

## Purification, Characterization and Inhibition of Alanine Racemase from a Pathogenic Strain of *Streptococcus iniae*

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Submitted 21 April 2019, revised 10 July 2019, accepted 10 July 2019

### Abstract

*Streptococcus iniae* is a pathogenic and zoonotic bacteria that impacted high mortality to many fish species as well as capable of causing serious disease to humans. Alanine racemase (Alr, EC 5.1.1.1) is a pyridoxal-5'-phosphate (PLP)-containing homodimeric enzyme that catalyzes the racemization of L-alanine and D-alanine. In this study, we purified alanine racemase from *S. iniae* that was isolated from an infected Chinese sturgeon (*Acipenser sinensis*), as well as determined its biochemical characteristics and inhibitors. The *alr* gene has an open reading frame (ORF) of 1107 bp, encoding a protein of 369 amino acids, which has a molecular mass of 40 kDa. The enzyme has optimal activity at a temperature of 35°C and a pH of 9.5. It belongs to the PLP-dependent enzymes family and is highly specific to L-alanine. *S. iniae* Alr (SiAlr) could be inhibited by some metal ions, hydroxylamine and dithiothreitol (DTT). The kinetic parameters  $K_m$  and  $V_{max}$  of the enzyme were 33.11 mM, 2426 units/mg for L-alanine, and 14.36 mM, 963.6 units/mg for D-alanine. Finally, the 50% inhibitory concentrations ( $IC_{50}$ ) values and antibiotic activity of two alanine racemase inhibitors (homogentisic acid and hydroquinone), were determined and found to be effective against both Gram-positive and Gram-negative bacteria employed in this study.

Key words: *Streptococcus iniae*, alanine racemase, inhibitors, homogentisic acid, hydroquinone and peptidoglycan

### Introduction

*Streptococcus iniae* is a Gram-positive and most commonly reported Streptococcal pathogen of fish responsible for high economic losses of aquaculture industries around the world. The zoonotic bacteria was also reported to cause bacteremia, cellulitis, meningitis, and osteomyelitis in human (Guo et al. 2018; Tavares et al. 2018). Vaccines and antibiotics were currently employed for minimizing the impact of the disease. However, recent studies revealed that the bacteria has so far developed resistance against many potential antibiotics (Tavares et al. 2018). As such, additional efforts for developing more effective vaccines and antibiotics are necessary steps for circumventing the threat of its infection (Saavedra et al. 2004).

Alanine racemase (Alr; E.C. 5.1.1.1) is an enzyme that catalyzes the interconversion of L-alanine and D-alanine using a pyridoxal 5-phosphate (PLP) as a cofactor (Tassoni et al. 2017). It provides D-alanine for the synthesis of peptidoglycan of the bacterial cell wall, D-alanine is directly involved in cross-linking

of adjacent peptidoglycan strands and also present in lipoteichoic acids of Gram-positive bacteria (Liu et al. 2018; Ray et al. 2018). There are two isoforms (non-homologous) of the alanine racemase genes (*alr* and *dadX*). The *alr* gene, which is constitutively expressed encodes for an essential enzyme for cell wall synthesis. While the expression of *dadX* is induced in the presence of high concentrations of L- or D-alanine. *DadX* is required for L-alanine catabolism, forming a substrate for D-alanine dehydrogenase (*dadA*) (Duque et al. 2017). The bacterial cell wall is indispensable for the survival and viability of bacteria (Liu et al. 2019) and has always been an attractive target for many antibiotics and antimicrobial agents (Anthony et al. 2011). Alanine racemase is ubiquitous among bacteria and rare in eukaryotes but absent in humans (Kawakami et al. 2018); hence, it emerges as an attractive and potential therapeutic target for the antimicrobial drugs development (Wang et al. 2017).

Numerous inhibitors were identified as able to affect the activity of alanine racemase (Kim et al. 2003a; Kim et al. 2003b). Many of these inhibitors were structural

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analogs of alanine; they interact with the enzyme-bound PLP, covalently bound to some eukaryotic PLP-dependent enzymes and lead to cellular toxicity (Toney 2005). PLP-related off-target effects could be overcome by using enzyme inhibitors that are not substrate analogs. Structure-based approach and molecular modeling have been employed to discover novel alanine racemase inhibitors, which are devoid of affinity for the PLP and hence off-target effects (Lee et al. 2013; Azam and Jayaram 2018).

In this study, we identified and purified the alanine racemase from *S. iniae* HNM-1 strain that was previously isolated from an infected Chinese sturgeon (*A. sinensis*). We have characterized its enzymatic properties, substrate specificity, and kinetic parameters. We have also explored the potentiality of the enzyme as an attractive antimicrobial target against *S. iniae*. We determined the 50% inhibition concentrations ( $IC_{50}$ ) of two alanine racemase inhibitors (homogentisic acid and hydroquinone) and their antimicrobial susceptibility against six opportunistic pathogens including *S. iniae*, in quest of providing the possible solutions against antibiotics resistance and bacterial infections.

## Experimental

### Materials and Methods

**Strains, plasmids, and growth conditions.** The characteristics of bacterial strains and plasmids used in this context were summarized in Table I. *S. iniae* HNM-1 was cultured at 35°C in the Tryptone soy yeast extract (TSYE) medium. *Escherichia coli* DH5 $\alpha$ , *E. coli* BL21 strains, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* were cultured in Luria Bertani (LB) medium at 37°C or 35°C. 100 g/ml final concentration of ampicillin (Amp) was used in this study.

