Appl Biochem Biotechnol
DOI 10.1007/s12010-015-1647-6

In Silico Identification for α-Amino-ε-Caprolactam Racemases by Using Information on the Structure and Function Relationship

Wisarut Payoungkiattikun[1,2] · Seiji Okazaki[2,3] · Shogo Nakano[2,3] · Atsutoshi Ina[2,3] · Aran H-Kittikun[1] · Yasuhisa Asano[2,3]

Received: 16 February 2015 / Accepted: 21 April 2015

Abstract *In silico* identification for enzymes having desired functions is attractive because there is a possibility that numerous desirable enzymes have been deposited in databases. In this study, α-amino-ε-caprolactam (ACL) racemases were searched from the NCBI protein database. Four hundred thirteen fold-type I pyridoxal 5′-phosphate-dependent enzymes which are considered to contain sequences of ACL racemase were firstly obtained by submitting the sequence of ACL racemase from *Achromobacter obae* to the database. By identifying Lys241 as a key amino acid residue, 13 candidates for ACL racemase were selected. Then, putative ACL racemase genes were synthesized as codon-optimized sequences for expression in *Escherichia coli*. They were subcloned and expressed in *E. coli* BL21 and underwent His-tag purification. ACL and amino acid amide racemizing activities were detected among ten of the candidates. The locus tags Oant_4493, Smed_5339, and CSE45_2055 derived from *Ochrobactrum anthropi* ATCC49188, *Sinorhizobium medicae* WSM 419, and *Citreicella* sp. SE45, respectively, showed higher racemization activity against D- and L-ACLs rather than that of ACL racemase from *A. obae*. Our results demonstrate that the newly discovered ACL

Electronic supplementary material The online version of this article (doi:10.1007/s12010-015-1647-6) contains supplementary material, which is available to authorized users.

✉ Aran H-Kittikun
aran.h@psu.ac.th

✉ Yasuhisa Asano
asano@pu-toyama.ac.jp

[1] Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat-Yai 90112, Thailand

[2] Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan

[3] Asano Active Enzyme Molecule Project, ERATO, JST, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan

Published online: 06 May 2015

racemases were unique from ACL racemase from *A. obae* and might be useful for applications in dynamic kinetic resolution for D- or L-amino acid production.

Keywords *In silico* identification · α-Amino-ε-caprolactam · Racemase · Enzyme structure · Lys241 · PLP-dependent enzyme

Introduction

Enzymes play an important role in many bioprocesses and applications. They show high stereo- and regio-selectivities for their substrates [1]. They contribute to safer work conditions, because they are active under mild conditions and eliminate the use of hazardous chemicals. However, enzymes derived from microorganisms, plants, and animals have many limitations and do not satisfy industrial demand. An enrichment culture method has been an excellent procedure for discovering many new industrial enzymes [2, 3]. With the accumulation of genetic information, many enzymes have been engineered and also screened from new sources to fit specific industrial processes [4]. The rapidly increasing numbers of gene sequences are also attractive as targets for the search of new enzymes. These sequences contain genes which encode proteins with unknown functions, and this phenomenon can result in major problems, such as misleading interpretations of the function [5–7]. The identification of protein functions is therefore a necessary and challenging task. The first step for enzyme function identification is the use of enzyme commission (EC) numbering; however, this is not always reliable as some have a functional complex in metabolic pathways. The structural basis of protein has also been used as a prediction method of the function [8], but the accuracy of this approach for predicting a protein function is not yet completely satisfactory [9]. The analysis of structure/sequence-function relationships of transaminases, racemases, and decarboxylases was discussed by Steffen-Munsberg et al. [10]. They revealed that the amino acid patterns of a few active site residues (fingerprint) were correlated to enzyme function expression in different proteins.

α-Amino-ε-caprolactam (ACL) racemase (EC 5.1.1.15) was found in *Achromobacter obae*, *Achromobacter cycloclastes*, *Alcaligenes faecalis*, and *Flavobacterium arborescens* [11–13]. However, detailed properties of ACL racemase were studied only with ACL racemase from *A. obae* [14–19]. This enzyme was used in combination with L-ACL hydrolase from *Cryptococcus laurentii*, *Candida humicola*, or *Trichosporon cutaneum* for L-lysine production from D- and L-ACL on an industrial scale [20, 21]. Asano and Yamaguchi [22] discovered that the enzyme also catalyzed the racemization of other amino acid amides including L-2-aminobutyric acid amide, L-alanine amide, L-threonine amide, L-norvaline amide, L-norleucine amide, L-leucine amide, L-methionine amide, L-serine amide, and L-phenylalanine amide. ACL racemase can therefore be used in combination with D- or L-amino acid amidase for D- or L-amino acid production with 100 % yield [23, 24] (Fig. 1). The crystal structure of ACL racemase from *A. obae* revealed the active site residues, and these residues rationally explained the substrate binding mode [25] (Fig. 2). For the ACL racemase, two mechanisms have been proposed, a two-base mechanism (where two acid-base amino acid residues are situated on either side of the substrate-PLP complex) and a single-base mechanism (with a single base capable of accessing both faces). Ahmed et al. [26] found evidence for a single-base

