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# Biochemical characterization and mutational analysis of alanine racemase from *Clostridium perfringens*

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***Clostridium perfringens*** **is a gram-positive, anaerobic, pathogenic bacterium that can cause a wide range of diseases in humans, poultry and agriculturally important livestock. A pyridoxal-5-phosphate-dependent alanine racemase with a function in the racemization of D- and L-alanine is an attractive drug target for *C. perfringens* and other pathogens due to its absence in animals and humans. In this study alanine racemase from *C. perfringens* (CPAlr) was successfully expressed and purified in *Escherichia coli* and biochemically characterized. The purified CPAlr protein was a dimeric PLP-dependent enzyme with high substrate specificity. The optimal racemization temperature and pH were 40°C and 8.0, respectively. The kinetic parameters $K_m$ and $k_{cat}$ of CPAlr, determined by HPLC at 40°C were 19.1 mM and 17.2 $s^{-1}$ for L-alanine, and 10.5 mM and 8.7 $s^{-1}$ for D-alanine, respectively. Gel filtration chromatographic analysis showed that the molecular weight of mutant Y359A was close to monomeric form, suggesting that the inner layer residue Tyr359 might play an essential role in dimer-formation. Furthermore, the mutation at residues Asp171 and Tyr359 resulted in a dramatic increase in $K_m$ value and/or decreased in $k_{cat}$ value, indicating that the middle and inner layer residues Asp171 and Tyr359 of CPAlr might have the key role in substrate binding, catalytic activity or oligomerization state through the hydrogen–bonding interaction with the pentagonal ring waters and/or PLP cofactor.**



[**Key words:** *Clostridium perfringens*; Entryway corridor; Alanine racemase; Site-directed mutagenesis; Dimer interface]

*Clostridium perfringens* is a gram-positive, anaerobic, spore-forming and rod-shaped bacterium, which is usually inhabitant in the soil, manure and the gastrointestinal tract of humans and animals. This pathogen can cause a wide range of diseases such as food poisoning and gas gangrene in humans, necrotic enteritis in poultry, hemorrhagic enteritis, enterotoxaemia, and sudden death in agriculturally important livestock (1). In recent years, the antibiotic-resistant strains have become progressively more common, which can be challenging to treat and are often associated with increased mortality rates (2). Therefore, to implement alternative ways to target antimicrobial drugs against *C. perfringens* and other pathogens are highly desirable.

One of such target is the bacterial alanine racemase (Alr; EC 5.1.1.1), which is a member of the superfamily of fold type III enzymes and use PLP as a cofactor for its catalytic activity (3). The role of this enzyme is to catalyze the racemization of L-alanine to D-alanine for bacterial cell wall peptidoglycan layer biosynthesis (3). In most of the bacteria, alanine racemases are encoded by two independent genes named as *alr* and *dadX* (4,5). The *dadX* act as the catabolic gene to directly convert abundant of L- to D-alanine, which subsequently metabolized to pyruvate, and can be used by the cells as an alternative carbon and energy source for its survival (6,7). The expression of *dadX* is mostly induced with a high concentration of L- or D-alanine supplemented to the medium (8). However, the *alr* is expressing at a low level and serves as an anabolic gene, to provide enough D-alanine for the peptidoglycan layer of the bacterial cell wall (9). Knockout of this anabolic gene from *Mycobacterium smegmatis* strains revealed that an external supplement of D-alanine is necessary for its growth, which indicates the indispensable role of alanine racemase in the production of D-alanine (10). In some bacteria, there are two alanine racemase genes (*alr* and *dadX*) such as *Escherichia coli*, *Salmonella* and *Pseudomonas aeruginosa* (5,8) while others have only one gene such as *Bacillus pseudofirmus* OF4 (7).

Alr has long been considered as an imperative antimicrobial drug target due to its absence in most of the higher eukaryotes (11–13). Although several of alanine racemase inhibitors have been synthesized and most of them are structural analogs of D-alanine (14,15). However, the adverse side effects of these analogs limited their use in human chemotherapy, because they are not only active against alanine racemases but can also interact with other PLP-dependent enzymes, several of which have been expressed in humans (15). Recently, several non-substrate inhibitors have been identified for alanine racemase from *Aeromonas hydrophila* in which two inhibitors homogenistic acid and hydroquinone showed minimal cytotoxicity against HeLa cells; however, IC50 values of both compounds were about 2–3 folds higher than that of DCS (16). So, it is better to push forward an efficient inhibitor screening or designing strategy for alanine racemase.

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A recent progress in the structural studies of Alrs from different bacteria, such as *Bacillus anthracis* (Ames) (17), *Streptococcus pneumoniae* (18), *Staphylococcus aureus* (19) and *P. aeruginosa* PAO1 (20), revealed that Alr is a homodimeric enzyme in its native conformation and is composed of two symmetric monomers with a head-to-tail association. Based on the catalytic mechanisms and crystal structure analysis of alanine racemase, the entryway corridor to the active site is composed of three layers divided as inner, middle and outer layers (17–20). This corridor of alanine racemase from *B. anthracis* (Ames) contains 4 residues (Tyr270′, Tyr359, Tyr289′ and Ala172; primed residues found in the second monomer) in the inner layer and 4 residues (Arg314′, Ile357, Arg295′and Asp173) in the middle layer, which are highly conserved in many other bacteria (17). These conserved residues are charged near the entrance and generally hydrophobic near the active site (18). According to this specific structural feature, Priyadarshi propounded a practical approach for alanine racemase inhibitors designing, which using an inhibitor to bind to the substrate entryway and block the substrate accessibility to the active site (15). Therefore, a detailed biochemical characterization of the residues in the substrate entryway corridor of alanine racemase is crucial for drug development with greater specificity.

