PfmA, a novel quorum-quenching N-acylhomoserine lactone acylase from Pseudoalteromonas flavipulchra

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Abstract
Many bacteria, such as Proteobacteria, Cyanobacteria and Bacteroidetes, use N-acylhomoserine lactones (AHLs) as quorum-sensing (QS) signal molecules for communication. Enzymatic degradation of AHLs, such as AHL acylase and AHL lactonase, can degrade AHLs (quorum quenching, QQ) to attenuate or disarm the virulence of pathogens. QQ is confirmed to be common in marine bacterial communities. Many genes encoding AHL acylases are found in marine bacteria and metagenomic collections, but only a few of these have been characterized in detail. We have reported that the marine bacterium Pseudoalteromonas flavipulchra JG1 can degrade AHLs. In the present study, a novel AHL acylase PfmA, which can degrade AHLs with acyl chains longer than 10 carbons, was identified from strain JG1. Ultra-performance liquid chromatography (UPLC) and electrospray ionization mass spectrometry (ESI-MS) analysis demonstrated that PfmA functions as an AHL acylase, which hydrolysed the amide bond of AHL. The purified PfmA of P. flavipulchra JG1 showed optimum activity at 30°C and pH 7.0. PfmA belongs to the N-terminal nucleophile (Ntn) hydrolase superfamily and showed homology to a member of penicillin amidases, but PfmA can degrade ampicillin but not penicillin G. The residue Ser256 in PfmA is the active site according to site-directed mutagenesis. Furthermore, PfmA reduced AHL accumulation and the production of virulence factors in Vibrio anguillarum VIB72 and Pseudomonas aeruginosa PAO1, and attenuated the virulence of P. aeruginosa to increase Artemia survival, which suggested that PfmA can be considered as a therapeutic agent to control AHL-mediated pathogenicity.

INTRODUCTION
Quorum sensing (QS) is a cell-to-cell communication mechanism dependent on cell density, which allows bacterial populations to regulate the expression of genes responsible for a wide range of biological behaviours, such as the production of pigments, biofilm formation, antibiotic production, motility, adhesion, bioluminescence and secretion of virulence factors [1, 2]. In the QS process, bacteria rely on self-produced auto-inducers (AIs) as signal molecules [1], and N-acylhomoserine lactones (AHLs) are the most common signal molecules produced by Gram-negative bacteria [3]. Each AHL molecule is composed of an identical homoserine lactone ring and a transformable acyl chain, which varies in the number of carbons, saturation or side chain substitution [4]. These variations in the acyl chain make the signal molecule specific to certain bacteria [3]. Interference with QS, a process known as quorum quenching (QQ), may be a means already adopted by many organisms to counter potentially pathogenic or competitor bacteria [5, 6]. The most common approach to QQ is enzymatic degradation of the signal molecules to block signal transmission, which can help bacteria inhibit the expression of virulence and infection of host cells [7].

QQ enzymes are found in bacteria, archaea, fungi, plant cells, mammalian cells and soil metagenomic libraries [7, 8]. Two main groups of AHL-degrading enzymes, AHL lactonases and AHL acylases, have been well characterized. AHL lactonases hydrolyse lactones of AHLs, and AHL acylases hydrolyse the amide bond of AHLs [9]. Another unusual QQ enzyme, AHL oxoreductases, reduce the AHL carbonyl to hydroxyl [10]. It was reported that the frequency of QQ genes in marine metagenomes is high, and AHL acylase may have a wider distribution in the marine environments [11]. AHL acylase has an advantage over AHL lactonase in regard to bacterial...
growth, because the homoserine lactone (HSL) and fatty acyl moiety, which are the AHL degradation products of AHL acylase, can serve as sources of carbon, nitrogen and energy [12], while acyl homoserine, which is the product of AHL lactonase, exerts an inhibitory effect on bacterial growth to a certain extent [13]. In addition, the substrates of AHL acylases showed higher specificity than AHL lactonases and may affect the QS activity of various bacteria.

