Aii810, a Novel Cold-Adapted N-Acylhomoserine Lactonase Discovered in a Metagenome, Can Strongly Attenuate Pseudomonas aeruginosa Virulence Factors and Biofilm Formation

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The pathogen Pseudomonas aeruginosa uses quorum sensing (QS) to control virulence and biofilm formation. Enzymatic disruption of quorum sensing is a promising anti-infection therapeutic strategy that does not rely on antibiotics. Here, a novel gene (aii810) encoding an N-acylhomoserine lactonase was isolated from the Mao-tofu metagenome for the first time. Aii810 encoded a protein of 269 amino acids and was expressed in Escherichia coli BL21 (DE3) in soluble form. It showed the highest activity at 20°C, and it maintained 76.5% of activity at 0°C and more than 50% activity at 0–40°C. The optimal pH was 8.0. It was stable in both neutral and slightly alkaline conditions and at temperatures below 40°C. The enzyme hydrolyzed several ρ-nitrophenyl esters, but its best substrate was ρ-nitrophenyl acetate. Its $k_{cat}$ and $K_m$ values were 347.7 S⁻¹ and 205.1 µM, respectively. It efficiently degraded N-butyryl-L-homoserine lactone and N-(3-oxododecanoyl)-L-homoserine lactone, exceeding hydrolysis rates of 72.3 and 100%, respectively. Moreover, Aii810 strongly attenuated P. aeruginosa virulence and biofilm formation. This enzyme with high anti-QS activity was the most cold-adapted N-acylhomoserine lactonase reported, which makes it an attractive enzyme for use as a therapeutic agent against P. aeruginosa infection.

Keywords: biofilm, N-acylhomoserine lactonase, quorum sensing, Pseudomonas aeruginosa, cold adaptation

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium that thrives in diverse terrestrial and aquatic environments. It is also an opportunistic pathogen of several lower eukaryotes, plants, and animals (Schuster and Greenberg, 2006; Tettmann et al., 2016). In immunocompromised humans, P. aeruginosa lead to many severe diseases, such as urinary tract infections, cystic fibrosis (CF), conjunctival erythema, soft tissue infections, abscess, catheter-associated infections, corneal infections, and meningitis (Hentzer and Givskov, 2003). Biofilms are surface-associated communities enclosed within an extracellular matrix (O’Toole, 2003), mostly made up of polysaccharides, lipids, nucleic acids, proteins, and other macromolecules and chemicals...
(Wei and Ma, 2013). During infection, *P. aeruginosa* can form biofilms that are recalcitrant to antibiotic therapies and immune clearance. Classical antibiotics used to treat *P. aeruginosa* infections are required at concentrations 100- to 1,000-fold higher than the concentrations used to kill their planktonic counterparts (Hentzer et al., 2003). Moreover, a serious side effect of antibiotic therapy is antibiotic resistance (Flamm et al., 2004), because the treatment is based on compounds that aim to kill or inhibit bacterial growth (Hentzer and Givskov, 2003).

Cell–cell communication, also known as quorum sensing (QS), is a procedure of bacterial communication that collectively controls group behaviors and triggers target cells to alter gene expression (Fuqua et al., 1994; Waters and Bassler, 2005). The procedure relies on the production, release, and group-wide detection of small diffusible signal molecules, which are typically *N*-acyl *L*-homoserine lactones (AHLs) in gram-negative bacteria. AHLs are composed of a homoserine lactone ring linked via a saturated or unsaturated fatty acyl chain, and the chain lengths generally vary from 4 to 18 carbon atoms and in the substitution of a carbonyl at C3-position (Shaw et al., 1997). *P. aeruginosa* possesses two QS systems based on acyl homoserine lactone molecules to orchestrate synchronous production of biofilm formation and virulence factors (de Kievet, 2009). *P. aeruginosa* utilizes this system to regulate many extracellular virulence factors, such as pyocyanin, LasB elastase, LasA protease, and biofilm, which exert its important action in promoting infection (Wagner et al., 2003). AHL synthase LasI synthesizes *N*(3-oxododecanoyl)-*L*-homoserine lactone (OdDHL) (Gambello and Iglewski, 1991), which is a pivotal factor in producing several virulence factors and the maturation and differentiation of biofilm (Davies et al., 1998), as well as in the modulation of inflammatory responses (Kravchenko et al., 2008); RhlI AHL synthase synthesizes *N*-butyryl-*L*-homoserine lactone (BHL) (Ochsner et al., 1994). The observation that AHLs have been found to be one of the QS regulator in *P. aeruginosa* suggests that the enzymatic degradation of QS signal molecules (AHLs), also known as quorum quenching (QQ), has been proved to be most promising and applicable (Kalila and Purohit, 2011), because it is unlikely to pose selective pressure for the development of resistance.