Interestingly, the residues positioned at the entryway corridor of putative alanine racemase CPAlr from *C. perfringens* are somewhat different. The middle and inner layers of CPAlr are comprised of seven highly conserved residues and one non-conserved residue (Ser170). Functional analysis of CPAlr and its variants may provide some essential information about the role of the residues in the substrate entryway. Herein, a complementation analysis, cloning, expression, biochemical characterization and mutational analysis of putative alanine racemase gene were conducted from *C. perfringens*. In particular, the role of three residues (Asp171, Ser170, and Tyr359) in the middle and inner layer of entryway corridor were also analyzed.

## MATERIALS AND METHODS

**Bacterial strains, plasmids and media** *C. perfringens*, *E. coli* DH12S and BL21(DE3) (Novagen, Madison, WI, USA) were utilized for DNA manipulation and recombinant protein expression, respectively. The *alr*/*dadX* double-knockout *E. coli* MB2795 was used as a D-alanine auxotroph for complementation analysis (21). Ampicillin (100 μg $mL^{-1}$) or D-alanine (0.5 mM) was added to LB medium when required. Plasmid miniprep kit and bacterial genomic DNA extraction kit were obtained from Omega Bio-Tek (Doraville, GA, USA). Vector pMD18-T (Takara, Kyoto, Japan), and pET-22b (+) (Novagen) were severally used for TA cloning or expression. ExTaq DNA polymerase, T4 DNA ligase, and restriction enzymes were purchased from Takara. All materials for protein purification were purchased from GE Healthcare (Uppsala, Sweden). L/D-Amino acids were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used in this study were that of analytical grade.

**Gene cloning and construction of expression plasmids** Chromosomal DNA was extracted from *C. perfringens* with the genomic DNA extraction kit and used as a template for PCR amplification. The primer set (5′-gcatatggatatatgtgttagac-3′, and 5′-gctcgagaatatagtttattacct-3′) was designed according to the nucleotide sequence of the alanine racemase gene of *C. perfringens* ATCC 13124 (GenBank ID: ABG82585.1). The underlined sequences represent the restriction sites of *Nde*I and *Xho*I, respectively. PCR was performed by ExTaq DNA polymerase and carried out at 94°C for 5 min, followed by 25 cycles of 94°C for the 30s, 48°C for 40s, and 72°C for 1 min. After a final incubation step for 7 min at 72°C, the reaction was stopped. PCR-amplified products were cloned into TA cloning vector pMD18-T to obtain a constructed plasmid pMD-CPAlr. The plasmid was confirmed by DNA sequencing and digested with restriction enzyme *Nde*I and *Xho*I. The gene fragment encoding full-length CPAlr was then ligated into a pET-22b vector (Novagen) to obtain plasmid pET-CPAlr.

The plasmid pET-CPAlr was transformed into D-alanine auxotroph strain *E. coli* MB2795 to check the gene's functions mentioned before (7) and then introduced into *E. coli* BL21(DE3) by heat shock for protein expression.

**Site-directed mutagenesis** Based on sequence alignment results, three residues Tyr359, Ser170 and Asp171 in the inner and middle layer of entryway corridor were subjected to site-directed mutagenesis and constructed a set of five single-point mutants S170A, D171S, D171A, Y359H, Y359A, and three double point mutants D171A/Y359A, S170A/Y359H, S170A/Y359A, using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the instruction manual. Plasmid pET-CPAlr was used as a template for PCR amplification.

The oligodeoxynucleotides (ODNs) used in this study are listed in Table 1. The PCR products were digested with *Dpn*I and transferred into *E. coli* DH12S competent cells. The transformants were verified by DNA sequencing. The recombinant pET-CPAlr and eight mutant plasmids were then propagated into *E. coli* BL21(DE3) for protein expression and purification.

**Protein expression and purification** *E. coli* BL21(DE3) carrying plasmid pET-CPAlr or 8 mutants were cultured in 200 mL LB medium with 100 μg $mL^{-1}$ ampicillin at 37°C until the $OD_{600}$ reached 0.6–0.7. For protein expression induction, the medium was supplemented with 0.5 mM IPTG and the cells growth was continued at 28°C overnight. Further, the cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C. The protein was purified by Ni-NTA Superflow Cartridge (Qiagen, Valencia, CA, USA) according to the manufacturer's handbook (7). The elution was combined and performed on FPLC by gel filtration chromatography as described previously (9,22). The protein fraction was combined and dialyzed against the phosphate buffer (20 mM, pH 8.0) containing 0.5 mM EDTA, 0.01% (v/v) 2-mercaptoethanol and 20 μM PLP using an Amicon Ultra-15 Centrifugal Filter Units (MWCO 10 kDa, Millipore, Billerica, MA, USA).