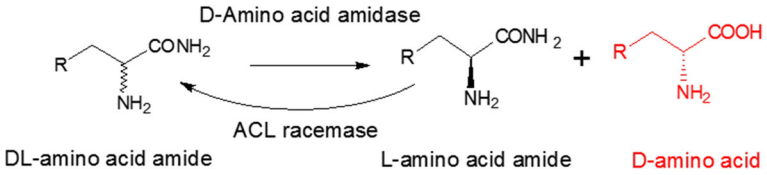

Fig. 1 Strategy for the synthesis of optically pure D-amino acid using D-amino acid amidase coupled with ACL racemase. By using ACL racemase in combination with a D-stereospecific amino acid amidase, such as D-aminopeptidase (DAP), D-amino acid amide was hydrolyzed by D-aminopeptidase. The remaining L-amino acid amide was racemized by ACL racemase. From this reaction, 100 % theoretical yield of D-amino acid was obtained

mechanism, which is at odds with the two base mechanism described by Okazaki et al. [25] which was proposed based on structural information [10]. The substrate binding site is assigned between Trp49 and Tyr137, and the carboxyl group of Asp210 is speculated to recognize the nitrogen atom of a lactam or amide of a substrate. Moreover, the N^{ζ} of Lys241 is speculated to form a hydrogen bond with the carbonyl O group of substrate. However, the physiological roles and the natural distribution of ACL racemase remain unknown. For a greater understanding of the applications of the enzyme, detailed studies of ACL racemases are required.

To identify further ACL racemases, the *in silico* identification method was attempted. The pyridoxal 5′-phosphate (PLP)-dependent racemases identified to date are classified into fold types I, II, or III [27]. ACL racemase from *A. obae* belongs to the fold-type I group of PLP-dependent enzymes. In this study, 413 PLP-dependent fold-type I enzymes were identified by submitting the sequence of ACL racemase from *A. obae* to the protein database. The newly developed computational program [28] was then used to identify key amino acid residues of ACL racemase. Using this program, Lys241 of ACL racemase from *A. obae* was predicted as a key amino acid residue. The predicted ACL racemases were selected, and their genes synthesized and cloned. The function of the expressed enzymes was also evaluated.

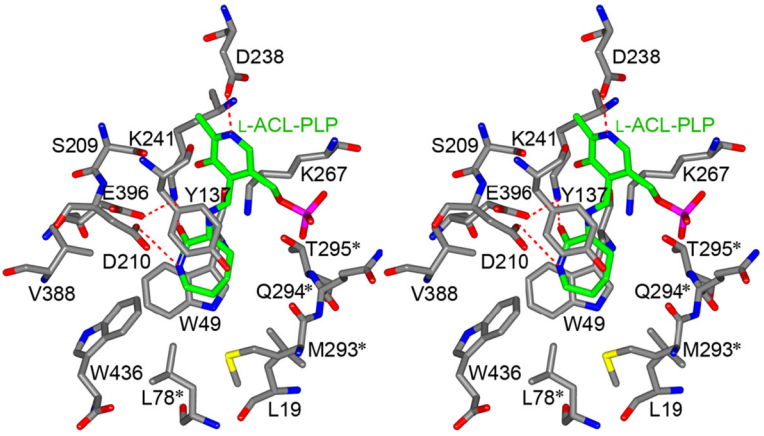

Fig. 2 Active site structure of ACL racemase. Chain B of PDB ID 3DXV, drawn with gray C atoms, red O atoms, and blue N atoms with the model of the external aldimine intermediate (L-ACL-PLP; green). Noteworthy, hydrogen bonds are shown as *red broken lines*. Residues contributed by neighboring subunit are labeled with an *asterisk* (color figure online)

Materials and Methods

Materials, Enzymes, and Chemicals

Restriction endonucleases were obtained from Takara Shuzo, Toyobo (Osaka, Japan), and New England Biolabs (Beverly, MA, USA). DNA Ligation Kit was purchased from Takara Shuzo. Crownpak CR (+) column was from Daicel Chemical Industries, Ltd. (Tokyo, Japan). Bio-Scale™ Mini Profinity™ IMAC purification system was purchased from Bio-Rad (CA, USA). ÄKTA purification system equipped with HisTrap HP column or Ni-sepharose fast flow column was purchased from GE Healthcare (NJ, USA). Bacto yeast extract and Bacto tryptone were obtained from Difco (Detroit, USA). All other chemicals were purchased from commercial sources and used without further purification.