Until now, many AHL-degrading enzymes have been discovered from the metagenome and from several bacteria. On the basis of their catalytic mechanisms, AHL-degrading enzymes could be divided into three families: AHL-lactonases (Dong et al., 2000; Bijtenhoorn et al., 2011b; Fan et al., 2012; Mayer et al., 2015; Morohoshi et al., 2015), AHL-acylases (Lin et al., 2003), and AHL-oxidases (Bijtenhoorn et al., 2011a). AHL-lactonases degrade AHLs by breaking the lactone bond (Dong et al., 2000); AHL-acylases hydrolyze AHLs by degrading the amide linkage and separating the AHL into homoserine lactone and fatty acid (Lin et al., 2003); AHL-oxidases degrade signal molecules by oxidizing the u-1, u-2, and u-3 carbons of the acyl chain of AHL (Chowdhary et al., 2007). Furthermore, some enzymes have shown anti-QS activity against *P. aeruginosa* infection (Bijtenhoorn et al., 2011a; Miigiyama et al., 2013; Tang et al., 2015; Valera et al., 2016). Very few reports are available on lactonases with novel characteristics, such as cold adaptation and high hydrolytic activity, because such enzymes are rare. Cold-adapted enzymes exhibit high catalytic efficiency at moderate and low temperatures and are thus versatile biocatalysts in many applications (Rahman et al., 2016).

Mao-tofu, a renowned traditional food in south-central China, is a type of fermented tofu. The primary fermenting agent is *Mucor* spp., which can degrade the proteins, starches, and lipids of soybean (Zhao and Zheng, 2009). Presumably, several Mao-tofu microbes excrete hydrolytic enzymes. Here, we reported the molecular cloning, identification, and biochemical characterization of one novel cold-adapted *N*-acylhomoserine lactonase derived from the Mao-tofu metagenome. This enzyme showed high activity at moderate and low temperatures. It efficiently degraded BHL and OdDHL, and significantly decreased the production of virulence factors and biofilm in *P. aeruginosa* PAO1.

### MATERIALS AND METHODS

#### Chemicals and Materials

All ρ-nitrophenyl esters, BHL, and OdDHL were purchased from Sigma. T4 DNA ligase, restriction endonuclease, and DNA polymerase were purchased from TaKaRa (Dalian, China) and used according to manufacturer recommendations. E.Z.N.A. Plasmid Mini Kit and E.Z.N.A. Gel Extraction Kit were purchased from OMEGA (Norcross, GA, United States). All other chemicals and reagents were of analytical grade and were purchased from commercial sources, unless otherwise stated.

#### Bacterial Strains and Plasmids

*Escherichia coli* DH5α and *E. coli* BL21 (DE3) (Novagen, Madison, WI, United States) were used as the host for molecular cloning. The pUC118 (TaKaRa) and pET-28a (+) (Novagen) were used to construct metagenomic libraries and express the target protein, respectively.

#### Metagenomic Library Construction and N-Acylhomoserine Lactonase Screening

One metagenomic library was constructed using microbes from Mao-tofu. The total DNA was extracted on the base of the method described before (Zhou et al., 1996; Fan et al., 2012). The purified DNA was incompletely digested with EcoRI. DNA fragments within 3.0–10 kb were ligated into EcoRI-digested pUC118, and the ligation mixtures were transformed into *E. coli* DH5α. The transformed products were plated onto Luria-Bertani (LB) tributyrin agar plates adding 100 µg/mL ampicillin, 0.1 mM isopropyl-β-D-thiogalactoside (IPTG), 0.1% (v/v) tributyrin, and 0.1 mg/ml rhodamine B. After incubation at 37°C overnight, clones with hydrolysis zones were selected. Then positive clones were further tested for the ability to hydrolyze BHL and OdDHL, which was confirmed by high-performance liquid chromatography analysis. One clone with AHL-lactonase activity was screened. The recombinant plasmid (pUC118 No.1) was sequenced on ABI 377 DNA sequencer.
Cloning, Expression, and Purification of N-Acylhomoserine Lactonase
The putative AHL-lactonase gene Aii810 was amplified from the pUC118No.1 plasmid by using the primers. BamHI and HindIII restriction sites were introduced for cloning in the pET-28a (+). The following primers were used: fw (5′-CGCGGATCC ATGTTTTTCAAGTTAACAACCACG-3′; the BamHI cutting site is underlined) and rv (5′-CCCAGCTTCTAGCCCTCGAGG-3′; the HindIII cutting site is underlined). The procedure was carried out according to the method of Fan et al. (2012). After inductive expression with 0.75 mM IPTG at 30°C for 7 h, cells were harvested and disrupted by sonication. The interest protein was purified by a Ni-NTA His•Bind column. The purified protein Aii810 were collected and stored at –20°C.

Determination of Molecular Mass
The molecular mass of the denatured protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 12% SDS-PAGE was prepared by the previous method (Laemmli, 1970). Proteins were stained with Coomassie brilliant blue G-250.

Determination of Substrate Specificity
General substrates of esterases/lipases were used to determine substrate specificity of Aii810 as per the study (Fan et al., 2012), with slight modification. The reaction was performed substrates and incubated at 20°C, with final concentration of 50 mM were as follows: citric acid-Na2HPO4 buffer (pH 5.5–8.0) and barbital sodium-hydrochloric acid buffer (pH 6.8–9.5). Overlapping pH values were used to test that there were no buffer effects on substrate hydrolysis. The pH stability was evaluated after incubation of Aii810 for 12 h at 0°C in the previously discussed solution.