The molecular masses of CPAlr and 8 mutants were determined by gel filtration chromatography using a Superdex 200 10/300 GL column (1.0 × 30 cm; Amersham Biosciences, Piscataway, NJ, USA) following the method as described previously (9,22). Cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa) were used as molecular mass standard (Sigma). The purity of the enzyme was analyzed by SDS-PAGE. Protein concentration was evaluated using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

**Racemization assay** The enzymatic activity of CPAlr and its mutants were conducted following the published protocol (9) with some modifications. The racemization mixture was composed of Britton–Robinson buffer (20 mM, pH 8.0), PLP (10 μM), L-alanine (50 mM) and cell lysate or purified protein in a final volume of 200 μL. Buffer instead of enzyme was used as a blank control. The enzymatic reaction was carried out at 40°C for 10 min. D-Amino acid oxidase was used to determine the D-forms of amino acids (9,23). The spectral analysis at 550 nm was recorded using Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). One unit (U) of the enzyme was defined as the amount of enzyme to catalyze the formation of 1 μmol of D- or L-alanine from either enantiomer per minute.

**Biochemical characterization of CPAlr** The optimal pH of the recombinant protein CPAlr was determined at 40°C in $Na_2HPO_4$–$KH_2PO_4$ buffer (20 mM, pH 4.92–9.18), Barbitone-HCl buffer (20 mM, pH 6.8–9.6), $NaHCO_3$–$Na_2CO_3$ buffer (50 mM, pH 9.16–10.8), Britton–Robinson buffer (20 mM, pH 1.81–11.0), $NaHCO_3$–NaOH buffer (12.5 mM, pH 9.6–11.0), $Na_2HPO_4$–NaOH buffer (12.5 mM, pH 11.0–12.0), and KCl–NaOH buffer (25 mM, pH 12.0–13.0). The optimum temperature was analyzed by using 20 mM Britton–Robinson buffer (pH 8.0) from 10°C to 60°C. The pH-dependent stability was detected by measuring the residual activity after incubation of the protein CPAlr in 20 mM Britton–Robinson buffer (pH 7.0, 8.0, or 9.0) at 40°C for 0–60 min. The thermostability of CPAlr was evaluated by assaying the residual activity after pre-incubation of the enzyme in Britton–Robinson buffer (20 mM, pH 8.0) at 30°C, 40°C, and 50°C, for 0–60 min.

The effects of various chemical reagents and metal ions on the enzyme activity were assessed by adding 0.1 mM, 1 mM, 5 mM and 10 mM of various additives ($Zn^{2+}$, $Cu^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Co^{2+}$, $Pb^{2+}$, DTT and EDTA) in the reaction mixture and incubated at optimal conditions for 10 min.

Substrate specificity of CPAlr was quantitated by assaying the enzyme activity on various L-amino acids (50 mM) in Britton–Robinson buffer (20 mM, pH 8.0) at 40°C for 10 min.

**TABLE 1.** Primers used in this study.

| Primer | Sequence (5′-3′) | Description |
|---|---|---|
| CPAlr-F01 | GCATATGGATATATGTGTTAGAC | $alr_{CP}$ gene cloning |
| CPAlr -R01 | GCTCGAGAATATAGTTTATTACCT | |
| S170A-F01 | TCTCAACA**GCA**GATGAAAAAGA | Ser-170 → Ala-170 |
| S170A-R01 | TCTTTTTCATC**TGC**TGTTGAGA | |
| D171S-F01 | CTCAACATCA**TCT**GAAAAAGAT | Asp-171 → Ser-171 |
| D171S-R01 | ATCTTTTTC**AGA**TGATGTTGAG | |
| D171A-F01 | CTCAACATCA**GCT**GAAAAAGA | Asp-171 → Ala-171 |
| D171A-R01 | TCTTTTTC**AGC**TGATGTTGAG | |
| Y359H-F01 | ACAATAAAC**CAC**GAAATACT | Tyr-359 → His-359 |
| Y359H-R01 | AGTATTTC**GTG**GTTTATTGT | |
| Y359A-F01 | ACAATAAAC**GCC**GAAATACT | Tyr-359 → Ala-359 |
| Y359A-R01 | AGTATTTC**GGC**GTTTATTGT | |

The underlined sequences are *Nde*I and *Xho*I restriction sites. Bold letters are the mutated nucleotides.

Kinetic parameters of CPAlr and mutant proteins were quantitatively estimated for the amounts of D- or L-alanine using fluorescence and HPLC methods (9,24). The reaction mixture (40 μL) was composed of 20 mM Britton–Robinson buffer (pH 8.0), 10 μM PLP, and D- or L-alanine in different concentrations. The enzymatic reaction was performed at 40°C for 10 min, and the alanine derivatives were separated using a Nova-Pack C18 column (4 μm, 3.9 × 300 mm; Waters, Milford, MA, USA) at 40°C and detected by a spectrofluorometer (RF10A, Shimadzu, Kyoto, Japan). The kinetic parameters, $K_m$ and $V_{max}$ were calculated by nonlinear regression using Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

**Sequence and modeling analysis** An ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA) was used to confirm the nucleotide sequences of the constructed plasmids of wild-type and mutants. Multiple sequence alignment was performed using the ClustalX program (25).

## RESULTS

**Gene cloning, homology analysis and protein purification** A DNA fragment of 1158 bp was successfully amplified by PCR and inserted into vector pMD18-T to construct the plasmid pMD-CPAlr. The nucleotide and the amino acid sequence of gene $alr_{CP}$ severally share 99.91% similarity and 100% identity (one synonymous mutation at Val160: GTG → GTA) with the putative alanine racemase gene from *C. perfringens* ATCC13124 (GenBank: ABG82585.1). The plasmid pMD-CPAlr was double digested by restriction enzymes and put into the expression vector pET-22b (+) to yield the plasmid pET-CPAlr. Complementation analysis experiment showed that the D-alanine auxotroph strain MB2795 harboring the plasmid pET-CPAlr grew well on the LB media without D-alanine, indicating that the cloned gene $alr_{CP}$ was a functional alanine racemase (26).