Bacterial Strains, Plasmids, and Culture Conditions

Escherichia coli JM109 and *E. coli* BL21 (DE3) were purchased from Takara Shuzo and were used as a host for the recombinant plasmids. Synthetic predicted genes were ligated in pUCIDT plasmid by synthesizing from the Integrated DNA Technologies, Inc. (Illinois, USA). Synthetic ACL racemase gene from *A. obae* which ligated in pET15b (pET15ACL) plasmid was obtained from Okazaki et al. [25]. The pET-28a(+) plasmid was used as an expression vector and was purchased from Novagen (Darmstadt, Germany). Recombinant *E. coli* was cultivated in a test tube containing Luria-Bertani (LB) medium (1 % Bacto tryptone, 0.5 % Bacto yeast extract, and 1 % sodium chloride (NaCl), pH 7.0) supplemented with 30 μg/ml kanamycin (for pET-28a(+) vector) or 80 μg/ml ampicillin (for pET15ACL) for 12 h at 37 °C with 200 rpm shaking speed. A 1.5 % of seed medium was transferred to 500 ml fresh LB medium containing appropriate antibiotic and cultivated at 37 °C with 150 rpm shaking. After OD_{600} of the culture reached 0.8–1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM final concentration, and the culture was further cultivated at 30 °C for 5 h.

Substrates

A two-necked reaction flask equipped with magnetic stirrer is charged with D- or L-lysine (5 g), xylene (450 ml), hexamethyldisilazane (50 ml), and 2 ml cholotrimethylsilane. Then, a two-necked reaction flask was equipped with reflux condenser carrying a valve and gas inlet tube. The mixture is heated in oil bath under reflux under a gentle stream of dry nitrogen for 3 days, and then a two-necked reaction flask was cooled in ice and poured into absolute ethanol (1000 ml) and evaporated under vacuum to dryness. The residue is taken up with chloroform, the mixture is filtered through a celite pad on a sintered glass funnel, and the filtrate is evaporated under reduce pressure. The residue is dissolved in dioxin (70 ml), treated with 2.5 normal hydrogen chloride (13.7 ml) in isopropanol, and the precipitate of D- or L-α-amino-ε-caprolactam hydrochloride was collected [29].

In Silico Identification of ACL Racemases Using the Information from Key Amino Acid Residues

The sequence of ACL racemase from *A. obae* was used for the NCBI protein database search to obtain fold-type I PLP-dependent enzymes, considered to contain candidate sequences of ACL racemase. The newly developed computational program [28] was then used to identify the key

amino acid residues of ACL racemase. This program requires two files to run: the sequence of the target protein (STP; ACL racemase from *A. obae*) and a library consisting of sequences of proteins in the same family (fold-type I PLP-dependent enzymes). It then assigns consensus residues for the STP. The occurrences of the amino acid residues are calculated for each position in the STP after marker amino acid residues have been defined. A key amino acid residue for ACL racemase was assigned by focusing the high occurrences (%) of other active site residues of ACL racemase (Table S1). The coexistence of the residues Lys241 and Asp210 was considered to be required for functional expression of ACL racemase activity. When using Lys241 as a mark, sequences of putative ACL racemases were selected from previously mentioned fold-type I PLP-dependent enzymes.

Purification of Predicted ACL Racemases and ACL Racemase from *A. obae*

The preparation of *E. coli* lysate was performed based on the instruction manual of Bio-Scale™ Mini Profinity™ IMAC Cartridges with some modification. The cells were suspended in Profinia lysis buffer containing 250 mM sucrose, 20 μM PLP, and 5 mM 2-mercaptoethanol. They were then disrupted by sonication for 20 min (200 kHz, Insonator model 201 M; Kubota Co. Ltd., Tokyo, Japan). Cell debris was removed by centrifugation (4700×*g*, 15 min) at 4 °C. The ACL racemase from *A. obae* was purified based on method of Asano and Yamaguchi [22, 23]. The predicted ACL racemases were passed directly through the ÄKTA purification system, equipped with a HisTrap HP column or Ni-sepharose fast flow column. The proteins were then repurified using the Profinia protein purification system, equipped with 5 ml affinity cartridge (native IMAC column). The gradient elution was performed using an elution buffer containing 20 mM sodium phosphate, 1 M NaCl, and 500 mM imidazole (pH 7.4). The purified ACL racemase from *A. obae* and predicted ACL racemases were dialyzed against the purification buffer and kept at −20 °C before further study.

Assay of ACL Racemase Activity

ACL racemization was assayed by using the method of Asano and Yamaguchi [23]. The reaction mixture (100 μl) contained 100 mM potassium phosphate buffer (KPB), pH 7.0 (for ACL racemase from *A. obae*), pH 7.5 (for ACL racemase from *Citreicella* sp. SE45) or Tris-HCl buffer pH 8.0 (for ACL racemase from *Ochrobactrum anthropi* IA NCIMB 41129), 20 μM PLP solution, and 100 mM substrate (D- or L- ACL). The reaction was performed at 30 °C for 10 min and stopped by the addition of 20 μl 2 N $HClO_4$. The amount of formed product was determined with a high-performance liquid chromatography (HPLC) equipped with a Crownpak CR(+) column (150 mm× 4 mm×5 μm) at a flow rate of 0.3 ml/min, 30 °C using the solvent system of 60 mM $HClO_4$. Absorbance of the eluent was monitored at 200 nm. One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per min.

