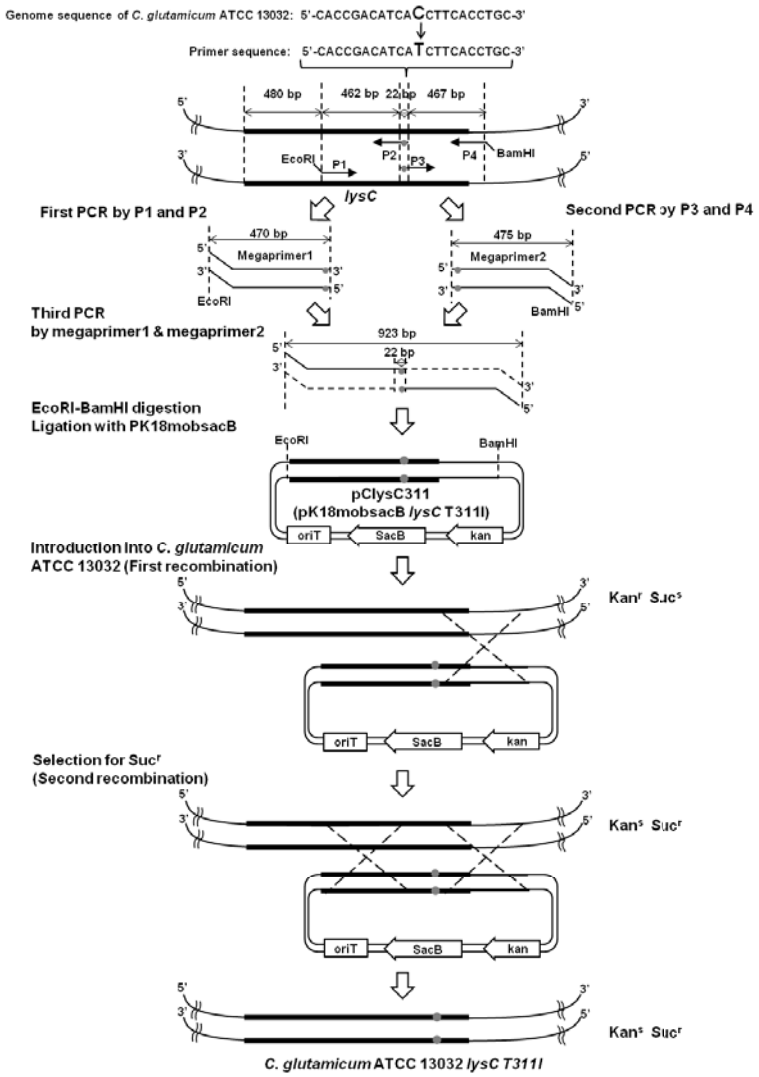


Fig. III-I-1. Construction strategy for allelic replacement of the *lysC* T311I in the chromosome of *Corynebacterium glutamicum* ATCC 13032.

Effects of overexpression of an arginine racemase of *Pseudomonas taetrolens* NBRC 3460 into *C. glutamicum* ATCC 13032. The expression of the recombinant arginine racemase from *P. taetrolens* NBRC 3460 in *C. glutamicum* ATCC 13032 cells was confirmed by SDS-PAGE and MALDI-TOF MS. The *C. glutamicum* ATCC 13032 cells harboring pEKEx3, pPargR, or pPSPargR was grown in a CGXII medium plus spectinomycin, and their growth was measured spectrophotometricaly at 600 nm. The growth rate of *C. glutamicum* ATCC13032 harboring a pPargR decreased to 0.35 h^{-1} as compared with that of *C. glutamicum* ATCC 13032 harboring a pEKEx3 (0.39 h^{-1}) or a pPSPargR (0.40 h^{-1}), respectively (Fig. III-I-2). Similar lag phase was observed in a growth curve of a *C. glutamicum* ATCC 13032 *lysC* T311I harboring a pPargR.

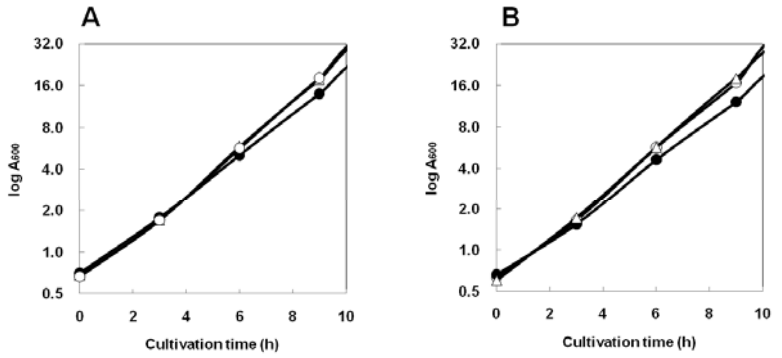


Fig. III-I-2. Growth curves of *C. glutamicum* ATCC 13032 and *C. glutamicum* ATCC 13032 *lysC* T311I harboring the arginine racemase of *P. taetrolens* NBRC 3460.