**Cloning of alanine racemase gene.** Primers were designed based on the *alr* gene sequence of *S. iniae* 89353 strain (NCBI accession number CP017952.1). The genomic DNA of *S. iniae* HNM-1 was extracted and amplified using the following primers, Alr-F-(5'-GCACCATGGATGATTTCAAGTTTG-3') and Alr-R-(5'-TCACTCGAGATCCCCGATAAAGC-3'), with *Nco*I and *Xho*I restriction sites, underlined respectively. PCR product was cloned in pMD19-T cloning vector to construct pMD-*alr* and transformed to *E. coli* DH5 $\alpha$ . The *alr* gene was digested with restriction endonucleases and cloned into expression vector pET-22b (+), forming recombinant plasmid pET22b-*alr*. The deduced amino acid sequence of the ORF was analyzed by the Blast software. Multiple amino acid sequence alignment and phylogenetic relationships among alanine racemase of *S. iniae* and other bacteria were constructed with Clustal Omega.

The evolutionary distances of the phylogenetic tree were computed using the p-distance method and are reported in the units of the number of amino acid differences per site. The bootstrap consensus tree inferred from 500 replicates was used to represent the evolutionary history (Felsenstein 1985). The analysis involved 10 amino acid sequences from *S. iniae* (NCBI Genbank accession number: APD32491.1), *P. aeruginosa* PAO1 (NCBI Genbank accession number: AF165882), *Streptococcus agalactiae* (NCBI Genbank accession number: WP\_037624882), *Enterococcus faecalis* (NCBI Genbank accession number: AFO43552.1), *Streptococcus pyogenes* (NCBI Genbank accession number: MGAS10750), *Staphylococcus aureus* (NCBI Genbank accession number: CAG41139.1), *Corynebacterium glutamicum* (NCBI Genbank accession number: AAL77207.1), *Aeromonas hydrophyla* (NCBI Genbank accession number: ABK36160.1), *Streptococcus pneumoniae* (NCBI Genbank accession number: AAL00344.1), and *E. coli* (NCBI Genbank accession number: YP\_002407858).

Table I  
Strains and plasmids used in this study.

Strains/Plasmids	Description	Source
<b>Strains</b>		
<i>Streptococcus iniae</i> HNM-1	Isolated from infected <i>A. sinensis</i>	This study
<i>Escherichia coli</i> DH5 $\alpha$	Used for cloning and propagation of plasmids	Novagen
<i>Escherichia coli</i> BL21(DE3)	Used for protein expression	Invitrogen
<i>Salmonella typhimurium</i>		This study
<i>Staphylococcus aureus</i>		This study
<i>Acinetobacter baumannii</i>		This study
<i>Pseudomonas aeruginosa</i>		This study
<b>Plasmids</b>		
pMD19-T	Carries ampR gene; used for cloning PCR product with A at 3' ends	Takara
pET 22b (+)	Carries ampR gene; used for expressing <i>S. iniae</i> Alanine racemase	Novagen

**Expression and purification of alanine racemase.** *E. coli* BL21 (DE3) was transformed with expression vector pET22b-Alr for protein expression, a single colony of the transformed *E. coli* was inoculated in 100 ml LB medium at 35°C. Protein expression was induced when the OD<sub>600</sub> reaches 0.6 by addition of IPTG at a final concentration of 1 mM, and re-incubated overnight at 16°C or at 35°C for 5 hours. Cells were collected and resuspended in 20 ml binding buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 300 mM NaCl, and 10 mM imidazole), lysed on ice by sonication for 40 minutes, and centrifuged at 8000 g, 4°C for 10 minutes. The supernatant was collected and purified using Nickel ion affinity chromatography (Qiagen), according to the manufacturer's protocol. The protein solution was dialyzed against phosphate buffered saline (PBS, pH 7.4). Protein purity and concentration were determined by SDS-PAGE and BCA protein assay kit (Takara), respectively. Western blotting was conducted using a monoclonal antibody against the poly-Histidine tag attached to the Alr protein as described previously (Liu et al. 2015).

**Enzyme assay.** Alanine racemase racemization assay was conducted in two coupled enzyme reactions, using a standard racemization mixture (Wang et al. 2017). The reaction was initiated by addition of the suitable concentration of SiAlr in the final reaction volume of 200 µl, incubated at 35°C for 10 minutes, terminated by addition of 25 µl of 2 M HCl and neutralized with 25 µl of 2 M sodium hydroxide. Then, the reaction mixture was centrifuged at 14 000 rpm, 4°C for 10 minutes. The concentration of D-alanine was analyzed in the second reaction containing 200 mM Tris- HCl pH: 8.0, 0.2 mg/ml 4-aminoantipyrine, 0.2 mg of N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methyl aniline, 1 unit of HRP, and 0.1 unit of D-Amino acid oxidase. The absorbance was measured using a microplate reader at 550 nm after 20 min incubation at 37°C.

**Effect of temperature and pH on enzyme activity and stability.** The influence of temperature was determined according to a standard enzyme assay by measuring the initial rate of reaction at various temperatures (10°C to 50°C), while the effect of pH was determined by measuring the initial rate of reaction in Britton-Robinson buffer (pH 2.0 to 12.0) at the optimum temperature. The relative residual activity was calculated with the highest activity as 100%. The thermal stability of the enzyme was examined by preincubation in the reaction buffer without substrate at temperatures of 30°C, 35°C, and 40°C for 2 h followed by addition of L-alanine as a substrate for determination of the relative residual enzyme activity. The pH stability of the enzyme was examined by incubation of the enzyme in the reaction buffers without substrate at pH ranging from 8.5 to 11.0 on ice for 2 hours, and subsequently,

L-alanine was added and the relative residual enzyme activity was measured.

**Substrate specificity of alanine racemase.** The substrate specificity of SiAlr was determined according to the standard racemization reaction mixture using 18 kinds of L-amino acids as the substrates and incubated at the optimum temperature for 10 minutes.

**Effect of metal ions, reducing agents and PLP on the enzyme activity.** The influence of some metal ions and chemical compounds on the activity of SiAlr were determined by incubating the enzyme with the chemical compounds in the reaction mixture for 30 min, afterward, the substrate was added and the relative residual activity was determined according to the standard protocol (Liu et al. 2015).