Protein Concentration Determination

The protein concentration was determined by the method of Bradford [30], using bovine serum albumin as the standard.

Protein Structure Representation

All the ACL racemase structure graphics was created by using *CCP*4*mg* program [31].

Results and Discussion

In Silico Identification of Predicted ACL Racemases Using Structural Information

Active site residues, considered to be required for functioning as ACL racemase, were identified by the crystal structure from *A. obae* (Fig. 2) [25]. The substrate binding site was typically located between Trp49 and Tyr137. Lys241 N^{ζ} was considered to be important for recognizing the carbonyl O of the substrate. Lys241 also formed a salt bridge with Glu396. Other fold-type (II or III) PLP-dependent racemases are considered to employ a two-base racemization mechanism [32] where two acid-base amino acid residues are situated on either side of the substrate-PLP complex [10]. Because the mutation of either Asp210 or Lys267 to alanine that abolished the racemization activity for ACL (unpublished data), Asp210 and Lys267 are two plausible acid/base catalytic candidate residues, situated on the *re* and *si* faces of the PLP ring, respectively. Therefore, the racemization of ACL racemase proceeds via a two-base mechanism [25]. The location of the side chain of Asp210 is suitable for approaching the substrate by interaction with the nitrogen of amides or lactams. This consideration is consistent with the observation that ACL racemase from *A. obae* does not exhibit activity toward D, L-amino acids but does exhibit activity toward substrates containing the nitrogen of amides or lactams such as D- and L-amino acid amides or D- and L-cyclic amino lactam [16, 17, 22]. Asp238 is also considered to play a role in stabilizing the electron sink properties of PLP by protonation of the nitrogen atom, consistent with the basic racemization mechanism that proceeded through a quinonoid intermediate [33]. Trp436 would be related to the substrate specificity because of the locational proximity to the active site.

Using ACL racemase protein sequence from *A. obae* as the query sequence, 413 fold-type I PLP-dependent enzymes were obtained from the protein database. These predicted enzymes were grouped into 14 particular residue categories of active site residues of ACL racemase from *A. obae*, and the occurrences (%) of 15 active site residues were examined in each category (Table S1). Only the category constructed by 14 enzymes which contain Lys and Glu corresponding to Lys241 and Glu396 of ACL racemase from *A. obae* simultaneously had both Asp and Lys corresponding to Asp210 and Lys241 of ACL racemase from *A. obae*. This may be required for functional expression of ACL racemase activity. This result implied that all 14 enzymes in this category had ACL racemase activity. Interestingly, this category showed the highest average occurrences (89.1 %) in all 14 categories (Table S1). This ACL racemase key amino acid residues information was very similar to the amino acid fingerprint residue suggested by Steffen-Munsberg et al. [10]. They suggested that Asp185 and Lys216 which corresponded to Asp210 and Lys241 of this result were believed to be essential for substrate recognition in the enzyme and indicated as the active site pattern of identifying the ACL racemase. They reported 18 protein sequences in ornithine transaminase-like database (OrnTL DB) as ACL racemases [10]. Multiple sequence alignments between these 14 enzymes are shown in Fig. 3. The protein sequence similarities (%) between ACL racemase from *A. obae* and these 13 enzymes are shown in Table S2. Oant_4493 had 84 % sequence similarity with the ACL racemase of *A. obae*, while the other enzymes had 46–54 %.

Confirmation of ACL Racemase Activities of Predicted Enzymes

In the next step, putative ACL racemase genes were synthesized as codon-optimized sequences for expression in *Escherichia coli*. They were subcloned and expressed in *E. coli*