A: *C. glutamicum* ATCC 13032, B : *C. glutamicum* ATCC 13032 *lysC* T311I

○, growth curves of *C. glutamicum* ATCC 13032 or *C. glutamicum* ATCC 13032 *lysC* T311I harboring a pEKEx3; ●, growth of *C. glutamicum* ATCC 13032 or *C. glutamicum* ATCC 13032 *lysC* T311I harboring a pPargR; and △, growth of *C. glutamicum* ATCC 13032 or *C. glutamicum* ATCC 13032 *lysC* T311I harboring a pPSPargR. (n=3)

D-Lysine production by *C. glutamicum* ATCC 13032 cells harboring an arginine racemase gene of *P. taetrolens* NBRC 3460. *C. glutamicum* ATCC 13032 *lysC T311I* harboring a pPargR produced 9.6 mM of D-lysine in 56 h-cultivation, and *C. glutamicum* ATCC 13032 *lysC* T311I harboring a pPSPargR produced 10.6 mM of D-lysine in 47 h-cultivation. But *C. glutamicum* ATCC 13032 *lysC* T311I harboring a pEKEx3 didn't produce D-lysine at all (Fig. III-I-3).

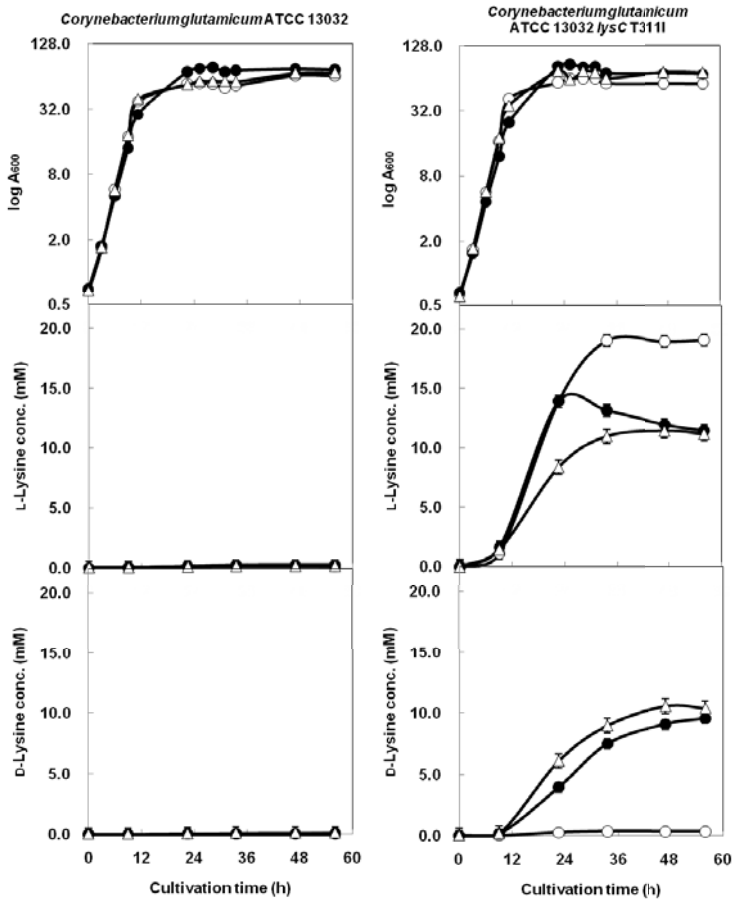


Fig. III-I-3. Comparison of growth, extracellular D-lysine and L-lysine concentration, of *C. glutamicum* ATCC 13032 *lysC* T311I and *C. glutamicum* ATCC 13032.

○, cells transformed with pEKEx3; ●, cells transformed with pPargR; and △, cells transformed with pPSPargR (n=3).

Secretion of D-lysine from *C. glutamicum* ATCC 13032 cells. In order to confirm the possibility of D-lysine secretion via an L-lysine exporter, *C. glutamicum* ATCC 13032 was cultivated in a CGXII medium plus spectinomycin and 2 mM L-Lys-L-Ala, or D-Lys-L-Ala. L-Lysine was secreted into the medium, but D-lysine was not detected. Accordingly, I cannot confirm that D-lysine is secreted via an L-lysine exporter of this microorganism (Fig. III-I-4).

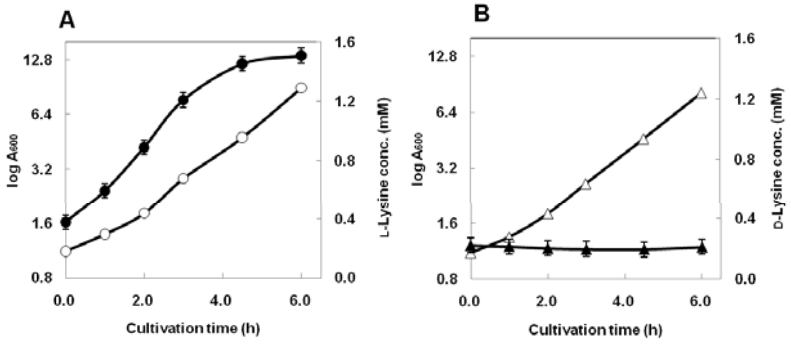


Fig. III-I-4. Investigation of secretion of L-lysine and D-lysine from *C. glutamicum* ATCC 13032 cells by using L-Lys-L-Ala and D-Lys-L-Ala.