Effect of Temperature and pH on Activity and Stability of Recombinant Aii810
The optimum temperature was determined by testing esterases/lipases activity at pH 6.8 in temperature ranges of 0–70°C as per the study (Fan et al., 2012), with slight modification. Thermostability was evaluated by pre-incubation of the purified Aii810 for intervals in 50 mM of potassium phosphate buffer (pH 6.8) at 0, 10, 20, 35, 40, and 45°C. Then, the residual enzyme activity was tested. The optimum pH of Aii810 was tested using ρ-nitrophenyl acetate (C2) as a substrate at 20°C. The buffers with final concentration of 50 mM were as follows: citric acid-Na2HPO4 buffer (pH 5.5–8.0) and barbital sodium-hydrochloric acid buffer (pH 6.8–9.5). Overlapping pH values were used to test that there were no buffer effects on substrate hydrolysis. The pH stability was evaluated after incubation of Aii810 for 12 h at 0°C in the previously discussed solution.

High-Performance Liquid Chromatography Analysis (HPLC)
Aii810 was measured for degrading of BHL and OdDHL by high-performance liquid chromatography analysis (HPLC). The temperature of this hydrolysis reaction was 20°C. A pH 6.8 potassium phosphate buffer (50 mM) with 5% methanol was used to prepare BHL and OdDHL with the final concentrations of 1 mM. Then, the enzyme samples (31 µg) were added into 1-ml substrates and incubated at 20°C for 30 min. The HPLC method used was described previously (Fan et al., 2012). In each test, the effect of non-enzymatic hydrolysis of BHL and OdDHL was considered and subtracted from the value measured when Aii810 was added.

Effect of Aii810 on the Formation of Virulence and Biofilm in P. aeruginosa PAO1
The method used was described previously, with some modifications (Ishida et al., 1998; Tang et al., 2015; Valera et al., 2016). Briefly, P. aeruginosa PAO1, which was grown overnight in LB, was diluted to 10⁶ CFU/ml in a 12-well cell culture plate with different concentrations of sterile Aii810 (0.05, 0.5, or 5 U/ml). Thereafter, one sterile microscope glass coverslip (18 mm × 18 mm × 0.15 mm) was placed in a 12-well cell culture plate, and the plate was incubated for 24 h at 37°C in static conditions. The cell density of the planktonic cells was spectrophotometrically measured at 600 nm. The supernatants were filtered and then used for virulence assays immediately or stored at –20°C. The coverslips were washed mildly with PBS three times to remove non-adhered bacteria and were used for biofilm quantification.

The extracellular LasA protease activity of PAO1 cultivated with different concentrations of Aii810 (0, 0.05, 0.5, or 5 U/ml)

FIGURE 1 | Arrangement of predicted open reading frames on the original genomic clone pUC118No.1. The scale represents a 1-kb length of nucleotides.
was measured as per the study (Ayora and Gotz, 1994). The amount of digested azocasein was spectrophotometrically measured at 415 nm. The pyocyanin production of PAO1 was measured according to the method of Essar et al. (1990), with slight modifications. A 1-ml sample was extracted with 1.5 ml chloroform and then reextracted into 500 µl of 0.2 N HCl. LasB elastase was tested using Elastin Congo Red (Ohman et al., 1980). PAO1 supernatant (1 ml) was mixed with 1 ml Elastin Congo Red (20 mg/ml, pH 7.5), and then incubated for 18 h at 37°C with shaking. Insoluble Elastin Congo Red was removed by centrifugation, and then the supernatant was measured at 495 nm. Quantitative analysis of alginate was measured by the method of Yasuda et al. (1993).

The inhibition of biofilm formed by P. aeruginosa PAO1 was tested using crystal violet staining assay (Dong et al., 2001). The coverslips were washed thrice with saline to discard the planktonic cells and air dried for 20 min. Subsequently, the wells were stained with 1% crystal violet solution for 20 min. The stained wells were washed thrice with saline and dried for 20 min, and then the stained...
crystal violet was dissolved with 95% ethanol. Ten minutes later, the solubilized crystal violet of each coupon was detected by measuring absorbance of crystal violet at 540 nm. The inhibition of biofilm formation against P. aeruginosa PAO1 was also characterized by scanning electron microscopy (SEM) as described by Grover et al. (2016). The biofilms samples were coated with platinum and analyzed using SEM (Hitachi SU3500, Krefeld, Germany) using the secondary electron emission mode at 1 kV. Images were collected at 5000× magnifications.

The H$_2$O$_2$ sensitivity disk assay was carried out as per the study (Hassett et al., 1999), with some modifications. Briefly, P. aeruginosa PAO1 pre-incubated in LB broth overnight were centrifuged and resuspended in BHI medium to OD$_{600}$ = 1. PAO1 was cultivated at 37°C in BHI medium for 6 h adding Aii810 (0, 0.05, 0.5, and 5 U/ml). Then, 100 µl culture was mixed with 3 ml of LB soft agar at 40°C in 0.6% (wt/vol) agar and poured on LB agar plates. Sterile filter paper disks were placed on the soft solid agar, and then spotted with 8 µl of 30% H$_2$O$_2$. Plates were incubated at 37°C for 8 h, and the diameter of the zone of growth inhibition was measured.