The protein encoded by the gene $alr_{CP}$ consisted of 386 amino acid residues with a calculated molecular weight of 42,902 Da (without a $His_6$-tag at N- or C-terminus). Amino acid sequence alignment results indicated that CPAlr is highly conserved among different bacterial alanine racemases and displayed the highest sequence homology to the deposited alanine racemases in Protein Data Bank (PDB) from *Clostridium difficile* 630 (identity 47%; PDB ID: 4LUS) (27), *Thermoanaerobacter tengcongenesis* MB4 (identity 43%; PDB ID: 4Y2W) (28), *S. pneumoniae* (identity 36%; PDB ID: 3S46) (18), and *E. coli* (identity 29%; PDB ID: 2RJG) (29), and contain the characteristic motifs among bacterial alanine racemases: pyridoxal phosphate binding site (VVKANAYGHG), two catalytic amino acid residues (Lys38 and Tyr267′), and the entrance corridor (inner, middle and outer layers) making up of a set of strictly conserved residues to the active site (17,30). The entryway to the active site of CPAlr consisted of 7 highly conserved amino acid residues in the inner (Tyr267′, Tyr286′ and Tyr359) and middle layers (Asp171, Arg292′, Arg312′ and Ile357) as the most member of the alanine racemase family, however, there is one exception in the inner layer (Ser170) (Fig. 1).

Plasmid pET-CPAlr and 8 mutants were introduced into *E. coli* BL21(DE3) for protein expression and purification. The apparent molecular weight of purified recombinant proteins with a $His_6$-tag at C-terminus was about 44 kDa evaluated by SDS-PAGE and coincided with the theoretically predicted molecular mass of CPAlr and its mutants (Fig. 2).

**Optimal reaction pH, buffer and temperature** The CPAlr showed a maximum activity for L-alanine as the substrate at pH 8.0 and 40°C in Britton–Robinson buffer (Fig. 3A, B, 1.85 μg of enzyme). The activity of CPAlr revealed a relatively wide range of reaction temperature and pH, which were approximately 10% at 0°C and 20% at 60°C together with over 50% in the pH range of 6.0–9.5 when compared with the maximum activity. The CPAlr showed relatively higher activity in Britton–Robinson buffers in comparison with other buffers, such as a $Na_2HPO_4$–$KH_2PO_4$ buffer, Barbitone-HCl buffer, $NaHCO_3$–$Na_2CO_3$ buffer, $NaHCO_3$–NaOH buffer, $Na_2HPO_4$–NaOH buffer and KCl–NaOH buffer (Fig. 3A). The enzyme relative activities gradually increased as the temperature rose up to 40°C, and then a sharp decline in relative activity was observed when the temperature was over 40°C (Fig. 3B).

Fig. 3C shows the thermostability of recombinant CPAlr (1.85 μg of protein). The residual activity of CPAlr retained almost 100% of the initial activity during the first 10 min incubation at 30°C or 40°C, and then gradually decreased thereafter. However, the enzyme revealed a drastic decline in activity for 20 min incubation followed by a gradual reduction to baseline over the next 40 min treatment. The half-life of CPAlr was less than 15, 30 and 40 min at 50°C, 40°C and 30°C, respectively.

Fig. 3D shows the pH dependence of the enzyme stability of protein CPAlr (1.85 μg). The recombinant CPAlr showed a little bit stability at pH 8.0 with almost no activity lost for the first 10 min incubation at 40°C. Subsequently, the residual activity of protein CPAlr gradually inactivated with time and showed a similar trend of reduction with a treatment at pH 7.0 and 9.0. The half-life of CPAlr was severally about 15, 20 and 25 min at pH 9.0, 7.0 and 8.0, respectively.

**Substrate specificity and effects of metal ions and chemicals** The substrate specificity of CPAlr against various L-amino acids was detected at 40°C and pH 8.0 in Britton–Robinson buffer. The CPAlr revealed high specificity to L-alanine (100.0 ± 0.8%) and very low specificity to other L-amino acids, such as L-valine (5.2 ± 0.2%), L-phenylalanine (3.5 ± 0.1%), L-lysine (2.4 ± 0.1%), and L-proline (2.3 ± 0.0%) (Fig. 3E), which indicated that the protein CPAlr possessed relatively strict substrate specificity towards L-alanine.

The influences of various additives including metal ions and chemicals on the enzyme activity of CPAlr are listed in Table 2. Most of the ions and chemical reagents showed obvious effects on CPAlr activity. The divalent ions $Mg^{2+}$ (5.0–10 mM) and $Mn^{2+}$ (0.1 mM) effectively promoted the enzyme activity of CPAlr. And the enzyme activity was dramatically improved with the increase in $Mg^{2+}$ concentration from 1.0 mM to 10 mM. In contrast, it was declined evidently along with the increasing concentration of $Mn^{2+}$, $Ca^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Pb^{2+}$, DTT, and EDTA (0.1–10 mM). The activity of CPAlr was almost lost by the addition of $Cu^{2+}$, $Zn^{2+}$ and DTT at 5.0–10 mM.