Cells were grown in CGXII containing 2 mM L-Lys-L-Ala (A), and D-Lys-L-Ala (B) (n=3). ○and △, A_{600}; ● and ▲, Lysine conc. (mM)

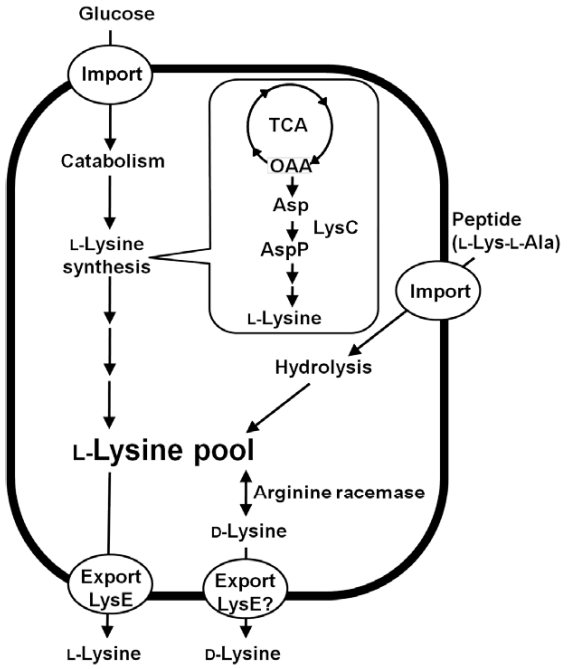


Fig. III-I-5. Proposed biochemical pathway of D-lysine production by *Corynebacterium glutamicum* ATCC 13032 *lysC* T311I harboring a pPargR.

TCA, TCA cycle; OAA, oxaloacetate; Asp, L-aspartate; AspP, L-β-aspartyl phosphate; LysC, aspartokinase

lysE was known as the gene encoding the L-lysine export carrier (85). In view of the finding that L-lysine is accepted as a transport substrate of LysE, I studied a possibility that D-lysine is exported via LysE by comparing (Fig. III-I-5), the growth of *C. glutamicum* ATCC 13032 and *C. glutamicum* ATCC 13032::*lysE*. When 2 mM of Lys-Ala was added to the medium, the only growth of *C. glutamicum* ATCC

13032::*lysE* delayed in an initial growth phase (Fig. III-6). Similarly *C. glutamicum* ATCC 13032::*lysE* with a pPargR was examined under the same conditions, and the growth also delayed. These observations well agreed with the growth-inhibition caused by high intracellular L-lysine concentrations reported so far (85). Accordingly D-lysine intracellularly produced probably shows the similar growth inhibition effect on *C. glutamicum* ATCC 13032.

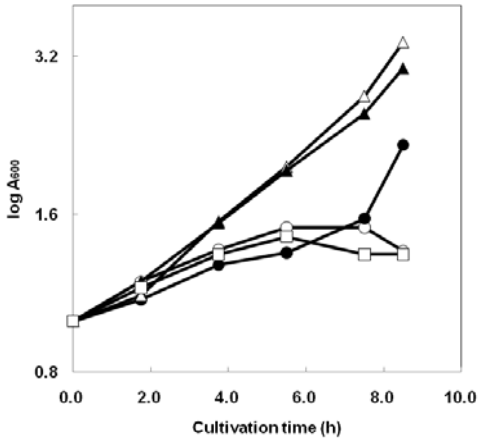


Fig. III-I-6. D-Lysine exporter of *Corynebacterium glutamicum* ATCC 13032.

○, *C. glutamicum* ATCC 13032::*lysE* with pPargR in a CGXII medium plus L-Lys-L-Ala; ●, *C. glutamicum* ATCC 13032::*lysE* with pPargR in a CGXII medium; △, *C. glutamicum* ATCC 13032 with pPargR in a CGXII medium plus L-Lys-L-Ala; ▲, *C. glutamicum* ATCC 13032 with pPargR in a CGXII medium; □, *C. glutamicum* ATCC 13032::*lysE* in a CGXII medium plus L-Lys-L-Ala (n=3).

DISCUSSION

A mutation of an aspartate kinase gene, the *lysC* in *Corynebacterium glutamicum* ATCC 13032, leads to the elimination of the feedback inhibition by L-lysine in the biosynthetic pathway from L-aspartic acid to L-lysine, and L-lysine is overproduced by the engineered *C. glutamicum* ATCC 13032 (80, 81). The *C. glutamicum* ATCC 13032 *lysC* T311I mutant produced about 19 mM of D-lysine, when an arginine racemase gene of *Pseudomonas taetrolens* NBRC 3460 was heterologously expressed into the mutant. The D-lysine production reached about half of the total amount of lysine in the culture broth.

The signal peptide locates at the *N*-terminus of the arginine racemase from *P. taetrolens* NBRC 3460, which functions in exporting the enzyme to a periplasm (53). I found that the growth of the *C. glutamicum* ATCC 13032 harboring an arginine racemase gene without the gene encoding the signal peptide delayed, and the reason for this phenomenon was discussed as follows. The arginine racemase is a periplasmic enzyme, and the enzyme activity decreased when it was expressed in a cytoplasm as compared with the activity detected in a periplasm (54). Probably, when the enzyme is expressed in a cytoplasm, the disulfide bond in its primary structure and other conformational changes could not be occurred well without chaperones and foldases.