**RESULTS AND DISCUSSION**

**Screening for N-Acylhomoserine Lactonases from a Metagenomic Library**

Metagenomic strategy has been developed and successfully applied to exploit novel enzymes (Handelsman, 2004). In this study, the total DNA was extracted from Mao-tofu microbes. A metagenomic library containing approximately 13,200 transformants was constructed to screen AHL-lactonase genes. Restriction digestion analysis of recombinant plasmids from 20 randomly picking clones demonstrated that the average size of insert fragments was 4.0 kb, and sizes ranged from 2.5 to 7.5 kb. Out of about 13,200 transformants, several transformants displayed hydrolysis zones on tributyrin plates, which was based on esterase and lipase activity. One E. coli DH5α clone harboring the pUC118No.1 showed AHL-degrading activity.

**Genetic Characterization**

The complete insert DNA sequence of pUC118No.1 was sequenced. Sequence analysis showed that the 6,522-bp nucleic acid fragment consist of four significant open reading frames (ORFs) (Figure 1). The third ORF (ORF3, aii810) is speculated to encode an enzyme of 269 amino acids, which was related to members of the alpha/beta-hydrolase fold family, with a predicted molecular mass (Mr) of 29.6 kDa. The pI is predicated to be 6.86 (Gasteiger et al., 2005). To measure if ORF3 encodes an N-acylhomoserine lactonase, ORF3 was amplified by polymerase chain reaction and ligated into the pET28a. E. coli BL21 harboring ORF3-pET28a displayed AHL-degrading activity. Therefore, ORF3 has been named aii810 (auto-inducer inactivation gene of 810 bp length). This study is the first report on an AHL-degrading gene derived from Mao-tofu.
microbes, which provided new potential enzyme resources for AHL-degrading genes. The deduced amino acid sequence of Aii810 was used to perform a BLAST program provided by the NCBI and Swiss-Prot databases. There was moderate identity between Aii810 and other hydrolases. The highest identity were the alpha/beta hydrolase fold family protein from Comamonas testosteroni (226/267, 85% identity). Multiple sequence alignment of Aii810, putative homologs, and other known AHL-lactonases revealed the typical catalytic triad of active site serine (S97) motif G-X-S-X-G (Figure 2), conserved aspartic acid (D218), and histidine (H249) residue motif in the encoded protein (Piper et al., 1993), similar to most lipase/esterases (Fan et al., 2012). This DNA sequence has been submitted to GenBank under accession number (KY783477).

**Heterologous Expression and Purification of Recombinant Aii810**

Aii810 was expressed in *E. coli* BL21 (DE3) and purified. SDS-PAGE analysis demonstrated that the purified enzyme migrates as a single band with a molecular mass a little higher than 35 kDa (Figure 3), in accordance with its predicted molecular mass of 35.2 kDa, containing the 269 amino acids and a fusion of 51 amino acids corresponding to polyhistidine tag (His-tag), a unique thrombin cleavage site (Thrombin). The expression quantity was about 0.21 mg/mL (OD$_{600}$ = 3.6). The content of recombinant Aii810 reached up to 50% of the total soluble protein quantified by Quantity One software (Bio-Rad laboratories Inc., Hercules, CA, United States).
Substrate Specificity and Activity of Aii810
Aii810 belongs to the alpha/beta hydrolase. In our research, it revealed a typical catalytic triad of active site serine (S97) motif G-X-S-X-G. For convenience, general substrates of esterases and lipases were chosen to measure substrate specificity of this enzyme. The activity on several \( \rho \)-nitrophenyl esters was tested at \( 20^\circ C \) and pH 6.5. Aii810 displayed the highest activity toward \( \rho \)-nitrophenyl acetate among all tested substrates, and showed lower activity toward with longer acyl esters (Figure 4). We calculated the \( k_m \) and \( k_{cat} \) values by fitting the data to the Michaelis–Menten equation. Using the optimal substrate, the \( k_{cat} \) and \( k_m \) values of Aii810 were 347.7 S\(^{-1}\) and 205.1 \( \mu \)M, respectively. Low \( k_m \) value demonstrated that Aii810 shows positive affinity toward the substrate. High activity is a very attractive property of enzymes for practical applications.

Effect of pH and Temperature on Recombinant Aii810 Activity and Stability
To measure the optimum temperature of Aii810, the catalytic activity was estimated at different temperatures ranging from 0 to 70\(^\circ C \) with \( \rho \)-nitrophenyl acetate as a substrate. Aii810 showed the highest activity at 20\(^\circ C \) (Figure 5A), and retained 76.5% of the maximum activity at 0\(^\circ C \) and more than 90% at 5\(^\circ C \). This remarkable activity at cold temperatures indicates that this enzyme could be a cold-adapted protein. Moreover, it maintained more than 50% of its maximal activity at 0–40\(^\circ C \), indicating that Aii810 possessed good adaptability at moderate and low temperatures. Fermentation of Mao-tofu is generally carried out at 20–30\(^\circ C \) for 5–6 days (Wu et al., 2015). This demonstrated that Aii810 is endowed with a habitat-specific characteristic (i.e., cold adaptation), because ecological conditions have been found to modulate enzyme characteristics in most cases (Martínez-Martínez et al., 2013).