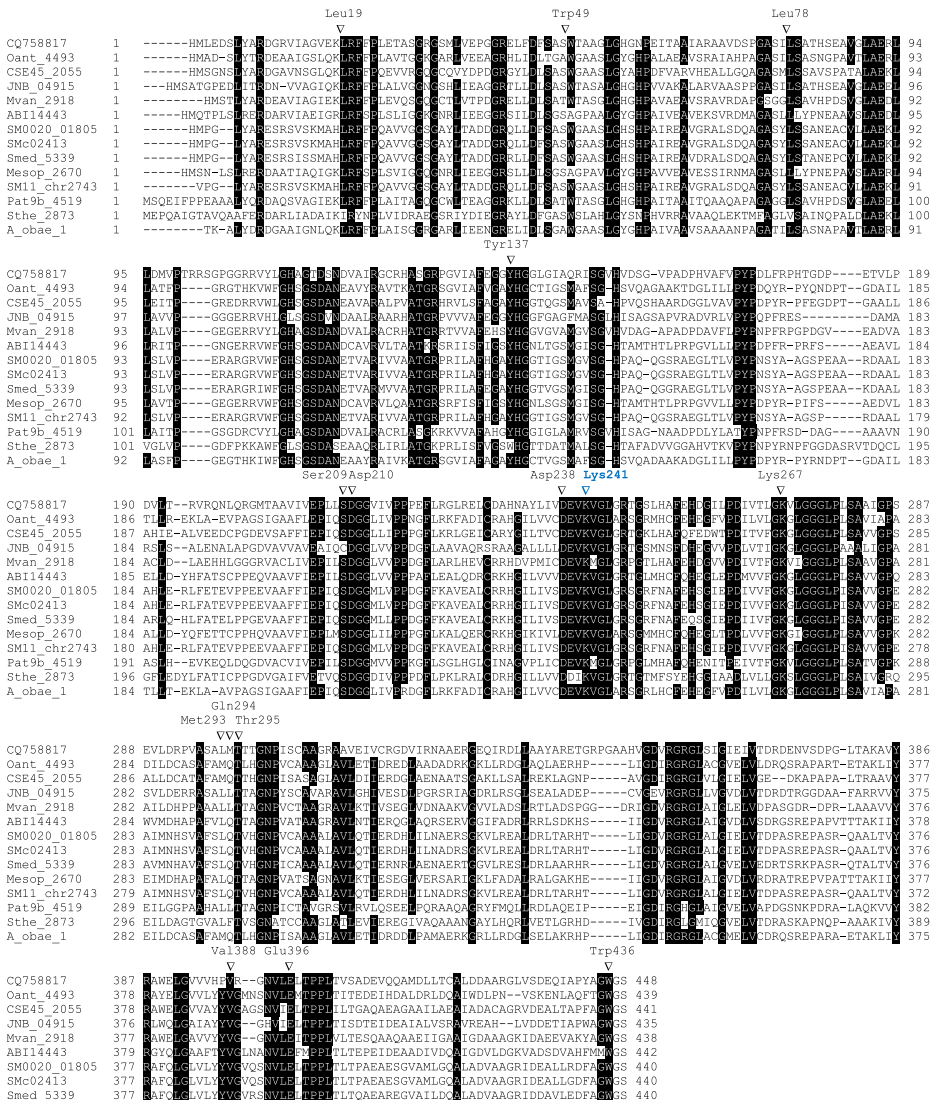

Fig. 3 Multiple alignment of amino acid sequences of ACL racemase from *A. obae* and predicted and observed ACL racemases. The source of predicted and observed ACL racemases were *A. nicotianae* NCIMB41126, *O. anthropi* ATCC49188, *Citreicella* sp. SE45, *Janibacter* sp. HTCC2649, *M. vanbaalenii* PYR-1, *O. anthropi* IA NCIMB41129, *S. meliloti* CCNWSX0020, *S. meliloti* 1021, *S. medicae* WSM419, *M. opportunism* WSM2075, *S. meliloti SM11*, *Pantoea* sp. At-9b, and *S. thermophilus* DSM20745. Key amino acid residue Lys241 is indicated in *blue triangle*, and other 14 active site residues are indicated in *black triangle* (color figure online)

BL21 and then subjected to His-tag purification. After purification of the predicted ACL racemases (Fig S1), the enzymes were investigated with respect to their activity toward D- and L-ACL and various L-amino acid amides. Ten out of the 13 predicted ACL racemases were

Table 1 Racemization activity of purified ACL racemases

Microorganism	Locus tag	Substrate									
		D-ACL U/mg	L-ACL U/mg	L-Alanine NH_2	L-Serine NH_2	L-Leucine NH_2	L-Valine NH_2	L-Methionine NH_2	L-Phenylalanine NH_2	L-Phenylglycine NH_2	L-Tyrosine NH_2
Achromobacter obae	A_obae_1	627	443	+	+	+	+	+	+	+	−
Arthrobacter nicotianae NCIMB41126	CQ758817	422	182	+	+	+	+	+	+	+	+
Citreicella sp. SE45	CSE45_2055	772	681	+	+	+	+	+	+	+	−
Janibacter sp. HTCC2649	JNB_04915	47	63	+	+	−	+	+	+	+	−
Mesorhizobium opportunism WSM2075	Mesop_2670	133	39	+	+	+	+	+	+	+	+
Mycobacterium vanbaalenii PYR-1	Mvan_2918	94	20	+	−	−	+	+	+	+	−
Ochrobactrum anthropi ATCC 49188	Oant_4493	1,225	444	+	+	−	+	+	+	+	+
O. anthropi IA NCIMB41129	ABI14443	63	11	+	+	−	+	+	+	+	−
Sinorhizobium medicae WSM 419	Smed_5339	1,072	520	+	+	−	+	+	+	+	+
S. meliloti 1021	SMc02413	105	68	+	+	−	+	+	+	+	−
S. meliloti CCNWSX0020	SM0020_01805	260	107	+	+	+	+	+	+	+	+