Much amount of unfolded arginine racemase accumulated may cause the growth inhibition of this microorganism, and the enzyme activity decreased. And another reason to be considered is that the difference of export rate between L-lysine and D-lysine. In order to clarify the difference, *C. glutamicum* ATCC 13032 was grown in a CGXII medium in the presence of L-Lys-L-Ala or D-Lys-L-Ala, and D-Lys did not secret into a medium. These results suggest that a peptidase of *C. glutamicum* ATCC 13032 probably does not act on D-Lys-L-Ala or D-Lys-L-Ala in a medium is not incorporated in this microorganism. Therefore, the difference of the export rate between D- and L-Lys is still unknown.

Various L-amino acids have been produced such as L-arginine, L-ornithine, L-alanine, L-serine, and L-tryptophan as well as L-lysine, and L-glutamate by *C. glutamicum* ATCC 13032. Accordingly, the D-amino acid production system by using the L-amino acid overproducer of *C. glutamicum* ATCC 13032 plus various amino acid racemases is widely applicable for production of various D-amino acids. At least, the arginine racemase can be used in productions of D-arginine and D-ornithine. In addition, various amino acid racemases such as alanine racemase (EC 5.1.1.1) (62), glutamate racemase (EC 5.1.1.3) (13), ornithine racemase (EC 5.1.1.12) (12), aspartate racemase (EC 5.1.1.13) (91), and serine racemase (EC 5.1.1.18) (27) have been reported

previously. Thus various D-amino acid could be produced by combination of the *C. glutamicum* ATCC 13032 L-amino acid overproducer with the amino acid racemases.

Summary

I constructed the *C. glutamicum* ATCC 13032 *lysC* T311I mutant strains for L-lysine production, and produced about 19 mM of D-lysine when the arginine racemase gene of *Pseudomonas taetrolens* NBRC 3460 was heterologously expressed in it. The amount of D-lysine produced reached a half of total lysine in a culture broth. *lysE* was known as the gene encoding the L-lysine exporter of *C. glutamicum* ATCC 13032. In view of the finding that L-lysine is accepted as a transport substrate of LysE, I studied the possibility that D-lysine is exported via LysE. The growth of *C. glutamicum* ATCC 13032 and *C. glutamicum* ATCC 13032::*lysE* were compared in the presence of L-Lys-L-Ala. When 2 mM of L-Lys-L-Ala was added to the medium, only the growth of *C. glutamicum* ATCC 13032::*lysE* delayed. *C. glutamicum* ATCC 13032::*lysE* with a pPargR was also examined under the same conditions, and the growth of this strain also delayed. These observations well agreed with the growth-inhibition caused by high intracellular L-lysine concentrations. D-Lysine intracellularly produced probably shows the similar growth inhibition effect on *C. glutamicum* ATCC 13032.

CHAPTER III / SECTION II

Detection of D-ornithine extracellularly produced by *Corynebacterium glutamicum* ATCC 13032::*argF*

INTRODUCTION

Corynebacterium glutamicum ATCC 13032 is a facultative anaerobe that is useful in the industrial production of various amino acids, including L-glutamic acid, L-lysine, L-threonine, and L-valine (30, 37). In addition, *C. glutamicum* ATCC 13032::*argF*, whose structural gene encodes the ornithine carbamoyltransferase (*argF*, accession no. AAC24816) when the chromosome is disrupted, produces L-ornithine as reported by Hwang *et al* (33). Hence various researchers have studied the enzymes in various L-amino acids biosynthetic pathways of *C. glutamicum* ATCC 13032 and their genes related to the regulation of various forms of L-amino acid production (16, 47, 69, 74). However, D-amino acids production of *C. glutamicum* ATCC 13032 has not been reported so far. During the course of biochemical studies of *C. glutamicum* ATCC 13032::*argF*, I found that it extracellularly produces D-ornithine in addition to L-ornithine. Here we describe the D-ornithine production of *C. glutamicum* ATCC 13032::*argF* as compared with that of the wild-type strain.

MATERIALS AND METHODS

Materials. D-Ornithine was purchased from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). L-Ornithine was purchased from Wako Chemical Co. (Osaka, Japan). All other chemicals used were from Kanto Kagaku Co. (Tokyo, Japan), Kishida Chemical Co. (Osaka, Japan), Sigma-Aldrich Co. (St. Louis, MO, USA), or Tokyo Kasei Kogyo (Tokyo, Japan), unless otherwise stated, and were of the best commercially available grade.

Bacterial strains. *Corynebacterium glutamicum* ATCC 13032 was obtained as an NBRC 12168, from the type-culture collection of the Japanese National Institute of Technology and Evaluation (Ciba, Japan). *C. glutamicum* ATCC 13032::*argF*, the ornithine carbamoyltransferase deletion mutant (accession no. AAC24816) of *C. glutamicum* ATCC 13032, was prepared by a modification of the method of Hwang *et al.* using *Escherichia coli* JM110 instead of *E. coli* DH5α for non-methylation of a vector transformed into *C. glutamicum* ATCC 13032 (33).