Thermostability is an important factor to consider in enzyme applications. To analyze thermostability, the proteins were pre-treated at different temperatures, and then the residual activity toward \( \rho \)-nitrophenyl acetate was recorded. Incubation at 0–40\(^\circ C \) for 12 h reduced the Aii810 activity slightly, but pre-treatment at 45\(^\circ C \) for 12 h decreased the activity drastically (Figure 5B), suggesting that Aii810 was stable at temperatures below 40\(^\circ C \). Only a few cold-adapted microbial enzymes have shown catalytic activity at such low temperatures. Aii810 has the same optimal temperature as with \( Lp \)\_2631 from *Lactobacillus plantarum* (Esteban-Torres et al., 2014), slightly lower than other cold-adapted esterases and lipases (Yu et al., 2011; Mander et al., 2012). In the light of cold activity, Aii810 displayed better performance (76.5% at 0\(^\circ C \)) than other reported cold-active esterases, such as EstLiu from *Zunongwangia profunda* which showed 75% activity at 0\(^\circ C \) (Rahman et al., 2016), est10 from *Psychrobacter pacificensis* which showed 55% activity at 0\(^\circ C \) (Wu et al., 2013a), and Est12 from *Psychrobacter celer* 3Pb1 which showed 41% activity at 0\(^\circ C \) (Wu et al., 2013b). Another cold-active esterase, EstPc from *Psychrobacter cryohalolentis* K5T, showed 80% of activity at 0\(^\circ C \) (Novototskaya-Vlasova et al., 2012). Aii810 showed stronger cold adaptability than any other AHL-degrading enzymes reported previously, such as Est816 (Fan et al., 2012), AiiAAI96 (Cao et al., 2012), and AiiAB546 (Chen et al., 2010), which were most active at 30–60\(^\circ C \). A thermostable \( N \)-acylhomoserine lactonase AiiT showed maximal activity at higher temperatures ranging from 60 to 80\(^\circ C \) (Morohoshi et al., 2015). However, temperature properties of other \( N \)-acylhomoserine lactonases were not quantified (Dong et al., 2006; Cao et al., 2012; Mayer et al., 2015; Tang et al., 2015; Valera et al., 2016). This enzyme Aii810 was the first cold-adapted \( N \)-acylhomoserine lactonase, more suitable for application at moderate and low temperatures.

The optimum pH of Aii810 was 8.0. It exhibited poor activity in basic and acidic conditions (Figure 5C). Regarding pH stability, it was observed that Aii810 retained more than 70% activity in the pH range 7.0–9.5 but the activity abruptly fell after pH 6.5 (Figure 5C). Thus, the pH has an obvious influence on Aii810 activity, which is stable in both neutral and alkaline conditions.

High-Performance Liquid Chromatography Analysis of AHLs Degradation
The AHL-degrading activity of Aii810 was tested using BHL and OdDHL. BHL and OdDHL were chosen as substrates because *P. aeruginosa* uses them as signal molecules to regulate various processes.
FIGURE 8 | Scanning electron microscopy (SEM) images of biofilm formation by *P. aeruginosa* PAO1 on control glass coverslips without Aii810 at 1 days (A) and 2 days (C), and glass coverslips with Aii810 at 1 days (B) and 2 days (D). Images were collected at 5000 × magnifications.

FIGURE 9 | Zone of inhibition by H$_2$O$_2$ of *P. aeruginosa* PAO1 treated without (control) and with Aii810 at the concentration of 0.5 U/ml.

QS-mediated phenotypes (Pearson et al., 1994). In the natural environment, QS usually happens at 0–40°C. The temperature of this hydrolysis reaction was chose at 20°C. As known to all, AHLs were automatically degraded under alkaline conditions. Considering optimal reaction conditions and the practical application, pH 6.8 potassium phosphate buffer (50 mM) was used to prepare substrates. The degradation efficiency was tested for BHL and OdDHL by HPLC (Figure 6). The hydrolysis rates of BHL and OdDHL were 72.3 and 100%, respectively, similar to Est816 and its mutants (Fan et al., 2012; Liu et al., 2016), but distinct from AhlM. AhlM preferred to AHLs with long acyl-chains than short acyl-chains, but it did not degrade BHL (Park et al., 2005). These results suggest that Aii810 has high activity toward these two AHLs and possesses good adaptability at moderate and low temperatures, which may be useful in targeting the QS systems in *P. aeruginosa*, but these findings needs further exploration.