SiAlr and different concentrations of hydroxylamine (0.1, 1, and 10 mM) were added to the reaction mixture without the substrate, dialyzed in phosphate buffered saline for 40 min, and its activity was determined without the addition of PLP. The effect of Dithiothreitol (DTT) on the activity of SiAlr was also determined by incubating the enzyme in different concentrations of DTT (1 and 3 mM) for 30 minutes and the relative activity was measured. To confirm SiAlr is a PLP-dependent enzyme, the purified Alr was treated with 10 mM hydroxylamine and dialyzed to obtain the apoenzyme. The apoenzyme was incubated in different concentrations of PLP (0.01, 0.04, and 0.06 mM) and its relative activity was measured.

**Kinetic parameters.** The alanine racemase activity was determined by measuring the amount of both enantiomers of alanine by high-performance liquid chromatography (HPLC) using a fluorescence detector according to the method described earlier (Hashimoto et al. 1992). The reaction mixture comprised of 10 µM PLP, 200 mM carbonate buffer pH 9.5, and various concentrations of either L or D forms of alanine. The enzymatic reaction was initiated by adding the purified SiAlr, followed by 10 min of incubation at 35°C. The reaction was terminated by the addition of 40 µl of 2 M HCl on ice for 2 min, neutralized with 40 µl of 2 M NaOH, and centrifuged at 10 000 g, 4°C for 5 min. A 40 µl aliquot of the reaction was derivatized by addition of 280 µl of 0.4 M boric acid pH 9.0, 0.1% N-tert-butylloxycarbonyl-L-cysteine (Sigma), and 0.1% O-phthaldialdehyde. One unit of the enzyme was defined as the amount of the enzyme that catalyzed the formation of 1 µmol of L- or D-alanine from either enantiomer per minute. Graph Pad Prism 6.0 was used for results analysis.

**Enzyme IC<sub>50</sub> determination.** Inhibitory effects of homogentisic acid and hydroquinone on the activity of alanine racemase were determined as described previously (Wang et al. 2017). Fivefold dilution series (in DMSO) was prepared for the compounds, and the

solutions were added to the wells of a 96-well plate to yield the final inhibitory concentrations. Each concentration was tested in triplicate. The substrate was added after 30 min of incubation, and the fluorescence intensity was measured after the reaction. The negative control was prepared without adding chemicals to the control wells and the D-cycloserine (DCS) was used as a positive control. Percentage inhibition at each inhibitor concentration was calculated with respect to the negative control. Graph Pad Prism 6.0 was used for the calculation of the concentration that causes 50% inhibition ( $IC_{50}$ ).

**Antimicrobial susceptibility tests.** Minimum inhibition concentrations (MIC) of hydroquinone and homogentisic acid against both Gram-positive and Gram-negative bacteria were determined by microdilution assay according to the guidelines of the Clinical and Laboratory Standards Institute, document M31-A3 (CLSI 2008), as described previously (Dal Pozzo et al. 2011). An overnight culture was subcultured to  $OD_{600}$  of 0.3, diluted tenfold, five times. Aliquots were spread on agar plates in triplicate to determine the number of colony-forming units (CFU)/ml. Compounds were diluted in DMSO at concentrations of 200, 100, 80, 40, 20, or 10  $\mu$ g/ml. DMSO solvent was used as a negative control of growth inhibition and DMSO alone was used as the blank control. All tests were performed in triplicate. The inoculums were prepared in LB medium ( $1 \times 10^8$  CFU/ml) and incubated at 30°C for 20 h. Subsequently, 100  $\mu$ l ( $1 \times 10^5$  CFU) of the inoculums and the inhibitors were added to each microplates wells and incubated at 30°C for 48 h. The MIC values were determined as the lowest concentration of the inhibitors at which no growth of the bacteria was observed after incubation.

## Results

### Identification of *S. iniae* alanine racemase gene.

According to the genomic sequence of *S. iniae*, the bacteria have a single putative alanine racemase (*alr*) gene. The *alr* gene has an open reading frame of 1107 bp that encodes a 369 amino acids protein (SiAlr) with a molecular mass of 39.82 kDa. The nucleotide sequence of *alr* has been submitted to Genbank under accession number MK620909.

The deduced amino acid sequence has 76%, 67%, 63%, and 47% similarities with alanine racemase of *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*, respectively. Phylogenetic analysis of alanine racemase from different bacteria revealed an evolutionary relationship among them. The phylogenetic tree consists of two distinct clades. The enzyme is clustered with other *Streptococci* species, such as *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, and *E. faecalis*. These sequences, from

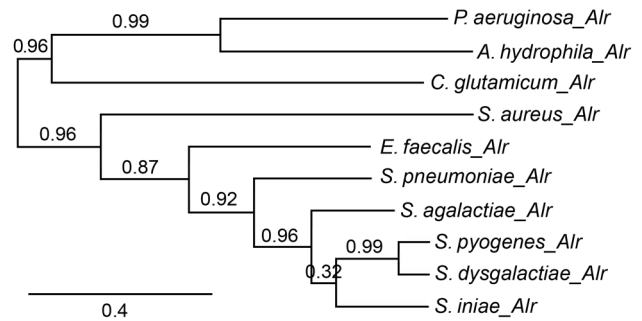


Fig. 1. Phylogenetic relationships of SiAlr and homologs alanine racemases from *Pseudomonas aeruginosa* PAO1, *Corynebacterium glutamicum* ATCC 13032, *Aeromonas hydrophila* ATCC 7966, *Staphylococcus aureus* ABFQT, *Enterococcus faecalis* D32, *Streptococcus pneumoniae* MDRSPN001, *Streptococcus agalactiae* 2603V/R, *Streptococcus pyogenes* MGAS10750 and *Streptococcus dysgalactiae* Kdys0611. The tree was constructed using Neighbor end-joining. Maximum likelihood tree based on complete coding sequences deposited in Genbank. The evolutionary distances were computed using the p-distance method and are reported in the units of the number of amino acid differences per site.