Course of D- and L-ornithine production by *C. glutamicum* ATCC 13032 and *C. glutamicum* ATCC 13032::*argF*. *C. glutamicum* ATCC 13032::*argF* was

pre-cultivated on a brain-heart infusion (BHI) agar medium (Difco, BD, France), and then a single colony on the medium was inoculated into 50 ml of a BHI medium in a 500-ml Erlenmeyer flask with a baffle and cultivated at 30°C for 24 h with shaking (150 rpm) until the growth of the microorganism reached stationary phase. After centrifugation at 5,500 X *g* for 15 min at 4°C, the collected cells were washed with an ice-chilled CGXII medium plus 1 mM L-arginine (38), and inoculated into 50 ml of the same medium at an A_{600} of 1.0. After 0, 3, 6, 9, 21, 24, 27, 30, 45, 48, 54, 69, and 93 h, 0.5 ml of the culture broth was taken from the flask and centrifuged at 16,100 X *g* for 15 min at 4°C. The supernatant was diluted with a 0.1 M sodium acetate buffer (pH 7.2) 1 or 10 times, and delivertized with *o*-phthalaldehyde after filtration with a GL chromatodisc (0.2 μm, GL Science, Tokyo). The sample was subjected to HPLC analysis. The course of D- and L-ornithine production by *C. glutamicum* ATCC 13032 was also studied by the method described above.

Preparation of peptideglycan fraction. *C. glutamicum* ATCC 13032::*argF* was cultivated under the conditions described above to determine the course of D- and L-ornithine production by *C. glutamicum* ATCC 13032::*argF*. After cultivation at 30°C for 30 h, the cells were collected by centrifugation at 5,500 X *g* for 15 min at 4°C.

A portion of the collected cells was suspended in a 10 mM Tris-HCl buffer (pH 8.0) plus 10% (v/v) TritonX-100, and disrupted at 4°C twice by French pressure cell press FA-078 (SLM Aminco, Urbana, IL, USA). After centrifugation at 5,500 X *g* for 15 min at 4°C, the supernatant solution obtained was used as a cell-free extract. Separately, a portion of the collected cells was suspended in a 10-mM Tris-HCl buffer (pH 8.0) and disrupted by the French press. After ultracentrifugation at 27,500 X *g* for 30 min at 30°C (P70AT Rotor; Himac CP70MX Preparative Ultracentrifuge, Hitachi Koki Co. Ltd., Tokyo, Japan), the insoluble fraction obtained was washed 4 times with deionised water and used as a peptideglycan fraction. A cell-free extract and a peptideglycan fraction of *C. glutamicum* ATCC 13032 were prepared by the method described above using a BHI and a CGXII medium without 1 mM L-arginine. After 24 h of cultivation, the absorbance at 600 nm of the culture broth of *C. glutamicum* ATCC 13032::*argF* and of *C. glutamicum* ATCC 13032 reached 54.20 ± 0.5 and 54.17 ± 0.2, respectively.

Hydrolyzation of peptydoglycan. A peptideglycan fraction was hydrolyzed with hydrogen chloride with a Pico-Tag Work Station (Waters, Tokyo, Japan) at 110°C for 20 h following the protocol of the manufacturer. The hydrolyzed samples were dried

under reduced pressure and resuspened in a mixture (95%, v/v) containing 5 mM disodium phosphate in deionized water and adjusted to pH 7.4 with 1 N NaOH and acetonitrile (5%, v/v). An aliquot of this solution was subjected to GC-MS analysis.

Analytical method. GC-MS analysis was carried out with a gas chromatography GC-2010 system equipped with a Model QP2010 mass spectrometer (GC-MS, Shimadzu, Kyoto, Japan). A fused silica capillary column, Chirasil®-L-Val (Varian, Darmstadt, Germany) was used to separate and quantify D- and L-amino acids by a method previously reported (1, 70, 71).

HPLC analysis was carried out with a high-performance liquid chromatography LC-10AD system (Shimadzu, Kyoto, Japan). A Hypersil ODS column (particle size, 5 μm, ϕ4 X 250 mm; Agilent, Santa Clara, CA, USA) was used to separate D- and L-ornithine method of Erbe *et al* (21). The column temperature was kept at 40°C, and the adsorbed sample was eluted with a linear gradient of a 0.1 M acetate buffer, pH 7.2 (solvent A) and methanol (solvent B). The column was equilibrated with solvent A containing 62% of solvent B at a flow rate of 0.8 ml min^{-1}, and then the concentration of solvent B was increased to 70% from 0 to 13 min. After elution of D- and L-ornithine, the concentration of solvent B was increased to 85% from 13 to 17 min. Before

analysis of the next sample, the column was re-equilibrated with solvent A containing 62% of solvent B for 5.5 min. The sample for HPLC analysis was delivertized with *o*-phthalaldehyde and *N*-*tert*-butyloxycarbonyl-L-cysteine (BocC) by the method of Buck *et al* (9). A sample (10 μl) was mixed with methanol (0.1 ml) containing 7.5 mg of *o*-phthalaldehyde and 7.5 mg of *N*-*tert*-butyloxycarbonyl-L-cysteine (BocC), and then 5.0 ml of a 0.4 M sodium borate buffer (pH 10.4) containing 15 μl of Brij 30 was added to the mixture.

Other methods. The growth of the microorganism in the culture broth was measured photometrically at 600 nm with a UV-VIS photometer V-550 (Jasco, Tokyo, Japan).