Effect of Aii810 on the Production of Virulence and Biofilm in *P. aeruginosa* PAO1

To further explore the anti-QS potential of Aii810, the effects of Aii810 on the production of virulence factors and biofilm in *P. aeruginosa* PAO1 were evaluated in vitro. Figure 7 shows the results. The optimum concentration of Aii810 was 0.5 U/ml. At this concentration, the extracellular protease activity of *P. aeruginosa* PAO1 was reduced by up to 73.4% (Figure 7A), outperforming MomL, which decreased 50% extracellular protease activity (Tang et al., 2015). Similarly, adding Aii810 reduced pyocyanin production by *P. aeruginosa* PAO1 by 65.6% (Figure 7B). We further demonstrated that the enzyme had a significant effect on alginate production, exceeding an inhibition ratio of about 70.7% (Figure 7C).

The inhibition of biofilm formed by *P. aeruginosa* PAO1 was also tested. The growth of planktonic bacteria was not affected obviously (Figure 7D), whereas biofilm production was significantly reduced by 78.4% (Figure 7E), which demonstrated that Aii810 inhibited biofilm without affecting the growth of the bacteria, and unlikely put selective pressure for the development
of resistance. The result was better than the antibiofilm activity (70%) of the enzyme from endophytic Enterobacter aerogenes VT66 (Rajesh and Rai, 2015). The inhibition of biofilm formation against P. aeruginosa PA01 was also characterized by SEM (Figure 8A). On the first day, there were regions consisting of a dense network of bacteria on control coverslip (Figure 8A). Conversely, in the case of glass coverslip with 0.5 U/ml Aii810, only a few bacteria were observed (Figure 8B). On the second day, there was still strong inhibition on biofilm formation (Figures 8C,D). Antibiofilm activity of AHL-lactonase from SEM photograph (Figure 8) was in accordance with results of biofilm production used to determine inhibition of biomass (Figure 7E). Furthermore, electron micrographs of structured colonies showed that the control coverslip was composed of cells surrounded by extracellular matrix (ÓToole, 2003), which was similar to those results observed previously in the biofilms (Branda et al., 2005; Grover et al., 2016).

Quorum sensing signaling in P. aeruginosa based on AHLs activates synchronous production of biofilm formation and virulence factors. It also significantly regulated biofilm resistance to H2O2. Biofilms with less catalase activity would be more resistant to hydrogen peroxide treatment than planktonic bacteria (Hassett et al., 1999). In our result (Figure 7F), when exposed to H2O2 on solid agar, P. aeruginosa PA01 treated with Aii810 were much more susceptible than untreated controls. When the concentration of Aii810 was 0.5 U/ml, the zone of inhibition was the biggest one, as shown in Figure 9.

For most reported AHL-lactonases, effects on the production of many virulence factors and biofilm in P. aeruginosa have not been quantified (Dong et al., 2000; Park et al., 2003; Mei et al., 2010; Wang et al., 2012; Migiyama et al., 2013; Mayer et al., 2015). Excitingly, we identified a novel AHL-lactonase with cold adaptation and strong inhibition on P. aeruginosa virulence and biofilm. These features make it a potential candidate to disrupt QS in P. aeruginosa. Therefore, the inhibition of QS systems might be a more valuable approach than targeting a single particular virulence factor for therapeutic or prophylactic control of infections. The enzyme is of great value for the continued search for a mammalian model.

CONCLUSION

Quorum sensing seems to play a key role in the expression of virulence and the interaction with host protection, enzymatic disruption of QS pathways has been suggested to be important components of future anti-pseudomonal therapies (Hentzer and Givskov, 2003). Here, our work is the first report on a novel AHL-lactonase with cold adaptation and high hydrolytic activity from Mao-tofu metagenome. We further demonstrated that the enzyme drastically attenuated P. aeruginosa virulence and biofilm. These features make Aii810 a potential candidate for use as a therapeutic agent against P. aeruginosa infection. Whether this enzyme has an influence on P. aeruginosa virulence in tissue culture and animal models; these studies are currently in progress.

AUTHOR CONTRIBUTIONS

XF have performed gene cloning, enzyme characterization, and revised the manuscript. LW have performed anti-QS experiments. ML have screened metagenomic library. RC and HL have performed construction of metagenomic library. XL have conceived the study, supervised the experiments, and written the manuscript. All authors have read and approved the manuscript.

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REFERENCES

Ayora, S., and Gotz, F. (1994). Genetic and biochemical properties of an extracellular neutral metalloprotease from Staphyloccocus hyicus subsp. hyicus Mol. Gen. Genet. 242, 421–430. doi: 10.1007/BF00281792

Bijtenhoorn, P., Mayerhofer, H., Müller-Dieckmann, J., Upatel, C., Schipper, C., Hornung, C., et al. (2011a). A novel metagenomic short-chain dehydrogenase/reductase attenuates Pseudomonas aeruginosa biofilm formation and virulence on Caenorhabditis elegans. PLOS ONE 6:e26278. doi: 10.1371/journal.pone.0026278

Bijtenhoorn, P., Schipper, C., Hornung, C., Quischau, M., Grond, S., Weiland, N., et al. (2011b). BpiI805, a novel metagenome-derived hydrolyase acting on N-acylamino acid homoserine lactones. J. Biotechnol. 155, 86–94. doi: 10.1016/j.jbiotec.2010.12.016