Gram-positive bacteria, were classified into one group. The sequences from Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and *Corynebacterium glutamicum* were classified into another (Fig. 1). The evidence indicated that these alanine racemases evolved independently from a common ancestor and formed two isolated genes.

Multiple sequence alignment of SiAlr with sequences of other 10 species Alr suggested that some regions are conserved in SiAlr, which includes PLP binding motif near the N-terminus (AVVKANAYGHG) and the two catalytic amino acid residues of the active center (Lys 40 and Tyr 274). The eight residues making up the entry-way to the active site (inner layer: Ala 174, Tyr 273, Tyr 282 and Tyr 366; middle layer: Asp 166, Arg 298, Arg 318 and Ile 364) (Fig. 2).

**Expression and Purification of SiAlr.** The SiAlr protein was expressed in *E. coli* (DE3) incubated overnight at 16°C, and purified to homogeneity using Ni-agarose affinity chromatography. The protein has a relative molecular mass of 39.82 kDa as estimated by SDS-PAGE, which was similar to the calculated relative molecular mass. Western blotting analysis using the anti-poly-His antibody confirmed that 39.82 kDa protein is SiAlr (Fig. 3).

**Characterization of the enzyme.** The optimal temperature of SiAlr was approximately 35°C. The enzyme was found to be very stable at the temperature of 30°C and 35°C, with more than 50% residual activity. The optimal pH of SiAlr was approximately 9.5 at 35°C. The enzyme was found to be very stable, with more than 50% residual activity after incubation for 2 hours at a pH range of 8.5 to 9.5 (Fig. 4). Thus demonstrating that SiAlr is a basophilic enzyme.



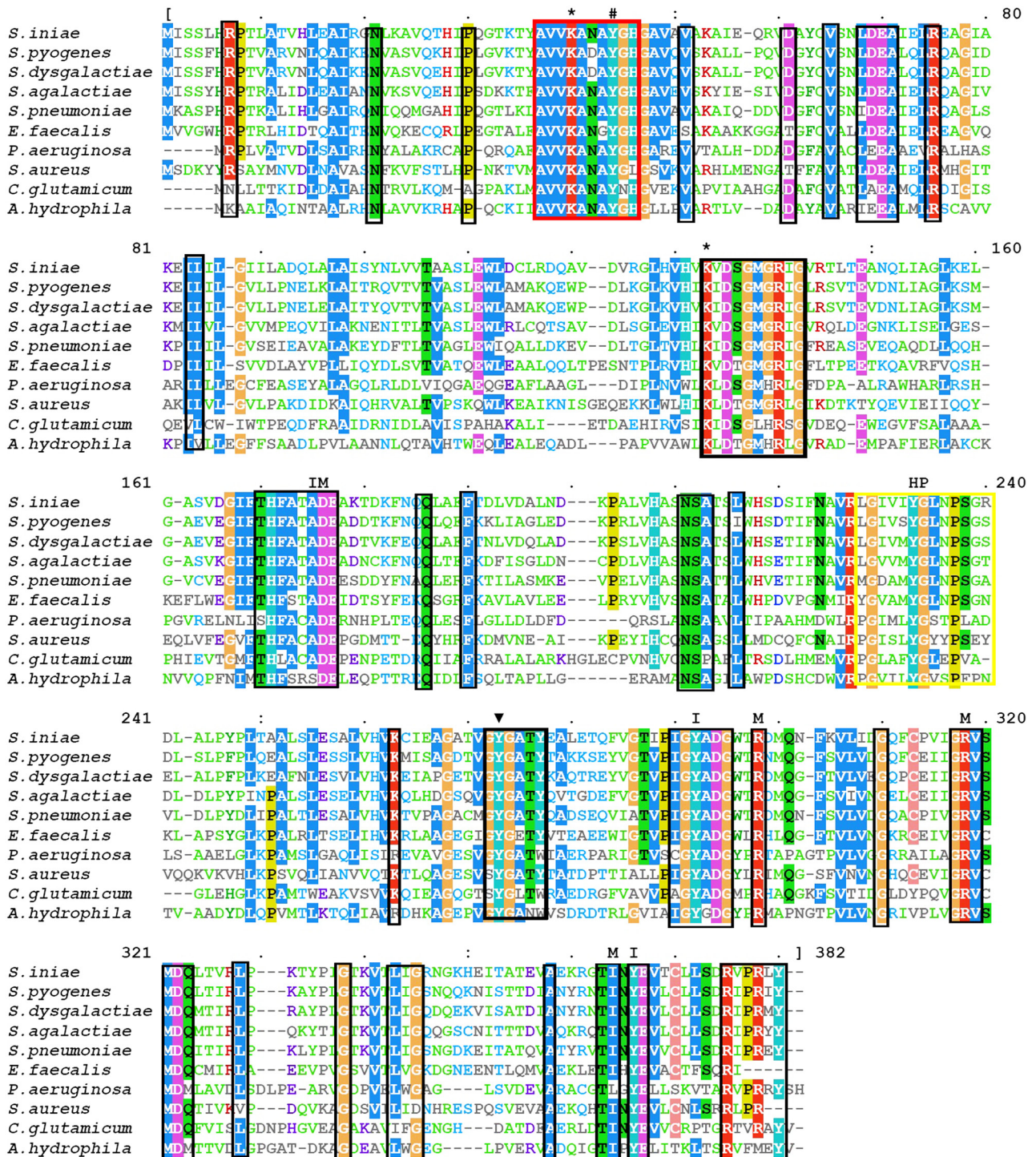


Fig. 2. Structure-based sequence alignment of alanine racemases sequences. The amino acid sequence of Alr from *Streptococcus iniae* was aligned with alanine racemases sequences of *Streptococcus pyogenes* MGAS10750, *Streptococcus dysgalactiae* Kdys0611, *Streptococcus agalactiae* 2603V/R, *Streptococcus pneumoniae* MDRSPN001, *Enterococcus faecalis* D32, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* ABFQ1, *Corynebacterium glutamicum* ATCC 13032 and *Aeromonas hydrophila* ATCC 7966. The red box enclosed the conserved PLP-binding sites; Lys40 (\*), Tyr44 (#). The catalytic Tyr residue was indicated by (+). Strictly conserved residues were enclosed in the black boxes, while the hydrophobic patch (HP) in the yellow box. Residues of the active site entryway are marked with either I (inner layer) or M (middle layer). Highly conserved residues were indicated by the box and strictly conserved with (\*).