**Mutational and oligomeric analysis** In order to explore the function and interaction of three residues Ser170 (inner), Asp171 (middle) and Tyr359 (inner) of CPAlr were separately replaced by alanine, serine and histidine using site-directed mutagenesis, and formed the plasmid with single or double point mutation, such as pET-S170A, pET-D171S, pET-D171A, pET-Y359A, pET-Y359H, pET-D171AY359A, pET-S170AY359A, and pET-S170AY359H.

The purified mutated proteins Y359A, S170A, S170A/Y359A, Y359H, S170A/Y359H, D171S, D171A and D171A/Y359H were all less active than the wild-type at 40°C and pH 8.0, exhibited approximately 79.6 ± 2.1%, 67.5 ± 0.3%, 46.9 ± 0.9%, 42.0 ± 1.3%, 32.9 ± 0.6%, 29.2 ± 0.4%, 25.1 ± 0.4%, and 20.3 ± 0.8% of the relative activity of CPAlr (100 ± 0.9%), suggesting that these residues are required for enzyme activity.

Oligomerization state of CPAlr and all mutated proteins were analyzed with size exclusion chromatography. Molecular weights were calculated by estimating the elution volumes of molecular mass standards. The molecular weight of CPAlr was approximately 74.2 kDa, indicating that the protein eluted as a dimer in solution (Fig. 3F, Table 3). The molecular masses of mutants Y359H and S170A were 70.4 kDa and 71.3 kDa, close to the calculated value of CPAlr. Mutants D171A, D171S, S170A/Y359H, and D171A/Y359H exhibited somewhat smaller retention volumes than wild-type, and the molecular weights were about 67.6 kDa, 61.7 kDa, 63.9 kDa, and 65.0 kDa, respectively, which exhibited a weak dissociation of dimerization. However, the mutants S170A/Y359A and Y359A

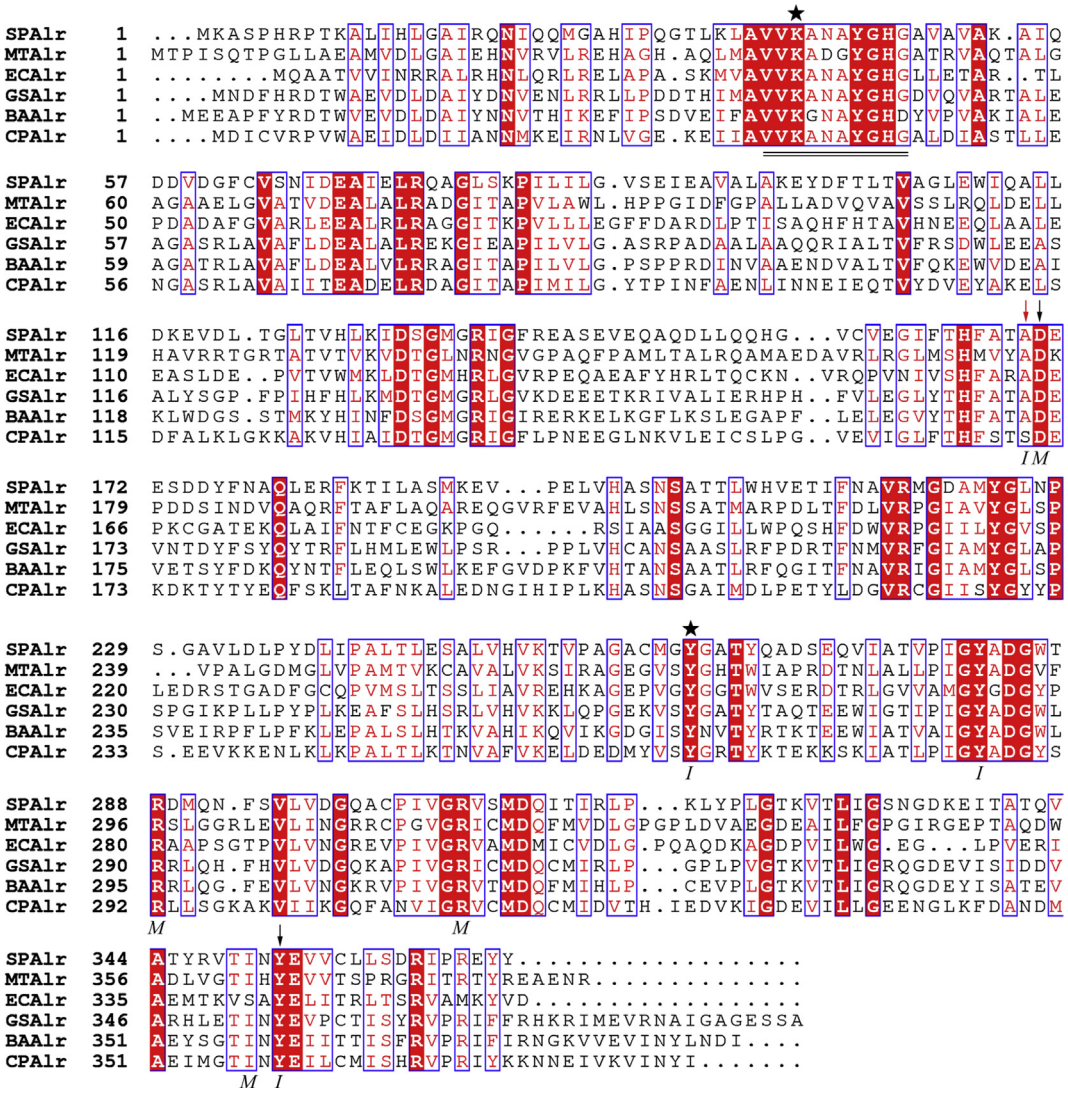


FIG.1. Structural based sequence alignment of alanine racemases from *S. pneumoniae* (SPAlr), *Mycobacterium tuberculosis* (MTAlr), *E. coli* (ECAlr), *B. anthracis* (BAAlr), *G. stearothermophilus* (GSAlr) and *C. perfringens* (CPAlr). The pyridoxal phosphate binding site (VVKANAYGHG) and key catalytic residues (Lys38 and Tyr267′) are marked with the double line and star, respectively. Letters *I* and *M* represent the residues located at the inner and middle layer. The arrow indicates the selected residues for this study.