− no racemizing activity detected, + racemizing activity detected

shown to have ACL racemase and amino acid amide racemase activities (Table 1). Although ACL and amino acid amide racemase activities of two enzymes from *O. anthropi* IA, NCIMB41129, and *A. nicotianae*, NCIMB41126, were already reported by Boesten et al. [34], these enzymes were not purified to homogeneity. On the other hand, ACL racemase from *A. obae* was purified to homogeneity by Asano and Yamaguchi [22]. In addition, the genomic DNA of *S. meliloti* SM11, *Pantoea* sp. At-9b, and *S. thermophilus* DSM20745 were obtained and cloned into pET-28a(+) plasmid. However, the native DNA sequences from the genomic DNA of these three bacteria could not be expressed in *E. coli.* The specific activity and substrate range were different among the ten predicted ACL racemases and ACL racemase from *A. obae* (Table 1). The locus tags Oant_4493, Smed_5339, and CSE45_2055 derived from *O. anthropi* ATCC49188, *S. medicae* WSM 419, and *Citreicella* sp. SE45, respectively, showed better racemization activity against D- and L-ACL than ACL racemase from *A. obae*. ACL racemase from *O. anthropi* ATCC49188 (protein locus tag: Oant_4493) showed the highest racemizing activity against D-ACL (1225 U/mg). ACL racemase from *Citreicella* sp. SE45 (protein locus tag: CSE45_2055) showed the highest racemizing activity against L-ACL (681 U/mg). These activities were higher than mutant ACL racemase (L19V/L78T) applied for enzymatic synthesis of chiral phenylalanine derivatives by dynamic kinetic resolution [35]. The racemizing activities of novel discovered ACL racemases might be unique from the ACL racemase of *A. obae* because of the variety of active site residues (Table 2). They might also be useful for expanding the method of chiral amino acid synthesis by dynamic kinetic resolution of amino acid amides.

Table S5 shows the up-to-date ACL racemase distribution in bacterial strains. In this study, seven strains of the order rhizobales and three strains of order burkholderiales are fallen into a group of rhizobia which was the largest group of ACL racemase producing microorganisms. Rhizobia are well known as plant symbionts which can fix nitrogen after becoming established inside root nodules of legumes. Two actinomycetales, *A. nicotinae* and *Janibacter* sp., also

Table 2 Putative active site residues of the predicted and observed ACL racemases

Locus tag	Residue number of active site residues of ACL racemase from *A. obae*														
	19	49	78	137	209	210	238	241	267	293	294	295	388	396	436
A_obae_1	L	W	L	Y	S	D	D	**K**	K	M	Q	T	V	**E**	W
CQ758817	L	W	L	Y	S	D	D	**K**	K	L	M	T	V	**E**	W
CSE45_2055	L	W	L	Y	S	D	D	**K**	K	M	Q	T	V	**E**	W
JNB_04915	L	W	L	Y	C	D	D	**K**	K	L	L	T	V	**E**	W
Mesop_2670	L	A	L	Y	S	D	D	**K**	K	L	Q	T	V	**E**	W
Mvan_2918	L	W	L	Y	S	D	D	**K**	K	L	L	T	V	**E**	W
Oant_4493	L	W	L	Y	S	D	D	**K**	K	M	Q	T	V	**E**	W
ABI14443	L	A	L	Y	S	D	D	**K**	K	L	Q	T	V	**E**	W
Pat9b_4519	L	W	L	Y	S	D	D	**K**	K	L	L	T	V	**E**	W
Smed_5339	L	W	L	Y	S	D	D	**K**	K	L	Q	T	V	**E**	W
SMc02413	L	W	L	Y	S	D	D	**K**	K	L	Q	T	V	**E**	W
SM0020_01805	L	W	L	Y	S	D	D	**K**	K	L	Q	T	V	**E**	W
SM11_chr2743	L	W	L	Y	S	D	D	**K**	K	L	Q	T	V	**E**	W

Key residues which determine the ACL racemase activity are indicated by *bold letters*

expressed ACL racemase activity. There is evidence that actinomycetes affected the growth of root symbiotic and plant parasitic soil fungi [36]. However, there is little concrete information on the physiological function of ACL racemase in microorganisms. The observations on the distribution of ACL racemase in microorganisms by this study revealed the possibility of a relationship with a plant root infection pathway.