Various chemicals and metal ions were reported to inhibit the activity of alanine racemases. The results revealed that the enzyme activity was inhibited by most of the metal ions, but markedly inhibited by  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Fe}^{2+}$  (Fig. 5).

**Effect of reducing agents on the activity of SiAlr.** Many inhibitors of alanine racemase have been discovered (Wang et al. 2017). The enzyme lost its activity after treatment with 1 or 10 mM hydroxylamine. Addition of 0.1 mM hydroxylamine reduced the activity of

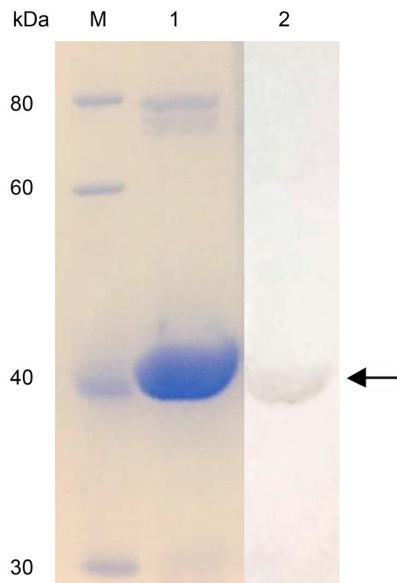


Fig. 3. Purification of *Streptococcus iniae* alanine racemase. The enzyme was purified using Nickel ion affinity chromatography, analyzed by SDS-PAGE and western blotting. M: molecular weight standards; 1; 40 kDa SiAlr. 2: Western blotting analysis of the purified protein.

the enzyme by 80%. Treatment of SiAlr with 1 mM of DTT resulted in a 70% loss of activity and completely inhibition at the concentration of 3 mM (Table II).

We examined the role of PLP in the activity of SiAlr by resolving the enzyme to Apo-enzyme by hydroxy-

Table II  
Effect of Hydroxylamine, DTT and PLP on SiAlr Activity.

Chemical	Concentration (mM)	Relative activity (%)
None		100 (0.7)
Hydroxylamine	0.1	21 (1.2)
	1	11 (0.8)
	10	9 (1.4)
DTT	1	27 (3.1)
	3	2 (0.8)
PLP	0.01	56 (2.4)
	0.04	83 (1.5)
	0.06	96 (2.7)

The data were presented as mean (SD) from three independent enzyme reactions

lamine treatment. The Apo-enzyme completely lost its activity after treatment with 10 mM hydroxylamine. Addition of 0.01, 0.04, and 0.06 mM of PLP make the enzyme regained up to 56%, 83 % and 96% of its activity, respectively. The result indicated that SiAlr is a PLP-dependent enzyme that requires more than 0.01 mM PLP to maintain its activity (Table II).

**Substrate specificity.** Alanine racemase is a highly conserved bacterial enzyme and known to be very specific to its substrate (Patrick et al. 2002). As shown in Fig. 6, the enzyme is highly specific to L-alanine and