existed as a protein with the relative molecular mass of about 55 kDa and 47.7 kDa, corresponding to the theoretical monomeric mass of 44 kDa (Fig. 3F). As we are known, residue Tyr359 located at the inner layer is involved in the dimer interface (18). Substitution of the conserved residue Tyr359 by alanine might weaken the intermonomer interaction. So, our studies suggest that the inner layer residue Tyr359 might play an important role in dimer-formation.

**Kinetic parameters** The kinetic parameters of CPAlr and 8 mutants were calculated and are shown in Table 3. The apparent

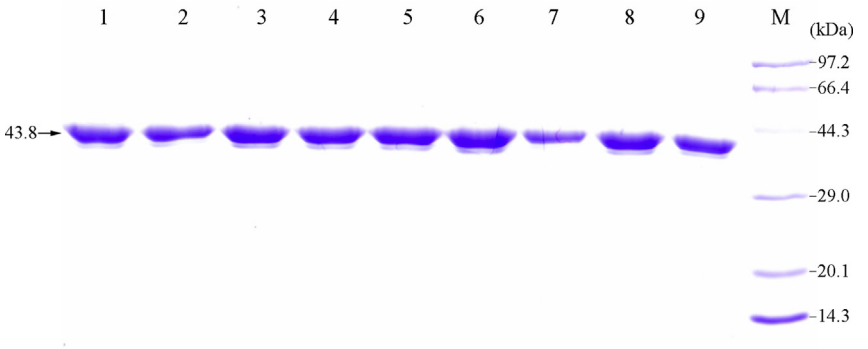


FIG. 2. SDS-PAGE analysis of purified protein CPAlr and mutants. Lanes 1–9 and M are proteins CPAlr, D171S, Y359A, S170A and Y359A, S170A, S170A and Y359H, Y359H, D171A and Y359H, D171A, and molecular weight standards.