Conclusions

Nowadays, enormous progress in sequencing technology in combination with metagenome libraries has led to an exponential increase in the number of sequence data. However, it is difficult to select the proteins which have desirable function from a large database. By identifying Lys241 as a key amino acid residue by the aid of developed computational program, newly 13 predicted ACL racemases were identified from 413 fold-type I PLP-dependent enzymes. They showed highest average occurrence (%) of amino acid residues on the corresponding positions of the active site of ACL racemase of *A. obae*. These residues were considered to be required and implicated for functioning as ACL racemase. Afterward, ACL racemase activity was confirmed in ten of these predicted ACL racemases. Moreover, some of them showed higher racemizing activity against ACL than the ACL racemase from *A. obae*. The discovery of novel ACL racemases will be useful for chiral amino acid synthesis by dynamic kinetic resolution of amino acid amides. The information of ACL racemases obtained by this study may also be useful for approaching unknown physiological function of ACL racemase.

Acknowledgments This work was financed by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/099/2551) and Japan Student Services Organization (JASSO). The authors greatly appreciated Asst. Prof. Ken-ichi Fuhshuku for the preparation of the chiral ACLs and enantiomerically pure amino acid amides.

Conflict of Interest None declared.

Ethical Approval Not required.

References

1. Gröger, H., & Asano, Y. (2012). Introduction-principles and historical landmarks of enzyme catalysis in organic synthesis. In K. Drauz, H. Gröger, & O. May (Eds.), *Enzyme catalysis in organic synthesis* (Vol. 2, pp. 3–42). Weinheim: Wiley-VCH Verlag & Co. KGaA.
2. Asano, Y. (2002). Overview of screening for new microbial catalysts and their uses in organic synthesis-selection and optimization of biocatalysts. *Journal of Biotechnology, 94*(1), 65–72.
3. Asano, Y. (2010). Tools for Enzyme Discovery -Industrial enzymes, biocatalysis and enzyme evolution. In H. B. Richard, E. D. Julian, & D. Arnold (Eds.), *Manual of Industrial Microbiology and Biotechnology* (pp. 441–452). USA: ASM press.
4. Bornscheuer, U. T., Huisman, G. W., Kazlauskas, R. J., Lutz, S., Moore, J. C., & Robins, K. (2012). Engineering the third wave of biocatalysis. *Nature, 485*, 185–194.
5. Gerlt, J. A., & Babbitt, P. C. (2000). Can sequence determine function? *Genome Biology, 1*(5), 0005.1–005.10.
6. Lee, D., Redfern, O., & Oregon, C. (2007). Prediction protein function from sequence and structure. *Nature Reviews Molecular Cell Biology, 8*, 995–1005.
7. Davids, T., Schmidt, M., Böttcher, D., & Bornscheuer, U. T. (2013). Strategies for the discovery and engineering of enzymes for biocatalysis. *Current Opinion in Chemical Biology, 17*, 215–220.