To date, several AHL acylases has been characterized in detail [13–21], all belonging to the N-terminal nucleophile (Ntn) hydrolase superfamily. AHL acylases showed a preference for long-chain AHLS, and the substituent at the 3’ position of the side chain did not affect their activity [22]. For example, AiiD from Ralstonia strain XJ12B only degraded AHLS with an acyl side chain at least eight carbons long [14], while AiiC from Anabaena sp. PCC7120 degraded a wide range of AHLS including short-chain AHLS such as C₆-HSL and C₈-HSL, but degraded long-chain AHLS more efficiently [17]. The best-studied AHL acylase, PvdQ, whose crystal structure was initially determined and reported, has a typical α/β heterodimeric Ntn hydrolase fold as well as a large hydrophobic binding pocket that can accommodate 3-Oxo-C₁₂-HSL. [23]. The SerB1 in PvdQ was identified as the nucleophile and the active site, which is essential for enzymatic degradation. Furthermore, AHL acylases were effective in quenching pathogen QS, and reducing their production of virulence factors. Expression of the aiiD from Ralstonia strain XJ12B decreased the ability of Pseudomonas aeruginosa PAO1 to swarm, to produce elastase and pyocyanin, and protected nematodes from infection by PAO1 [14]. It was also reported that the production of virulence factors in P. aeruginosa PAO1 was decreased by addition of the purified AhlM to the bacterial growth medium [16].

In previous studies, we demonstrated that Pseudoalteromonas flavipulchra JG1 can degrade long-chain AHLS (longer than 10 carbons in the acyl chain) and that the degradation process could not be reversed by acidification [24]. In the present study, we report the expression and characterization of a novel AHL acylase gene, pfmA, from P. flavipulchra. Also, the effects of the novel AHL acylase on the production of virulence factors in Vibrio anguillarum VIB72 and P. aeruginosa was investigated.

**METHODS**

**Bacterial strains, growth conditions, plasmids and chemicals used**

The bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. *P. flavipulchra* JG1 was grown on marine agar 2216 (MA; Difco) at 28 °C. P. aeruginosa PAO1 and *Escherichia coli* strain BL21 (DE3) were cultured at 37 °C in Luria–Bertani (LB) medium. Agrobacterium tumefaciens A136 (pCF218) (pCF372) [25] was used to detect AHLS (C₆-C₁₄) and was grown in AT minimal medium [26]. Chromobacterium violaceum CV026 [27] and VIR24 [28], which respond to short-chain (C₄, C₆) and long-chain (C₈, C₁₄) AHLS, respectively, were cultured in LB medium at 28 °C. Antibiotics were added to maintain the plasmids, at final concentrations of kanamycin 50 µg ml⁻¹, spectinomycin 50 µg ml⁻¹ and tetracycline 4.5 µg ml⁻¹. *Artemia* was incubated in glass bottles with shaking at 170 r.p.m. at 28 °C under light conditions. The AHLS used in this study, C₆-HSL, 3-Oxo-C₆-HSL and C₈-HSL, were purchased from Cayman Chemical Company (Ann Arbor, MI); 3-Oxo-C₈-HSL, C₁₀-HSL, 3-Oxo-C₁₀-HSL, C₁₂-HSL, 3-Oxo-C₁₂-HSL, C₁₄-HSL and 3-Oxo-C₁₄-HSL were purchased from Sigma-Aldrich (St. Louis, MO).

**Characterization of the AHL degradative activity of *P. flavipulchra* JG1**

The AHL degradative activities of JG1 were determined by A136 liquid X-Gal assay, which uses short-chain (C₆-HSL) and long-chain (C₁₂-HSL) AHLS as substrates, according to the method of Tang *et al.* [29]. Briefly, the entire culture of JG1 was centrifuged and divided into cells and supernatant parts. In addition, the degradative products were acidified to determine whether they could be reversed at lower pH [30]. Marine broth 2216 (MB) medium and AHL lactonase MomL were used as the negative and positive controls, respectively. The results were also confirmed by either CV026 or VIR24 plate assay [27, 28].