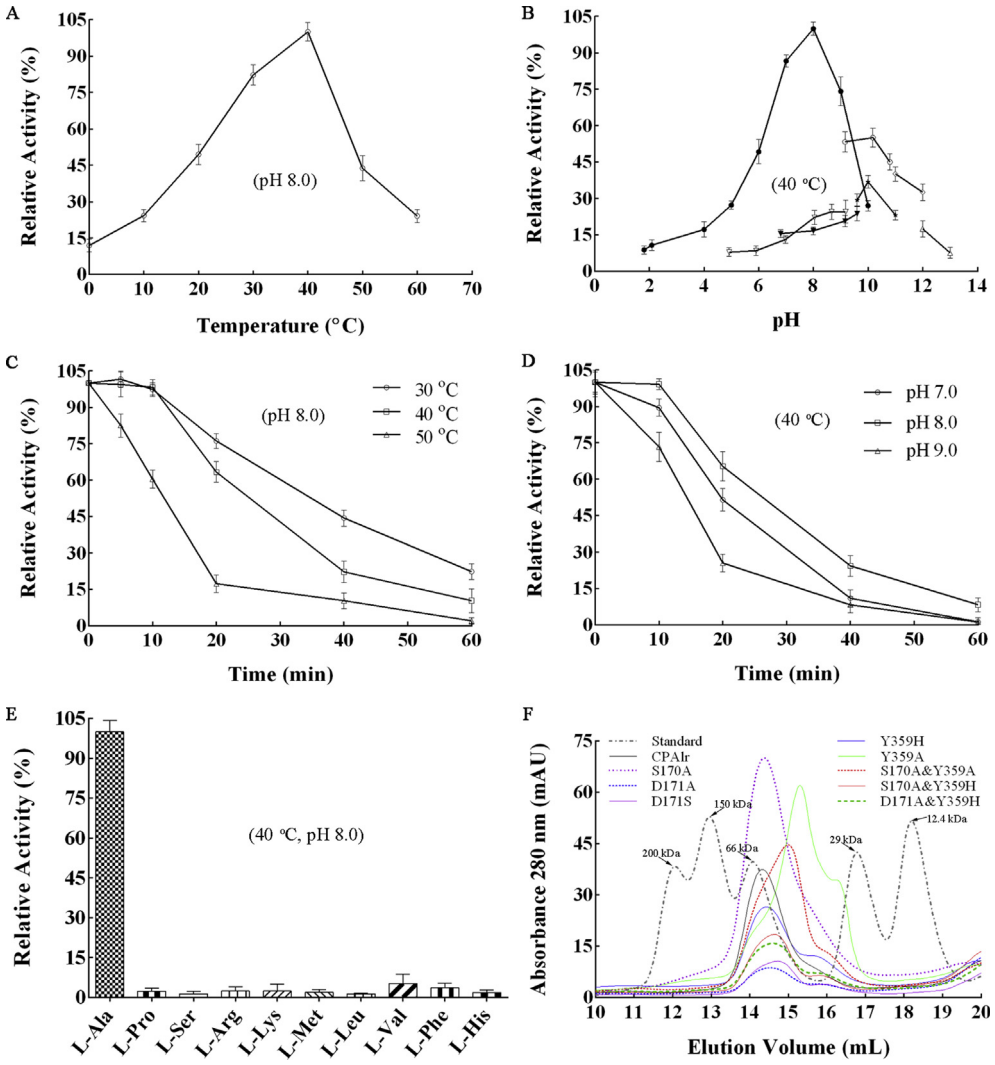


FIG. 3. Enzymatic properties of CPAlr from *C. perfringens*. (A) Optimal temperature of CPAlr at pH 8.0. The enzyme activity was determined in 20 mM Britton–Robinson buffer (pH 8.0) from 10°C to 60°C. (B) Optimal pH of CPAlr at 40°C. Closed circles, Britton–Robinson buffer (20 mM, pH 1.81–11.0); open inverted triangles, $Na_2HPO_4$–$KH_2PO_4$ buffer (20 mM, pH 4.92–9.18); closed inverted triangles, Barbitone-HCl (20 mM, pH 6.8–9.6); open circles, $NaHCO_3$–$Na_2CO_3$ buffer (50 mM, pH 9.16–10.8); stars, $NaHCO_3$–NaOH buffer (12.5 mM, pH 9.6–11.0); diamonds, $Na_2HPO_4$–NaOH buffer (12.5 mM, pH 11.0–12.0); and open triangles, KCl–NaOH buffer (25 mM, pH 12.0–13.0). (C) Thermo-stability of CPAlr in 20 mM Britton–Robinson buffer (pH 8.0) at 30°C (open circles), 40°C (open squares), and 50°C (open triangles). (D) pH-dependent stability at 40°C for 1h in 20 mM Britton–Robinson buffer. Open circles, pH 7.0; open squares, pH 8.0; open triangles, pH 9.0. (E) Substrate specificity of CPAlr. The relative activity of CPAlr for L-amino acids was determined at 40°C and pH 8.0. (F) The molecular weight evaluation of CPAlr and mutants using gel-filtration chromatography. Molecular mass standard (Sigma–Aldrich): β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa; and cytochrome *c*, 12.4 kDa. The data were presented as the mean values ± SD from triplicate independent enzymatic assays. Buffer without enzyme was set as a negative control.

velocity of all recombinant proteins was characterized at 40°C and pH 8.0 against various concentrations of both D and L enantiomers. The $K_m$ value of CPAlr was 19.1 mM for L-alanine and 10.5 mM for D-alanine, the $k_{cat}$ of CPAlr was 17.2 $s^{-1}$ $mM^{-1}$ for L-alanine and 8.7 $s^{-1}$ $mM^{-1}$ for D-alanine. All mutants displayed an appreciable decrease in catalytic efficiency ($k_{cat}/K_m$) and catalytic constant ($k_{cat}$) relative to the wild-type CPAlr. Furthermore, mutants Y359A, Y359H, D171A, D171A/Y359H and S170A/Y359A showed an elevated $K_m$ value for both substrates L-alanine and D-alanine, whereas mutants D171S and S170A/Y359H caused a little reduction in $K_m$ value. These studies indicated that the selected residues are essential for enzyme activity and might be involved in substrate binding.

**TABLE 2.** The effects of some metal ions and chemical reagents on enzyme activity.

| Metal ion and chemical | Relative activity (%) | | | |
|---|---|---|---|---|
| | 0.1 mM | 1.0 mM | 5.0 mM | 10.0 mM |
| Control | 100.0 ± 3.1 | | | |
| $Zn^{2+}$ | 16.3 ± 0.4 | 6.9 ± 0.2 | 4.3 ± 0.1 | 6.9 ± 0.2 |
| $Cu^{2+}$ | 30.5 ± 1.8 | 12.2 ± 0.4 | n.d. | n.d. |
| $Mg^{2+}$ | 102.7 ± 5.0 | 108.2 ± 7.6 | 136.8 ± 8.9 | 147.5 ± 9.1 |
| $Mn^{2+}$ | 139.2 ± 6.6 | 108.2 ± 4.0 | 78.3 ± 2.9 | 89.3 ± 4.2 |
| $Ca^{2+}$ | 107.6 ± 4.3 | 100.7 ± 4.2 | 73.9 ± 3.5 | 72.9 ± 2.9 |
| $Co^{2+}$ | 51.2 ± 2.3 | 31.1 ± 2.2 | 34.6 ± 2.2 | 39.9 ± 2.6 |
| $Pb^{2+}$ | 79.6 ± 2.5 | 48.8 ± 2.2 | 36.6 ± 2.2 | 59.2 ± 2.6 |
| EDTA | 42.6 ± 3.0 | 81.5 ± 3.0 | 78.6 ± 1.7 | 25.2 ± 1.5 |
| DTT | 86.0 ± 2.3 | 46.5 ± 1.8 | 0.5 ± 0.0 | 0.7 ± 0.0 |

ND, no detection.