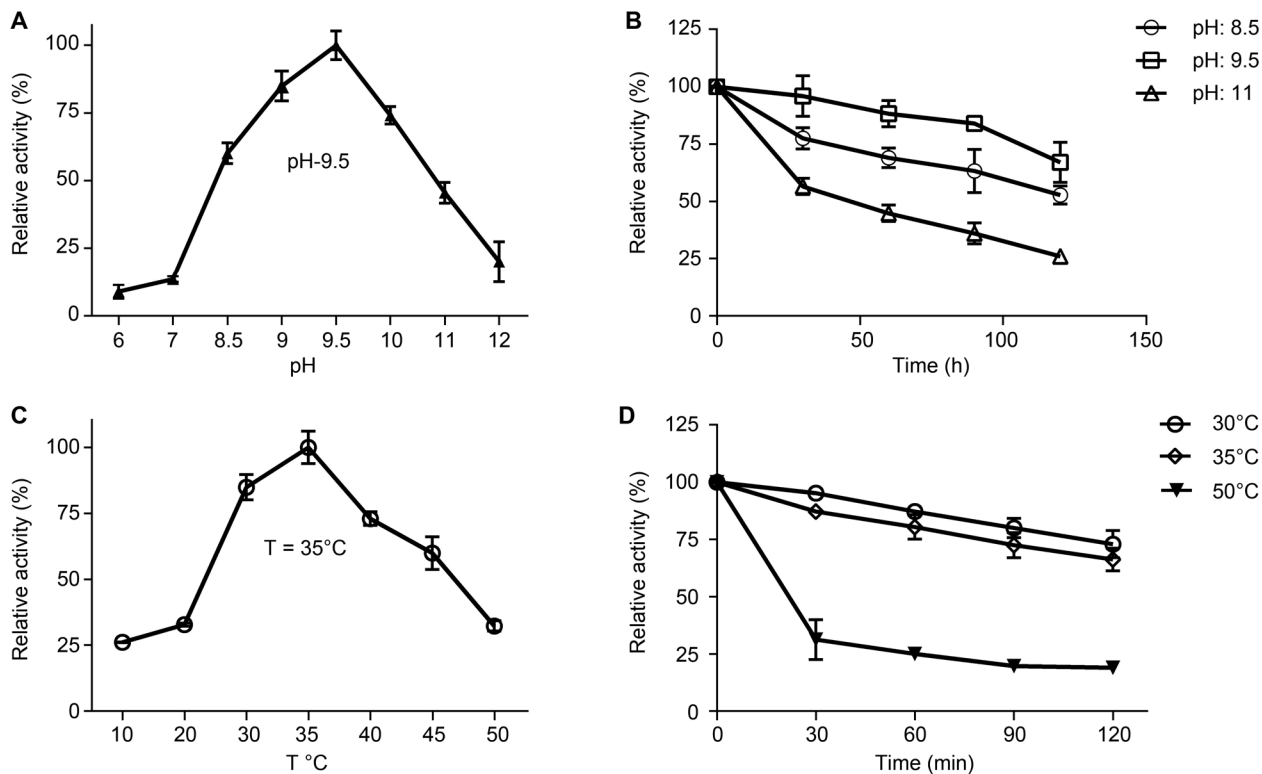


Fig. 4. Effect of pH and temperature on the activity of SiAlr. (A) Optimal pH, (B) pH stability, (C) Optimal temperature, (D) Thermal stability.

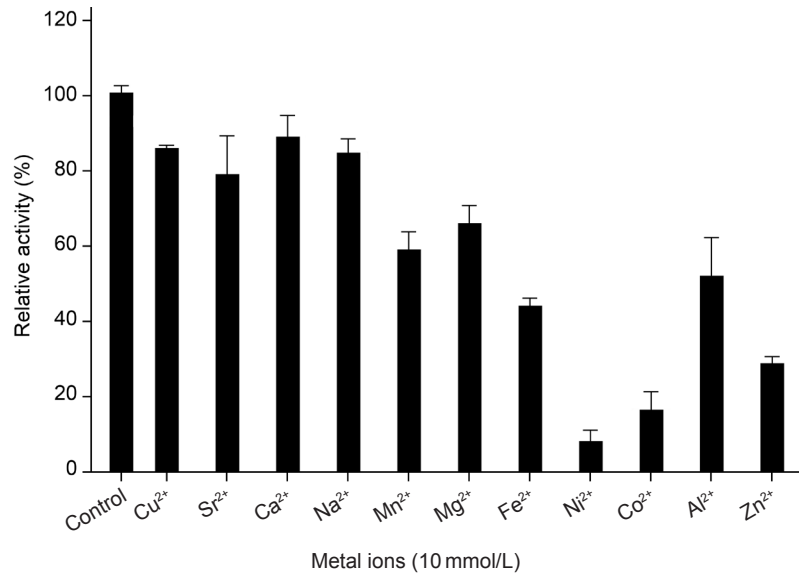


Fig. 5. Effect of metals on SiAlr activity. The metal ions were at a concentration of 10 mM/L. The data were presented as mean  $\pm$  SD from 3 independent determinations.

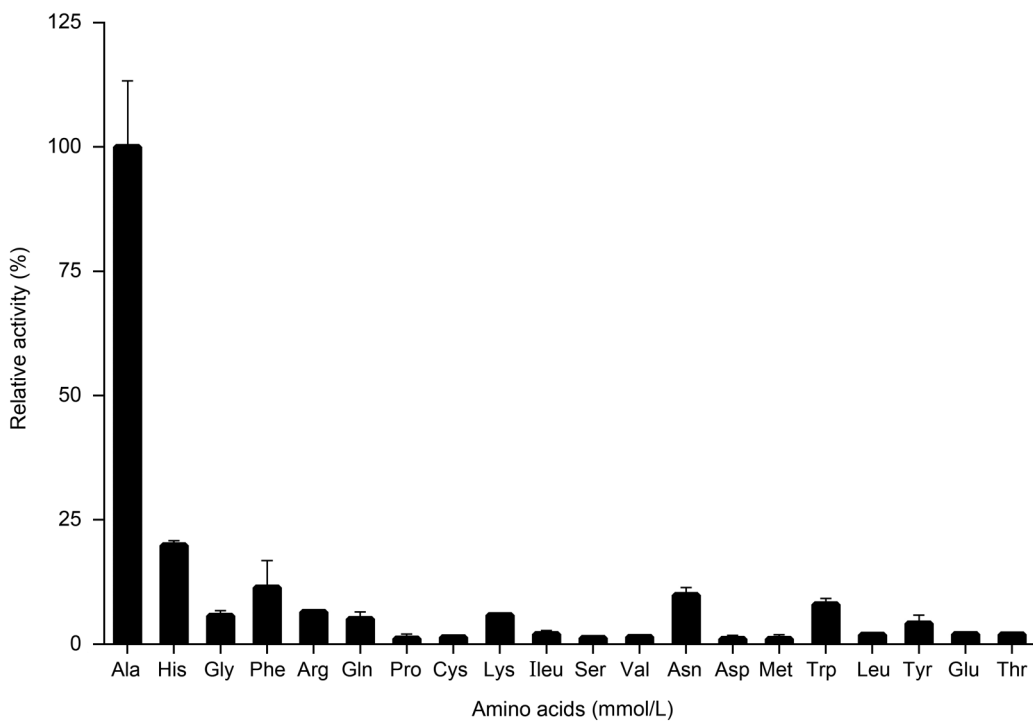


Fig. 6. The substrate specificity of SiAlr. The relative activity of SiAlr for various L-Amino acids was determined at optimum pH and temperature. The data were presented as mean  $\pm$  SD from 3 independent enzyme assays.

showed weak activity with L-phenylalanine (11%), L-Histidine (20%), and L-Asparagine (10%). This result indicates that SiAlr has strict substrate specificity.

**Kinetic parameters determination.** Kinetic parameters of SiAlr were determined using HPLC. The substrate affinity constant ( $K_m$ ) for L-alanine was 33.11 mM with a maximal velocity ( $V_{max}$ ) of 2426 units/mg, while the D-alanine  $K_m$  value was 14.36 mM with a  $V_{max}$  of 963.6 units/mg. The  $V_{max}$  of L-alanine was more than twice that of its enantiomer. These indicated that the

enzyme has a higher binding affinity for L-alanine than for D-alanine, and the conversion of L- to D-alanine was more rapid than the reverse conversion. The equilibrium constant ( $K_{eq}$  (L/D)) was 1.09, which is consistent with the reported theoretical equilibrium constant ( $K_{eq} = 1$ ) for alanine racemase (Liu et al. 2015).