Branda, S. S., Vik, A., Friedman, L., and Kolter, R. (2005). Biofilms: the matrix revisited. Trends Microbiol. 13, 20–26. doi: 10.1016/j.tim.2004.11.006

Cao, Y. N., He, S. X., Zhou, Z. G., and Yao, B. (2012). Orally administered thermostable N-acylamino acid homoserine lactonase from Bacillus sp. strain A96 attenuates Aeromonas hydrophila infection in zebrafish. Appl. Environ. Microbiol. 78, 1899–1908. doi: 10.1128/AEM.06139-11

Chen, R., Zhou, Z., Cao, Y., Bai, Y., and Yao, B. (2010). High yield expression of an AHL-lactonase from Bacillus sp. B546 in Pichia pastoris and its application to reduce Aeromonas hydrophila mortality in aquaculture. Microb. Cell Fact. 9:39. doi: 10.1186/1475-2859-9-39

Chowdhary, P. K., Keshavan, N., Nguyen, H. Q., Peterson, J. A., Gonzalez, J. E., and Haines, D. C. (2007). Bacillus megaterium CYP102A1 oxidation of acyl homoserine lactones and acyl homoserine. Biochemistry 46, 14429–14437. doi: 10.1021/bi701945j

Davies, D. G., Parsek, M. R., Pearson, J. P., Igleswki, B. H., Costerton, J. W., and Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280, 295–298. doi: 10.1126/science.280.3631.295

de Kievit, T. R. (2009). Quorum sensing in Pseudomonas aeruginosa biofilms. Environ. Microbiol. 11, 279–288. doi: 10.1111/j.1462-2920.2008.01792.x

Dong, Y. H., Wang, L. H., Xu, J. L., Zhang, H. B., Zhang, X. F., and Zhang, L. H. (2001). Quenching quorum-sensing dependent bacterial infection by an N-acyl homoserine lactonase. Nature 411, 813–817. doi: 10.1038/35081101
Dong, Y. H., Xu, J. L., Li, X., and Zhang, L. H. (2000). AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of Erwinia carotovora. Proc. Natl. Acad. Sci. U.S.A. 97, 3526–3531. doi: 10.1073/pnas.97.7.3526

Essar, D. W., Eberly, L., Hadero, A., and Crawford, J. P. (1990). Identification and characterization of genes for a second anthranilate synthase in Pseudomonas aeruginosa: interchangeability of the two anthranilate synthases and evolutionary implications. J. Bacteriol. 172, 884–900. doi: 10.1128/JB.172.2.884-900.1990

Esteban-Torres, M., Mancheño, J. M., de las Rivas, B., and Muñoz, R. (2014). Gambello, M. J., and Iglewski, B. H. (1991). Cloning and characterization of Grover, N., Plaks, J. G., Summers, S. R., Chado, G. R., Schurr, M. J., and Kaar, J. L. (1997). Ochsner, U. A., Koch, A. K., Fiechter, A., and Reiser, J. (1994). Isolation and characterization of a cold-adapted esterase produced by Lactobacillus plantarum sp. 20J. Cloning, purification, and characterization of a cold-adapted esterase produced by Psychrobacter cryohalolentis KST from Siberian cryopeg. FEMS Microbiol. Ecol. 82, 367–375. doi: 10.1111/j.1574-6941.2012.01385.x

Fan et al. Aii810 for Quorum Quenching

Mei, G. Y., Yan, X. X., Turak, A., Luo, Z. Q., and Zhang, L. Q. (2010). AidH, an alpha/beta-hydrolase fold family member from an Ochrobactrum sp. strain, is a novel N-acylhomoserine lactone lactonase. Appl. Environ. Microbiol. 76, 4933–4942. doi: 10.1128/AEM.00477-10

Migiyama, Y., Kaneko, Y., Yanagihara, K., Morohoshi, T., Morinaga, Y., Nakamura, S., et al. (2013). El Fimicb-08-01950 October 6, 2017 Time: 15:3 # 10 in a mouse model of acute pneumonia. Antimicrob. Agents Chemother. 57, 3653–3658. doi: 10.1128/AAC.00456-13

Morohoshi, T., Tominaga, Y., Someya, N., and Ikeda, T. (2015). Characterization of a novel thermostable N-acylhomoserine lactone from the thermophilic bacterium Thermoanaerobacter marianicus. J. Biosci. Bioeng. 120, 1–5. doi: 10.1016/j.jbiosc.2014.11.014

Novototskaya-Vlasova, K., Petrovskaya, L., Yakinis, S., and Gilchinsky, D. (2012). Cloning, purification, and characterization of a cold-adapted esterase produced by Psychrobacter cryohalolentis KST from Siberian cryopeg. FEMS Microbiol. Ecol. 82, 367–375. doi: 10.1111/j.1574-6941.2012.01385.x

Ochsner, U. A., Koch, A. K., Fiechter, A., and Reiser, J. (1994). Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in Pseudomonas aeruginosa. J. Bacteriol. 176, 2044–2054. doi: 10.1128/ JB.176.7.2044-2054.1994

Ohman, D. E., Crzy, S. J., and Iglewski, B. H. (1980). Isolation and characterization of Pseudomonas aeruginosa PA01 mutant that produces altered elastase. J. Bacteriol. 142, 836–842.