8. Höhne, M., Schätzle, S., Jochens, H., Robins, K., & Bornscheuer, U. T. (2010). Rational assignment of key motifs for function guides *in silico* enzyme identification. *Nature Chemical Biology, 6*, 807–813.
9. Loewenstein, Y., Raimondo, D., Redfern, O. C., Watson, J., Frishman, D., Linial, M., Orengo, C., Thornton, J., & Tramontano, A. (2009). Protein function annotation by homology-based inference. *Genome Biology, 10*(2), 207.1–207.8.
10. Steffen-Munsberg, F., Vickers, C., Kohls, H., Land, H., Mallin, H., Nobili, A., Skalden, L., Bergh, T. V. D., Joosten, H. J., Berglund, P., Höhne, M., & Bornscheuer, U. T. (2015). Bioinformatic analysis of a PLP-dependent enzyme superfamily suitable for biocatalytic applications. *Biotechnology Advances*. doi:10.1016/j.biotechadv.2014.12.012.
11. Fukumura, T. (1977). Bacterial racemization of α-amino-ε-caprolactam. *Agricultural and Biology Chemistry, 41*(8), 1321–1325.
12. Fukumura, T. (1977). Conversion of D- and DL-α-amino-ε-caprolactam into L-lysine using both yeast cells and bacterial cells. *Agricultural and Biological Chemistry, 41*(8), 1327–1330.
13. Fukumura, T. (1977). Partial purification and some properties of α-amino-ε-caprolactam-racemizing enzyme from *Achromobacter obae*. *Agricultural and Biological Chemistry, 41*(8), 1509–1510.
14. Ahmed, S. A., Esaki, N., Tanaka, H., & Soda, K. (1982). Production and stabilization of α-amino-ε-caprolactam racemase from *Achromobacter obae*. *Bulletin of the Institute for Chemical Research, 60*(5–6), 342–346.
15. Ahmed, S. A., Esaki, N., & Soda, K. (1982). Purification and properties of α-amino-ε-caprolactam racemase from *Achromobacter obae*. *FEBS Letters, 150*(2), 370–374.
16. Ahmed, S. A., Esaki, N., Tanaka, H., & Soda, K. (1983). Racemization of α-amino-δ-valerolactam catalyzed by α-amino-ε-caprolactam racemase from *Achromobacter obae*. *Agricultural and Biological Chemistry, 47*(5), 1149–1150.
17. Ahmed, S. A., Esaki, N., Tanaka, H., & Soda, K. (1983). Properties of α-amino-ε-caprolactam racemase from *Achromobacter obae*. *Agricultural and Biological Chemistry, 47*(8), 1887–1893.
18. Ahmed, S. A., Esaki, N., Tanaka, H., & Soda, K. (1984). L-α-Amino-β-thio-ε-caprolactam, a new sulfur-containing substrate for α-amino-ε-caprolactam racemase. *FEBS Letters, 174*(1), 76–79.
19. Ahmed, S. A., Esaki, N., Tanaka, H., & Soda, K. (1985). Mechanism of inactivation of α-amino-ε-caprolactam racemase by α-amino-δ-valerolactam. *Agricultural and Biological Chemistry, 49*(10), 2991–2997.
20. Fukumura, T. (1976). Screening, classification and distribution of L-α-amino-ε-caprolactam-hydrolysing yeasts. *Agricultural and Biological Chemistry, 40*(9), 1687–1693.
21. Fukumura, T. (1976). Hydrolysis of L-α-amino-ε-caprolactam by yeasts. *Agricultural and Biological Chemistry, 40*(9), 1695–1698.
22. Asano, Y., & Yamaguchi, S. (2005). Discovery of amino acid amides as new substrates for α-amino-ε-caprolactam racemase from *Achromobacter obae*. *Journal of Molecular Catalysis B: Enzymatic, 36*, 22–29.
23. Asano, Y., & Yamaguchi, S. (2005). Dynamic kinetic resolution of amino acid amide catalyzed by D-aminopeptidase and α-amino-ε-caprolactam racemase. *Journal of the American Chemical Society, 127*, 7696–7697.
24. Yamaguchi, S., Komeda, H., & Asano, Y. (2007). New enzymatic method of chiral amino acid synthesis by dynamic kinetic resolution of amino acid amides: use of stereoselective amino acid amidases in the presence of α-amino-ε-caprolactam racemase. *Applied and Environmental Microbiology, 73*(16), 5370–5373.
25. Okazaki, S., Suzuki, A., Mizushima, T., Kawano, T., Komeda, H., Asano, Y., & Yamane, T. (2009). The novel structure of a pyridoxal 5'-phosphate-dependent fold-type I racemase, α-amino-ε-caprolactam racemase from *Achromobacter obae*. *Biochemistry, 48*(5), 941–950.
26. Ahmed, S. A., Esaki, N., Tanaka, H., & Soda, K. (1986). Mechanism of α-amino-ε-caprolactam racemase reaction. *Biochemistry, 25*(2), 385–388.
27. Yoshimura, T., & Goto, M. (2008). D-amino acids in the brain: structure and function of pyridoxal phosphate-dependent amino acid racemases. *FEBS Journal, 275*(14), 3527–3537.
28. Nakano, S., & Asano, Y. (2015). Protein evolution analysis of S-hydroxynitrile lyase by complete sequence design utilizing the INTMSAlign software. *Scientific Reports, 5*, 1–10.
29. Pellegata, R., Pinza, M., & Pifferi, G. (1978). An improved synthesis of γ-, δ-, and ε-lactams. *Synthesis, 8*, 614–616.
30. Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.
31. McNicholas, S., Potterton, E., Wilson, K. S., & Noble, E. M. (2011). Presenting your structures: the *CCP4mg* molecular-graphics software. *Acta Crystallographica Section D: Biological Crystallography, 67*, 386–394.

32. Ito, T., Maekawa, M., Hayashi, S., Goto, M., Hemmi, H., & Yoshimura, T. (2013). Catalytic mechanism of serine racemase from *Dictyostelium discoideum. Amino Acids, 44*, 1073–1084.
33. Eliot, A. C., & Kirsch, J. F. (2004). Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. *Annual Review of Biochemistry, 73*, 383–415.
34. Boesten, W.H.J., Raemakers-Franken, P.C., Sonke, T., Euverink, G.J.W. & Grijpstra, P. (2003). Polypeptides having α-H-α-amino acid amide racemase activity and nucleic acids encoding the same. *WO International Patent Application, WO2003106691*.
35. Yasukawa, K., & Asano, Y. (2012). Enzymatic synthesis of chiral phenylalanine derivatives by a dynamic kinetic resolution of corresponding amide and nitrile substrates with a multi-enzyme system. *Advanced Synthesis & Catalysis, 354*(17), 3327–3332.
36. Maier, A., Riedlinger, J., Fiedler, H.-P., & Hampp, R. (2004). Actinomycetales bacteria from a spruce stand: characterization and effects on growth of root symbiotic and plant parasitic soil fungi in dual culture. *Mycological Progress, 3*(2), 129–136.