**IC<sub>50</sub> determination.** In our previous study, we found that homogentisic acid and hydroquinone are two alanine racemase inhibitors with minimal cytotoxicity against mammalian cells and can be utilized as potential



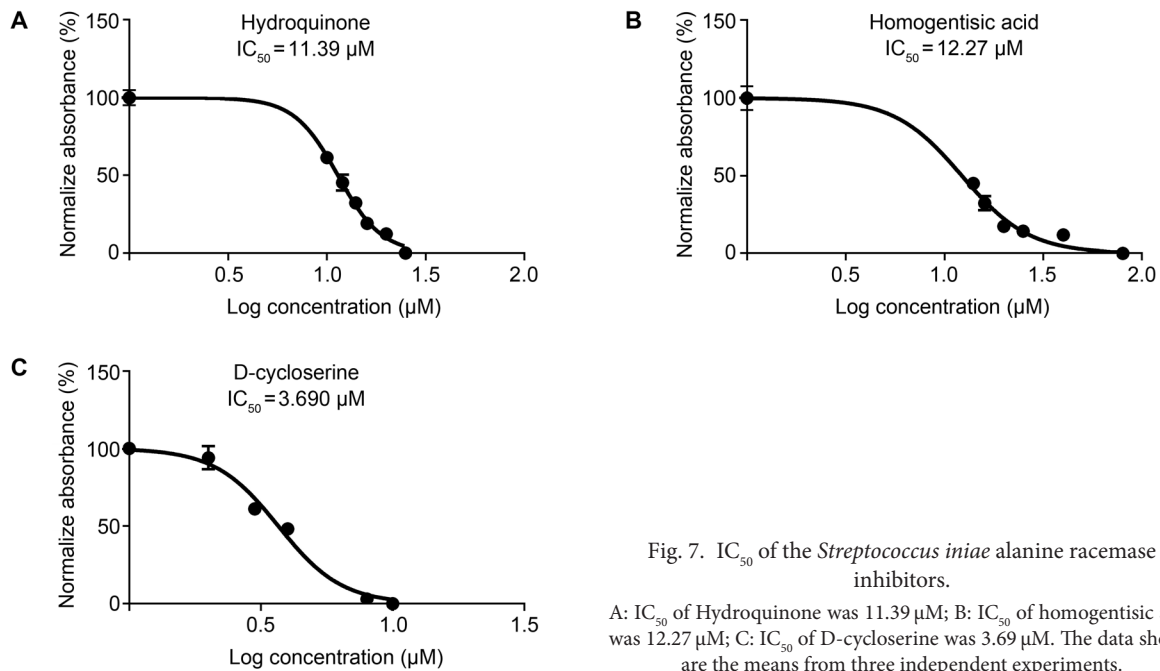


Fig. 7. IC<sub>50</sub> of the *Streptococcus iniae* alanine racemase inhibitors.

A: IC<sub>50</sub> of Hydroquinone was 11.39 µM; B: IC<sub>50</sub> of homogentisic acid was 12.27 µM; C: IC<sub>50</sub> of D-cycloserine was 3.69 µM. The data shown are the means from three independent experiments.

agents of antibiotics (Wang et al. 2017). In this study, we investigated the inhibitory effects of homogentisic acid and hydroquinone on SiAlr. DMSO was used as the blank control and DCS, a known alanine racemase inhibitor, as the positive control. The results showed that the IC<sub>50</sub> values of hydroquinone and homogentisic acid were 11.39 µM and 12.27 µM. The IC<sub>50</sub> values of hydroquinone and homogentisic acid were 3 and 3.3 times higher than that of DCS, respectively (Fig. 7).

**Antimicrobial activity of alanine racemase inhibitors.** The MIC was assayed to determine the antimicrobial activity of two alanine racemase inhibitors against *S. iniae* HNM-1 and several pathogenic bacteria (Table III). The results showed that hydroquinone and homogentisic acid have broad-spectrum antibiotic activities against both Gram-positive and Gram-negative bacteria. Hydroquinone showed good

antibiotic activity against *S. iniae* HNM-1 with MIC value of 25 µg/ml, however, it showed moderate antibiotic activity against other strains with MIC value of 130–210 µg/ml. Homogentisic acid demonstrated moderate antibiotic activity against the bacteria tested, with MIC value of 180–250 µg/ml. Interestingly, hydroquinone and homogentisic acid had no antibiotic activity against *P. aeruginosa*.

## Discussion

*Streptococcus iniae* is one of the pathogenic Gram-positive bacteria that causes morbidity and mortality of farmed and wild fish (Aruety et al. 2016). *S. iniae* HNM-1 strain was isolated from an infected Chinese sturgeon (*A. Sinensis*) after a disease outbreak that caused high morbidity and mortality. The classification of *S. iniae* HNM-1 was confirmed by molecular analysis of 16s rRNA gene sequence. The sequence was deposited at the NCBI Genbank database under accession number KY781829. The bacteria have a single putative alanine racemase (*alr*) gene. As reported earlier, most of the Gram-positive bacteria, including *Lactobacillus plantarum* (Palumbo et al. 2004), *Bacillus anthracis* (Couñago et al. 2009), *Mycobacterium tuberculosis* (Nakatani et al. 2017), and *Mycobacterium smegmatis* (Chacon et al. 2002), appear to possess only one alanine racemase gene.

The optimal pH and temperature of SiAlr were 9.5 and 35°C, respectively, which were similar to alanine racemase from *A. hydrophila* (Liu et al. 2015) and *Bacillus pseudofirmus* OF4 (Ju et al. 2009). Nearly all characterized alanine racemases have optimal pH of more

Table III

The results of antimicrobial activity of homogentisic acid and hydroquinone inhibitors against numerous isolates of Gram-positive and Gram-negative bacteria.