RESULTS

Detection of D-ornithine extracellularly produced by *C. glutamicum* ATCC 13032 and *C. glutamicum* ATCC 13032::*argF*. I found that *C. glutamicum* ATCC 13032::*argF* extracellularly produced large amount of an unknown amino acid when cultivated in a CGXII medium containing 1 mM L-arginine. The unknown amino acid produced was identified by GC-MS analysis and HPLC analysis. It was eluted at a retention time of 15.85 min on HPLC analysis, and this retention time coincided well with that of an authentic D-ornithine (Fig. III-II-1A). GC-MS analysis indicated that the unknown amino acid was eluted at a retention time of 32.25 min, and it showed an m/z value of 216 (Fig. III-II-1B). This m/z value agreed well with the theoretical m/z value of D-ornithine (216). Accordingly, the unknown amino acid produced by *C. glutamicum* ATCC 13032::*argF* was identified as D-ornithine. This is the first report that *C. glutamicum* ATCC 13032 or its mutant produces a D-amino acid extracellularly. Since Hwang *et al.* measured the amino acids extracellularly produced by *C. glutamicum* ATCC 13032::*argF* spectrophotometrically using a nynhydrin reaction, they probably did not notice D-ornithine production by this organism (33). Our finding also suggests that the D-ornithine intracellularly produced can be exported to a medium by *C. glutamicum* ATCC 13032 cells.

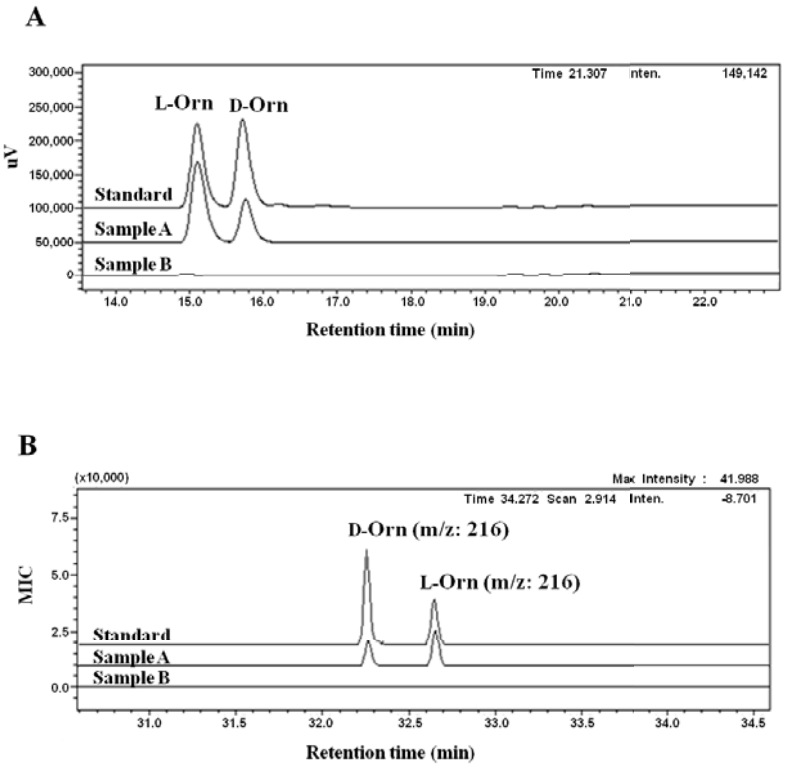


Fig. III-II-1. Detection and identification of D-ornithine in the culture broth of *Corynebacterium glutamicum* ATCC 13032::*argF*.

A, HPLC analysis. Sample A, D- and L-ornithine extracellularly produced by *C. glutamicum* ATCC 13032::*argF* after cultivation for 21 h. Sample B, D- and L-ornithine extracellularly produced by *C. glutamicum* ATCC 13032 after cultivation for 21 h. Standards, 10 mM D- and L-ornithine in deionized water. B, GC-MS analysis. Sample A, D- and L-ornithine extracellularly produced by *C. glutamicum* ATCC 13032::*argF* after cultivation for 21 h. Sample B, D- and L-ornithine extracellularly produced by *C. glutamicum* ATCC 13032 after cultivation for 21 h. Standards, 20 mM D- and L-ornithine in deionized water.

Secretion of L-lysine by the *C. glutamicum* ATCC 13032::*argF* in response to amino acid addition. After 45 h of cultivation of *C. glutamicum* ATCC 13032::*argF*, the D-ornithine and L-ornithine concentration reached 13 mM and, 21 mM, respectively (Fig. III-II-2A). In contrast, *C. glutamicum* ATCC 13032 produced only 0.07 mM D-ornithine and 0.17 mM L-ornithine in the same cultivation time (Fig. III-II-2B).