## DISCUSSION

In this comprehensive study, we carried out gene cloning, complementation analysis, oligomerization analysis, biochemical characterization and mutational studies of alanine racemase CPAlr (GenBank: ABG82585.1) from *C. perfringens*. The purified protein

**TABLE 3.** Kinetics parameters and molecular weights of CPAlr and its mutants.

| Mutants | M.W. (kDa) | L-Alanine | | | D-Alanine | | | $K_{eq}$ (L/D) |
|---|---|---|---|---|---|---|---|---|
| | | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}\cdot s^{-1}$) | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}\cdot s^{-1}$) | |
| CPAlr | 74.2 | 19.1 ± 0.9 | 17.2 ± 0.7 | 0.9 ± 0.0 | 10.5 ± 0.5 | 8.7 ± 0.4 | 0.8 ± 0.1 | 1.1 |
| D171A | 67.6 | 41.4 ± 2.4 | 7.8 ± 0.3 | 0.2 ± 0.0 | 20.1 ± 0.9 | 2.0 ± 0.1 | 0.1 ± 0.0 | 1.9 |
| D171S | 61.7 | 12.4 ± 0.4 | 3.6 ± 0.2 | 0.3 ± 0.0 | 10.8 ± 0.7 | 1.4 ± 0.1 | 0.1 ± 0.0 | 2.2 |
| S170A | 71.3 | 19.0 ± 1.1 | 12.6 ± 0.6 | 0.7 ± 0.0 | 11.6 ± 0.4 | 6.3 ± 0.3 | 0.5 ± 0.0 | 1.2 |
| Y359A | 47.7 | 68.7 ± 4.4 | 16.4 ± 0.8 | 0.2 ± 0.0 | 19.6 ± 1.4 | 6.8 ± 0.4 | 0.4 ± 0.0 | 0.7 |
| Y359H | 70.4 | 36.3 ± 2.5 | 12.0 ± 0.7 | 0.3 ± 0.0 | 13.4 ± 0.8 | 4.0 ± 0.3 | 0.3 ± 0.0 | 1.1 |
| S170A/Y359H | 63.9 | 12.8 ± 0.6 | 4.7 ± 0.3 | 0.4 ± 0.0 | 8.6 ± 0.4 | 3.2 ± 0.3 | 0.4 ± 0.0 | 1.0 |
| S170A/Y359A | 55.0 | 24.3 ± 1.5 | 5.6 ± 0.3 | 0.2 ± 0.0 | 16.6 ± 0.9 | 4.0 ± 0.3 | 0.2 ± 0.0 | 1.0 |
| D171A/Y359H | 65.0 | 20.8 ± 1.4 | 4.9 ± 0.3 | 0.2 ± 0.0 | 38.2 ± 2.0 | 3.4 ± 0.2 | 0.1 ± 0.0 | 2.6 |

CPAlr was a dimeric enzyme with relatively strict substrate specificity. The optimal enzyme reaction temperature and pH values of CPAlr were 40°C and 8.0 in Britton–Robinson buffers. The $K_m$ value for L-alanine was 19.1 mM and 10.5 mM for D-alanine, and the $k_{cat}$ of CPAlr was 17.2 $s^{-1}$ $mM^{-1}$ for L-alanine and 8.7 $s^{-1}$ $mM^{-1}$ for D-alanine. The calculated $K_{eq\ (L/D)}$ for alanine racemization of CPAlr was about 1.1 (Table 3), consistent with the theoretical value for the chemically symmetric reaction (31).

However, the $k_{cat}$ and $k_{cat}/K_m$ value of all mutants dropped significantly when compared with wild-type CPAlr, and the calculated $K_{eq\ (L/D)}$ value for all mutants varied widely. In particular, the $K_{eq\ (L/D)}$ value of several mutants (Y359A, 0.7; D171A, 1.9; D171S, 2.2; D171A/Y359H, 2.6) showed about half or more than twice that of wild-type CPAlr (1.1) because of the dramatically increased $K_m$ value and/or decreased $k_{cat}$ value of these mutants. Interestingly, the molecular weights of most of the mutants were decreased; especially the mutant Y359A, the molecular weight of Y359A was about 47.7 kDa, indicating that it exists as a monomer. These results showed that residues Asp171 and Tyr359 of CPAlr might have a vital impact on the substrate binding, catalytic activity or oligomerization state.

From the crystal structure-based sequence alignment, residues Asp171 and Tyr359 of CPAlr are positioned at the middle layer and inner layer of entryway corridor and highly conserved in many other bacteria. Crystal structures analysis demonstrated that the inner layer residues Tyr364 and Tyr271′ of MTAlr were essential for proper orientation of PLP cofactor through hydrogen bond interaction and acted as the entrance gate on the opposite direction for D-alanine (PDB ID: 1XFC) (32). And the two middle layer residues Asp164 and Arg280 of ECAlr formed a middle gate of the entrance for L-alanine to the PLP binding cavity (PDB ID: 2RJG) (29). Patrick reported that inner layer residue Tyr354 of alanine racemase from *Geobacillus stearothermophilus* played a crucial role in substrate specificity (33). Furthermore, Im speculated that residues Asp170 (middle layer) and Tyr352 (inner layer) of SPAlr were involved in the dimer interface and made direct hydrogen bonds with the pentagonal ring waters (18). Teeter reported that pentagonal rings of water molecules might have a role in stabilizing protein structure or in catalysis (18,34). Taken together, all these information reinforce our conjecture that residues Asp171 and Tyr359 of CPAlr, which involved in the interface surface and participated in substrate guidance, might have the important role in catalytic activity or oligomerization state through the hydrogen–bonding interaction with the pentagonal ring waters and/or PLP cofactor.

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