Organism	<sup>a</sup> MIC (µg/ml)	
	Hydroquinone	Homogentisic Acid
<i>Streptococcus iniae</i> HNM-1	25 (2.3)	200 (5.6)
<i>Escherichia coli</i> DH5α	130 (7.9)	210 (8.4)
<i>Salmonella typhimurium</i>	150 (8.7)	180 (11.4)
<i>Staphylococcus aureus</i>	210 (13.7)	250 (14.1)
<i>Acinetobacter baumannii</i>	180 (11.5)	210 (12.3)
<i>Pseudomonas aeruginosa</i>	0	0

<sup>a</sup> MIC, average values with standard deviations



than 8.0 (Seow et al. 1998; Francois and Kappock 2007). SiAlr is a mesophilic enzyme, stable at a temperature from 0°C to 40°C. Thermal stability of an enzyme is correlated with the host bacteria physiology and environment, thermophilic bacteria Alr are more stable than that of mesophilic and psychrotroph bacteria (Soda and Tanizawa 1990; Yokoigawa et al. 1993). SiAlr was inhibited by metal ions, such as Ni<sup>2+</sup>, Co<sup>2+</sup>, Zn<sup>2+</sup>, and Fe<sup>2+</sup>, which indicated that some divalent metal ions could influence the enzyme-substrate complex formation in such a way that favor decrease of enzyme activity. Similarly, treatment of SiAlr with 1 mM of DTT resulted in a 70% loss of activity and the complete inhibition at the concentration of 3 mM. However, the activity of alanine racemase from *Tolypocladium inflatum* has increased in the presence of DTT (Di Salvo et al. 2013). DTT reduces cysteine side chains and causes a reduction of intermolecular disulfide bonds, which may affect the enzyme conformation and eventually enhance or inhibit its activity. SiAlr requires exogenous PLP for its activity in contrast to Alr from *B. pseudofirmus* OF4 and *Thalassiosira* sp., which does not require exogenous PLP for optimal activity (Ju et al. 2009). The enzyme showed high substrate specificity to alanine, which is similar to most of the characterized alanine racemases (Kawakami et al. 2018).

Many studies have focused on alanine racemase to develop antibacterial drugs for multiple bacterial species (Scaletti et al. 2012; Shrestha et al. 2017). Although the MIC values of homogentisic acid and hydroquinone were higher than that of D-cycloserine, which is a cyclic analog of alanine and exerted its inhibitory effect through interaction with the enzyme-bound PLP cofactor (Batson et al. 2017), both homogentisic acid and hydroquinone are not structural analogs of Alr as such they are not interfering with other PLP dependent enzymes, but directly interact with the active sites of alanine racemase (Wang et al. 2017). According to the results of antimicrobial activity assay, the two inhibitors are capable of inhibiting both Gram-positive and Gram-negative bacteria with various efficacies, except *P. aeruginosa*. The reason the two compounds showed no antimicrobial activity against *P. aeruginosa* may be because homogentisic acid is a normal product of *P. aeruginosa* and it also contained hydroquinone oxidase that oxidized hydroquinone (Higashi 1958; Hunter and Newman 2010).

Several alanine racemases have been identified and characterized from the *Streptococcus* species. Alanine racemase from *Streptococcus faecalis* NCIB 6459 with the molecular weight of 42 kDa was the first one that was purified and characterized (Badet and Walsh 1985). Strych et al. (2007) isolated and characterized the alanine racemase gene from *S. pneumoniae*. They obtained preliminary crystals of *S. pneumoniae* Alr, and intend to

incorporate the enzyme into the structural-based drug design program. Im et al. (2011) solved the structure of *S. pneumoniae* Alr and identified three regions on the enzyme that could be targeted for the structure-based drug design. Qiu et al. (2016) provided the first evidence that D-alanine metabolism is essential for planktonic growth and biofilm formation of *Streptococcus mutans*. It would be possible to take Alr of *S. mutans* as an antibacterial target to screen and optimize the safety and effective specificity of agents. Wei Y. et al. (2016) confirmed that *alr* is an essential factor in maintaining the growth and cell wall integrity of *S. mutans*. A series of *in vivo* and *in vitro* experiments demonstrated that Alr is essential for the cariogenicity of *S. mutans*. Alr might represent a promising drug target to control the prevalence of cariogenic *S. mutans* in a multi-species microbial consortium and be a potential target for the prevention and treatment of caries (Liu et al. 2018). Therefore, Alr is regarded as a drug target for further investigation in the course of development of effective drugs against *S. iniae* and a subject of mutational studies for the growth of mutants with enhanced activity that can be utilized for industrial purpose. D-alanine is also widely used for the production of infusion solutions (Nachbauer et al. 1984), food additive (Awasthy et al. 2012), and in the manufacturing of artificial fibers (Teulé et al. 2009).

Purification and characterization of Alr from both Gram-positive and Gram-negative bacteria is an essential step towards an in-depth understanding of enzyme divers features, design new broad-spectrum antibiotics, and site-directed mutagenesis studies to improve the enzyme catalysis and stability. Hydroquinone and homogentisic acid are promising inhibitors of Alr that are capable of inhibiting the growth of both Gram-positive and Gram-negative bacteria. Future investigation will focus on finding the physiological role of Alr, exploring new novel antimicrobial agents against *S. iniae* and improving their efficacy by designing and analyzing their new derivatives that may have enhanced antimicrobial activity.

#### Acknowledgments

This work was supported by the Natural Science Foundation of Hebei Province (C2019205044); the Outstanding Youth Foundation of Department of Education of Hebei Province (YQ2014026); the Research Fund of Hebei Normal University (L2016Z03); the State Key Laboratory of Pathogen and Biosecurity (Academy of Military Medical Science) (SKLPBS1529); and the Science and technology research project of Hebei Normal University (ZD2018070).

#### Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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