L-Proline and L-glutamic acid are known as two of the precursors in the L-ornithine biosynthetic pathway of *C. glutamicum* ATCC 13032. I examined the effects of L-proline and L-glutamic acid addition to the medium on the production of D-ornithine by *C. glutamicum* ATCC 13032::*argF* and *C. glutamicum* ATCC 13032. As compared with L-ornithine production after 24 h-cultivation in a CGXII medium containing 1 mM L-arginine (15.1 mM), L-ornithine production increased to 17.3 and to 17.8 mM when 5 mM L-proline and 5 mM L-glutamic acid was added to a CGXII medium containing 1 mM L-arginine, respectively. But in the case of D-ornithine, only the addition of 5 mM L-glutamic acid to a CGXII medium containing 1 mM L-arginine increased D-ornithine production from 14.3 to 17.3 mM, and the addition of 5 mM L-proline to a CGXII medium containing 1 mM L-arginine decreased D-ornithine production from 14.3 to 9.2 mM. The addition of D- or L-ornithine to the medium showed no effect on D-ornithine production by *C. glutamicum* ATCC 13032::*argF* or *C. glutamicum* ATCC 13032. The

cell-free extract of *C. glutamicum* ATCC 13032::*argF* and of *C. glutamicum* ATCC 13032 also contained D-ornithine at concentrations of 0.20 ± 0.04 or 0.19 ± 0.05 mM, respectively.

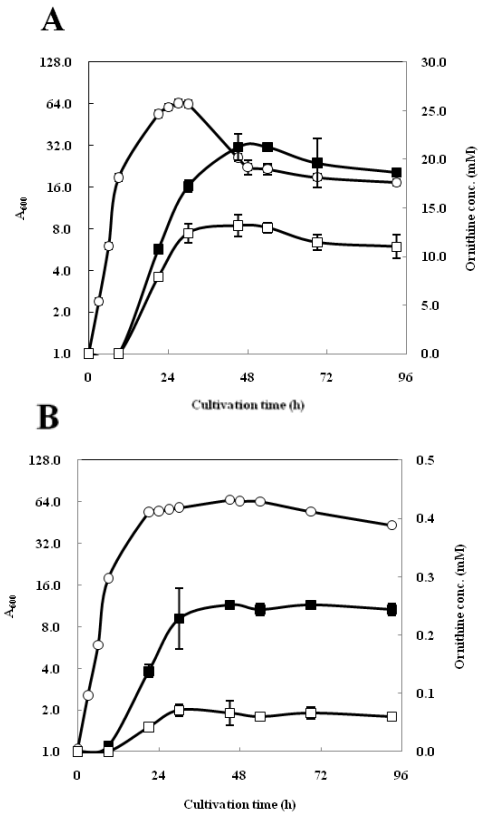


Fig. III-II-2. Course of D- and L-ornithine production by *Corynebacterium glutamicum* ATCC 13032::*argF* and *Corynebacterium glutamicum* ATCC 13032.

○, Absorbance at 600 nm; ■, L-Ornithine concentration; □, D-Ornithine concentration (n = 3). A, *Corynebacterium glutamicum* ATCC 13032::*argF*. B, *Corynebacterium glutamicum* ATCC 13032

Detection of D-amino acids in a peptideglycan fraction. GC-MS analysis of D-amino acids in the peptideglycan fraction indicated that peptideglycan fractions of both *C. glutamicum* ATCC 13032::*argF* and *C. glutamicum* ATCC 13032 contained only D-alanine (retention time, 8.25 min; m/z, 190) and D-glutamic acid (retention time, 31.75 min; m/z, 202), but not D-ornithine or other D-amino acids.

DISCUSSION

D-Ornithine has been reported previously to be a biochemically important molecule in several microorganisms. It was detected as the intermediate of the L-ornithine catabolic pathway in *Clostridium sticklandii* DSM 519 (23). It was also detected as an essential component of a bacteriocin and the cell walls of *Bacillus licheniformis* and *B. subtilis* (5, 19, 43). D-Ornithine produced in *C. sticklandii* DSM 519 was catalyzed by an ornithine racemase (23). In the case of *B. cereus* ATCC 10876, the putative ornithine racemase gene was found in the genomic DNA of *B. cereus* ATCC 10876 (accession no. ZP_04321077), but the function of its gene product remains unknown. The transamination activity of D-ornithine was also detected in *B. licheniformis* (5), but no enzyme related to D-ornithine catabolism or anabolism in *C. glutamicum* ATCC 13032 has been reported. Two amino acid racemase genes were found in the genomic DNA of *C. glutamicum* ATCC 13032: the alanine racemase gene (*alr*, accession no. YP_224879), and the glutamate racemase gene (*murI*, accession no. YP_226751) (40, 62). As we have reported, these amino acid racemases of *C. glutamicum* ATCC 13032 showed high substrate specificity, and probably do not act on ornithine. D-Amino acid dehydrogenase (*dadA*, accession no. YP_227262) and D-amino acid oxidase (*thiO*, accession no. YP_226279) genes also exist in the genomic

DNA of *C. glutamicum* ATCC 13032, but the functions of their gene products are still unknown.

I detected almost the same amount of D-ornithine (about 0.2 mM) in the cell-free extract of both *C. glutamicum* ATCC 13032::*argF* and *C. glutamicum* ATCC 13032, but only *C. glutamicum* ATCC 13032::*argF* extracellularly produced D-ornithine (13 mM after 45 h cultivation). This suggests that disruption of the ornithine carbamoyltransferase gene (*argF*) increases the L-ornithine production of *C. glutamicum* ATCC 13032. The genomic DNA of *C. glutamicum* ATCC 13032 encodes one protein partially similar to the ornithine racemase from *C. sticklandii* DSM 519 (accession no. CAQ42981) as found by a Psi- or Phi-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as an accession number of BA000036. This gene product is a candidate for the enzyme that catalyzes the conversion of L-ornithine to D-ornithine in *C. glutamicum* ATCC 13032. Using *C. glutamicum* ATCC 13032::*argF*, it is possible to produce D-ornithine easily at high concentration.