**Sequence analysis of putative AHL degradative protein of *P. flavipulchra* JG1**

The annotation of gene FaGL1422 of JG1 was performed with a BLASTP search against protein databases SwissProt (http://www.uniprot.org/) and NR (NCBI non-redundant database; http://www.ncbi.nlm.nih.gov/RefSeq/). The N-terminal signal peptide was predicted using SignalP server [31]. Multiple amino acid sequence alignment of PfmA with other AHL acylases and putative homologues was performed using ClustalW program in the MEGA 5.1 [32] software package, and enhanced by ESPript 3.0 [33]. A phylogenetic tree of AHL acylases based on amino acid sequences was constructed using the neighbour-joining method within the MEGA package. The branching pattern was analysed using 1000 bootstrap replicates.

**Cloning of the pfmA gene, expression in *E. coli* and purification of PfmA protein**

Genomic DNA was extracted from the cell masses of *P. flavipulchra* JG1 by standard methods [24]. The putative gene was amplified from genomic DNA by PCR using PrimeSTAR HS DNA polymerase (Takara) and the primer pairs AHL1422F-1, AHL1422R-1 and AHL1422F-2, AHL1422R-1 (Table 1). PCR was performed using the following cycling parameters: 95 °C for 30 s, 55 °C for 15 s and 72 °C for 150 s for 30 cycles. The PCR products were digested with *Nde*I and *Xho*I restriction enzymes and inserted into the corresponding sites of pET26b (+) plasmid for construction of pET26b (+)-pfmA. To express and purify the PfmA protein, the cultures of *E. coli* BL21 (DE3) strains harbouring pET26b (+)-pfmA were induced in LB medium containing...
kanamycin by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) until the OD_{600} reached 0.4–0.6, followed by cultivation at 16°C for 24 h. Cells were collected by centrifugation, resuspended in Tris-HCl buffer (pH 7.0), the suspension was sonicated, the cell-free extract was collected by centrifugation at 13,000 g for 30 min at 4°C, and loaded onto NTA-Ni (Qiagen). Proteins were purified according to the manufacturer’s recommendations. Expression and purification of the PfmA were assessed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The AHL degradative activities of the expressed proteins were detected using the VIR24 plate assay.

### UPLC and ESI-MS analysis

AHL degradation products of purified AHL degrading enzyme PfmA were analysed using ultra-performance liquid chromatography (UPLC) and electrospray ionization mass spectrometry (ESI-MS). Briefly, 100 µl deionized PfmA was mixed with 10 µM C12-HSL to yield a final concentration of 1 mg ml^{-1}. The reaction mixture was cultured at 28°C for 12 h and centrifugated at 10,000 g for 30 min using ultra-filtration centrifuge tubes (0.5 ml, 3 kDa) at 4°C. The samples were analysed by UPLC. The separated fractions were further analysed by ESI-MS.

### Characterization of PfmA enzymatic activity

The amount of violacein produced by C. violaceum is directly proportional to the amount of AHL remaining in the reaction mixture after enzymatic degradation. AHL degrading activity was determined by measuring residual AHL concentration using VIR24 plate assay [20]. The optimal temperature was determined by incubating the enzyme with 10 µM C12-HSL in PIPES buffer (1 M, pH 6.7) at various temperatures ranging from 20 to 70°C (10-degree intervals) for 1 h. The thermostability of PfmA was determined after the purified PfmA was preincubated at different temperatures for 2 h, then the residual activity was measured.