O’Toole, G. A. (2003). To build a biofilm. J. Bacteriol. 185, 2687–2689. doi: 10.1128/ JB.185.9.2687-2689.2003

Park, S. Y., Kang, H. O., Jang, H. S., Lee, J. K., Koo, B. T., and Yum, D. Y. (2005). Identification of extracellular N-acylhomoserine lactone acylase from a Streptomyces sp. and its application to quorum quenching. Appl. Environ. Microbiol. 71, 2632–2641. doi: 10.1128/AEM.71.5.2632-2641.2005

Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Rinehart, K. L., et al. (1997). Detection and characterizing N-acylhomoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. U.S.A. 94, 6036–6041. doi: 10.1073/pnas.94.12.6036

Mander, P., Cho, S. S., Simkhada, J. R., Choi, Y. H., Park, D. J., Ha, J. W., et al. (2012). An organic solvent-tolerant alkaline lipase from Streptomyces sp. CS268 and its application in biodiesel production. Biotechnol. Bioprocess. Eng. 17, 67–75. doi: 10.1016/j.procbio.2012.01.003

Martínez-Martínez, M., Alcaide, M., Tchigvintsev, A., Reva, O., Polaina, J., Bargiela, R., et al. (2013). Biochemical diversity of carboxyl esterases and lipases from Lake Arreo (Spain): a metagenomic approach. Appl. Environ. Microbiol. 79, 3553–3562. doi: 10.1128/AEM.00240-13

Mayer, C., Romero, M., Muras, A., and Otero, A. (2015). Aid20, a wide-spectrum thermostable N-acylhomoserine lactone from the marine bacterium Tenacibaculum sp. 20J, can quench AHL-mediated acid resistance in Escherichia coli. Appl. Microbiol. Biotechnol. 99, 9523–9539. doi: 10.1007/s00253-015-6741-8
Fan et al. Aii810 for Quorum Quenching

Tang, K., Su, Y., Brackman, G., Cui, F., Zhang, Y., Shi, X., et al. (2015). MomL, a novel marine-derived N-acyl homoserine lactonase from Maricatada olearia. Appl. Environ. Microbiol. 81, 774–782. doi: 10.1128/AEM.02805-14

Tettmann, B., Niewerth, C., Kirschhöfer, F., Neidig, A., Dötsch, A., Brenner-Weiss, G., et al. (2016). Enzyme-mediated quenching of the Pseudomonas quinolone signal (PQS) promotes biofilm formation of Pseudomonas aeruginosa by increasing iron availability. Front. Microbiol. 7:1978. doi: 10.3389/fmicb.2016.01978

Valera, M. J., Mas, A., Streit, W. R., and Mateo, E. (2016). GqqA, a novel protein in Komagataeibacter europaeus involved in bacterial quorum quenching and cellulose formation. Microb. Cell Fact. 15, 88. doi: 10.1186/s12934-016-0482-y

Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I., and Iglewski, B. H. (2003). Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol. 185, 2080–2095. doi: 10.1128/JB.185.7.2080-2095.2003

Wang, W. Z., Morohoshi, T., Someya, N., and Ikeda, T. (2012). AidC, a novel N-acylhomoserine lactonase from the potato root-associated Cytophaga-Flavobacteria-Bacteroides (CFB) group bacterium Chryseobacterium sp. strain StRB126. Appl. Environ. Microbiol. 78, 7985–7992. doi: 10.1128/AEM.02188-12

Waters, C. M., and Bassler, B. L. (2005). Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol. 21, 319–346. doi: 10.1146/annurev.cellbio.21.012704.131001

Wei, Q., and Ma, L. Z. (2013). Biofilm matrix and its regulation in Pseudomonas aeruginosa. Int. J. Mol. Sci. 14, 20983–21005. doi: 10.3390/ijms141020983

Wu, G., Wu, G., Zhan, T., Shao, Z., and Liu, Z. (2013a). Characterization of a cold-adapted and salt-tolerant esterase from a psychrotrophic bacterium Psychrobacter pacificensis. Extremophiles 17, 809–819. doi: 10.1007/s00792-013-0562-4

Wu, G., Zhang, S., Zhang, H., Zhang, S., and Liu, Z. (2013b). A novel esterase from a psychrotrophic bacterium Psychrobacter celer 3Pb1 showed cold-adaptation and salt-tolerance. J. Mol. Catal. B Enzym. 98, 119–126. doi: 10.1016/j.molcatb.2013.10.012

Yu, E. Y., Kwon, M. A., Lee, M., Oh, J. Y., Choi, J. E., Lee, J. Y., et al. (2011). Isolation and characterization of cold-active family VIII esterases from an arctic soil metagenome. Appl. Microbiol. Biotechnol. 90, 573–581. doi: 10.1007/s00253-011-3132-7

Zhou, J., Bruns, M. A., and Tiedje, J. M. (1996). DNA recovery from soils of diverse composition. J. Food. Sci. Technol. 52, 7353–7360. doi: 10.1007/s13197-015-1848-6

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