The peptideglycan of animal and human pathogenic *corynebacteria* such as *Corynebacterium diphteriae* and *C. glutamicum* ATCC 13032 contains a directly cross-linked *meso*-diaminopimelic acid (variation A1γ) as an essential component, but in the phytopathogenic *corynebacteria,* such as *Corynebacterium poinsettiae* NCPP 177,

Corynebacterium flaccumfaciens ATCC 6887, and *Corynebacterium betae* ATCC 13437, the peptideglycan contains D-ornithine instead of *meso*-diamino pimelic acid (variation B2β) (20). I measured the D-amino acids contents in a peptideglycan fraction of *C. glutamicum* ATCC 13032::*argF* and *C. glutamicum* ATCC 13032, but did not detect D-ornithine in either fraction. Accordingly, D-ornithine is not an essential component of the peptideglycan in *C. glutamicum* ATCC 13032, but may function as an intermediate or a precursor of a biochemically important compound in *C. glutamicum* ATCC 13032.

I am currently studying the D-ornithine production system of *C. glutamicum* ATCC 13032 with various biochemical systems.

SUMMARY

I found that *Corynebacterium glutamicum* ATCC 13032::*argF* extracellularly produced a large amount of D-ornithine when cultivated in a CGXII medium containing 1 mM L-arginine. This is the first report that *C. glutamicum* ATCC 13032 or its mutant produces a D-amino acid extracellularly. *C. glutamicum* ATCC 13032::*argF* produced 13 mM D-ornithine in 45 h of cultivation.

CONCLUSION

In this thesis, I demonstrated that arginine racemase is localized in a periplasm, and its translocation depends on the Sec system. I succeeded also for the first time to prepare the integrated mutant of arginine racemase gene (*argR*) in *Pseudomonas taetrolens* NBRC 3460. As further experiments showed, the arginine racemase is essential for the catabolism of D-lysine and for anabolism of D-methionine in a peptideglycan in *P. taetrolens* NBRC 3460. The osmotic stress strongly affected the survival rate of *P. taetrolens* NBRC 3460::*argR*, and it suggests that D-methionine is one of the essential components of the peptideglycan to protect the cells against osmotic stress. Accordingly, the arginine racemase is a bifunctional enzyme.

In the studies on structure, I found that the single intramolecular disulfide bond between the cysteines C47 and C73 exists in the primary structure of the arginine racemase from *Pseudomonas taetrolens* NBRC 3460, and this is the first example of a pyridoxal 5'-phosphate (PLP)-dependent amino acid racemase that contains a disulfide bond. Based on these studies, I can conclude that the disulfide bond of arginine racemase is essential for arginine racemase to fold and mature as an amino acid racemase with broad substrate specificity.

In the studies on application of the arginine racemase gene to D-amino acid

productions, I succeeded to produce 10 mM of D-lysine by using *C. glutamicum* ATCC 13032 *lysC* T311I harboring the arginine racemase gene of *P. taetrolens* NBRC 3460. In addition, I found that *Corynebacterium glutamicum* ATCC 13032::*argF* extracellularly produces a large amount of D-ornithine. These findings will bring new aspects in the research field of D-amino acid productions of microorganisms.

ACKNOWLEDGEMENTS

I wish to express my sincere thank to Dr. Tadao Oikawa, Professor of Department of Life Science and Biotechnology, Faculty of Chemistry, Materials and Bioengineering, Kansai University, for his clear suggestion, valuable discussion, and continuous warm encouragement throughout the course of this work.

I am also very grateful to Dr. Lothar Eggeling, Institute of Biotechnology 1, Forschungszentrum Juelich, Germany, for his valuable advice and warm encouragement.

My sincere thanks are due to Dr. Kenji Soda (Kansai University, Osaka, Japan), Dr. Noriaki Arakawa (Yokohama National University, Kanagawa, Japan), Dr. Roland Freudl (Forschungszentrum Juelich, Germany), Dr. Frank Lausberg (Forschungszentrum Juelich, Germany), Ms Norma Stäbler (Forschungszentrum Juelich, Germany), Dr. Andrea Tauch (Bielefeld University, Germany), Dr. Tino Polen (Forschungszentrum Juelich, Germany), Mr. Jan van Ooyen (Forschungszentrum Juelich, Germany), Dr. Takehiro Yokota (Mitsubishi chemical group, Science and Technology Research center, Inc., Tokyo, Japan) and Dr. Toru Ezure (Shimadzu Corp, Kyoto, Japan).

Special thanks are due to Dr. Tetsuaki Tsuchido, Dr. Yoshie Hasegawa, Dr.

Shinichi Uesato, Dr. Yasuo Nagaoka, and all other members of Department of Life Science and Biotechnology, Faculty of Chemistry, Materials and Bioengineering, Kansai University, for their kind help, suggestion and encouragement.

I also thank to the all members of Laboratory of Biomolecular Science and Technology, Kansai University, for their kindness, support, and encouragement.

Finally, but not the least, I would like to acknowledge the strong support and affectionate encouragement of my family throughout the course of this study.

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