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**Table 1. Strains, plasmids and primers used in this study**

<table>
<thead>
<tr>
<th>Strain, plasmid or primer</th>
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<td>pCF218, pCF372</td>
<td>[25]</td>
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<tr>
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<td>[27, 28]</td>
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<td>[39]</td>
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*Restriction enzyme sites in primers are underlined, and the mutagenic nucleotides are represented in lowercase.*
based on VIR24 plate assay. To determine the effect of pH on enzyme activity, PfmA was incubated at 30°C for 1 h using different buffers: 50 mM Na-acetate buffer (pH 4.0, 5.0), 50 mM phosphate buffer (pH 6.0, 7.0) and 50 mM Tris-HCl buffer (pH 8.0, 9.0). Furthermore, to determine the effect of metal ions on PfmA, the residual activity was evaluated after the purified PfmA protein was preincubated with 1 mM concentrations of different metal ions (Na²⁺, K⁺, Ca²⁺, Co²⁺, Ni²⁺, Fe³⁺, Mn²⁺, Zn²⁺ and Cu²⁺) or with 1 mM urea and SDS. The residual AHLs were detected using the AHL biosensor VIR24.

Site-directed mutagenesis of pfmA

Site-directed mutagenesis of pfmA was carried out following the method below. Briefly, the plasmid pET26b (+)-pfmA was used as a template. Primers used to obtain each mutant are listed in Table 1. The linear reconstructive plasmids were produced by PrimeSTAR GXL DNA polymerase (Takara), and DpnI was used to digest the parental DNA template and to select for mutation-containing synthesized DNA. The products were then phosphorylated and ligated to circular plasmids. The circular plasmids containing the desired mutation were then transformed into E. coli BL21 (DE3) competent cells. Each mutated protein was expressed and purified to measure its AHL-degrading activities, as described above. The relative activities of mutated proteins were obtained by comparison to that of purified PfmA.

Effect of enzyme on virulence factor production in pathogens

V. anguillarum VIB72 and P. aeruginosa PAO1 were cultured overnight with 10 µg ml⁻¹ purified PfmA in MB medium and LB medium, respectively. Heat-inactivated PfmA was used as the negative control. The cultures of pathogens were centrifuged at 12 000 g for 10 min at 4°C, and the supernatants were collected and incubated at 100°C for 10 min. The supernatants were extracted with ethyl acetate. After evaporation, the presence of AHLs in the extracts was tested using VIR24. In addition, the proteolytic activities of the cell-free culture supernatant in V. anguillarum VIB72 and P. aeruginosa PAO1, which were cultured in the presence of PfmA, were determined as previously described [35], with slight modifications. The cultures were spectrophotometrically measured at OD₆₀₀ and the amount of digested azocasein, which was used as substrate, was measured at OD₄₁₅. The protease activity was expressed as OD₄₁₅/OD₆₀₀. Pyocyanin was extracted from the supernatants of P. aeruginosa PAO1 cultured with PfmA and measured by the method previously described by Essar et al. [36]. Each assay was repeated three times.

Artemia survival assay

To assess whether the addition of PfmA had any effect on the pathogenicity of P. aeruginosa PAO1, the survival of Artemia after PAO1 infection with or without PfmA was determined. In brief, Artemia nauplii was incubated in aseptic seawater under light with shaking at 28°C for 24 h. Groups of 30 A. nauplii were transferred to a glass bottle with 20 ml aseptic seawater. P. aeruginosa PAO1 was cultured overnight, and the culture was then centrifuged, resuspended in aseptic seawater and normalized. Suspension was then added to each glass bottle while the aseptic seawater was added as negative control. The purified PfmA (10 and 20 µg ml⁻¹) was added to each test well. The assay bottles were incubated at 25°C, and the number of surviving A. nauplii were counted after two days. Each assay was repeated at least three times.

Nucleotide sequence accession number

The nucleotide sequence of pfmA (FaGL1422) from P. flavipulchra JG1 has been deposited in the GenBank database under accession no. KY328440.

RESULTS

P. flavipulchra JG1 possesses intracellular AHL acylase.

C₁₂-HSL degrading activity was found in the culture and cells of P. flavipulchra JG1, but no degradation activity could be detected in the cell-free supernatants (Fig. S1, available in the online Supplementary Material). There was no degradation activity in strain JG1 when C₆-HSL was used as the substrate. Additionally, acidified products of C₆⁻ and C₁₂⁻ HSL were not found to be completely recovered in strain JG1. These results suggest that JG1 may produce an intracellular AHL acylase and that this AHL acylase can only degrade AHLs possessing the long acyl chain.

In the genome of P. flavipulchra JG1, gene faGL1422 was predicted to encode a penicillin acylase that showed homology to PvdQ and QuiP in P. aeruginosa PAO1, which are well studied and demonstrated to degrade long-chain AHLS. The encoding protein was predicted to be 809 amino acids in length and 91 kDa in size. The amino acid sequence of FaGL1422 showed less than 30% identity to each of the known AHL acylases, and it exhibited 29.8% and 26.6% identity compared to AiiC and QuiP, respectively. Further sequence similarity searching against the NR database showed that its homologues mainly occur in the genus Pseudoalteromonas. FaGL1422 may represent a novel marine-derived AHL acylase and was thus termed PfmA (Pseudoalteromonas flavipulchra marine AHL acylase). Further second-structure analysis of PfmA showed that the protein includes an α subunit, a spacer sequence and a β subunit, and post-translational modification was performed prior to PfmA becoming an active enzyme (Fig. 1). When the amino acid sequence of PfmA was compared to that of known AHL acylases (Fig. 1), the Ser residues, the commonly conserved sequence of the known AHL acylases, were identified in PfmA and it was predicted as the catalytic site and the first amino acid of the β-subunit. In addition, PfmA was predicted to be intracellular and lacking an N-terminal signal peptide. The α subunit of PfmA was predicted to have seven α helices, two β folds and three random coils, while the β subunit was predicted to have 12 α helices, 19 β folds and eight random coils.
Expression and purification of PfmA

The *P. flavipulchra* pfmA gene was amplified by PCR from *P. flavipulchra* JG1 chromosomal DNA and cloned into plasmid pET26b (+), resulting in plasmid pET26b (+)-pfmA. The recombinant PfmA protein was expressed poorly when the full-length amino acid sequence was included. However, the recombinant PfmA protein was produced mainly in the soluble form when it was expressed with 20 amino acids reduced from the N-terminal sequence. Overexpression of pfmA in *E. coli* BL21 (DE3) resulted in the synthesis of a C-terminal His-tagged protein of about 68 kDa as shown by SDS-PAGE (Fig. 2a). The His-tagged protein PfmA was successfully purified using a Ni-based immobilized-metal-affinity chromatography protocol. SDS-PAGE analysis of the purified protein showed two protein bands, 68 and 26 kDa, which likely represents the β- and α-subunits, respectively (Fig. 2b). These results show that post-translational modification occurred after PfmA synthesis, and resulted in α- and β-subunits, as found in most proteins of the Nit hydrodrolase superfamily.

**PfmA is an AHL acylase**

The cells of strain JG1 degraded only those AHLs with a long acyl chain. The purified PfmA also degraded long-chain AHLs, including C10-HSL, C12-HSL, C14-HSL, 3-Oxo-...
C₁₂-HSL and 3-Oxo-C₁₄-HSL, but not short-chain AHLs (shorter than 10 carbons in the acyl chain) according to CV026 or VJR24 plate assay. The purified PfmA degraded weak 3-OH-C₁₄-HSL according to A136 plate assay. Gene pfmA was predicted to encode a penicillin acylase in the genome analysis, so we tried to determine whether PfmA could degrade penicillin G. It transpired that PfmA could not degrade penicillin G but ampicillin, which is an analogue of penicillin G, was degraded by this protein.

The reaction products of C₁₂-HSL that were digested by purified PfmA were analysed by UPLC and ESI-MS. The enzymatic digestion of C₁₂-HSL resulted in a product characteristic of AHL cleavage that was identified as HSL with a retention time of 0.298 min, as determined by UPLC analysis (Fig. 3a). ESI-MS analysis of the product revealed an abundance of HSL with M+H ions at a mass-to-charge ratio (m/z) of 101.02 and a retention time of 0.298 min, as determined by UPLC analysis.

Physical and chemical parameters affecting PfmA activity

The temperature, pH, SDS, urea and metal ions that potentially affect the AHL-degrading activity of PfmA were studied. The activity of purified PfmA was maximal at 30 °C but was drastically decreased at 60–80 °C (Fig. 4a). PfmA heated to 40 °C for 1 h retained 40 % activity, and the protein possessed no activity after being heated to 80 °C for 1 h (Fig. 4b). AHL acylase activity was enhanced at increased pH (>pH 4.0) and reached maximum at pH 7.0 (Fig. 4c). Cations including Na⁺, K⁺, Ca²⁺, Ni²⁺, Fe³⁺, Mn²⁺, Zn²⁺, and Cu²⁺ showed no effect on AHL acylase activity, while Co²⁺ increased it by 8.5 % (P<0.05) compared to the activity of PfmA. On the other hand, purified AHL acylase was partially inhibited by urea, which resulted in PfmA retaining 80 % (P<0.01) degrading activity, and was completely inhibited by SDS (P<0.01) (Fig. 4d).

The conserved serine residue is the active site essential for PfmA activity

To determine the action of several conserved and functional amino acids in PfmA, five amino acids of PfmA were subjected to site-directed mutagenesis. The PfmA residue Ser256 was replaced by neutral glycine, and the mutations resulted in an almost non-functional PfmA enzyme whose activity was reduced by 90 % (P<0.001) (Fig. 5), suggesting that the conserved serine is required for PfmA activity. When Arg528 was replaced by serine and asparagine the mutated proteins retained 84 % (P<0.001) and 80 % (P<0.001) of their degrading activities, respectively, and the mutated protein Asn324 that was replaced by valine retained 80 % (P<0.05) of its degrading activity. In addition, after His278 and Leu279 were replaced by serine and phenylalanine, respectively, the activity of mutated PfmA was decreased by 20 % (P<0.001) and 26 % (P<0.001), respectively (Fig. 5). These results suggested that Ser256 is the active site essential for the AHL-degrading activity of PfmA; meanwhile, Arg528, Asn324, His278 and Leu279 also played important roles in this process.

PfmA reduces the production of virulence factors by pathogens

The effect of PfmA on the production of virulence factors by various pathogens was investigated. The production of
protease in *V. anguillarum* VIB72 was reduced by 51% (*P*<0.01) with 20 µg ml$^{-1}$ PfmA (Fig. 6a). The presence of 10 and 20 µg ml$^{-1}$ PfmA significantly blocked the production of protease in *P. aeruginosa* PAO1 and led to a reduction of 25% (*P*<0.01) and 38% (*P*<0.01), respectively, in total protease activity (Fig. 6b). Similarly, the addition of 10 and 20 µg ml$^{-1}$ PfmA reduced the production of pyocyanin in *P. aeruginosa* PAO1 (Fig. 6c) by 20% (*P*<0.05) and 34% (*P*<0.01), respectively. The concentration of AHLs in *V. anguillarum* VIB72 and *P. aeruginosa* PAO1 was reduced when 10 µg ml$^{-1}$ PfmA was present (Fig. S2). These results show that the production of virulence factors in *V. anguillarum* VIB72 and *P. aeruginosa* PAO1 can be reduced in the presence of PfmA, which decreased the accumulation of AHLs in the two strains and impacted the targets of QS.

PfmA slightly increased the survival rate of infected *Artemia* but the difference was not significant (*P*>0.05). However, the addition of 20 µg ml$^{-1}$ PfmA significantly increased the survival rate of infected *Artemia* by 15% (*P*<0.05), demonstrating that a higher concentration of PfmA showed a better protective effect than a lower concentration (Fig. 6d).

**DISCUSSION**

QQ has been reported as a common feature among marine cultivable bacteria [11]. To date, there over 30 marine bacteria have been reported to have QQ activity, including members of the α- and γ-Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes [37]. *Pseudoalteromonas* belongs to the γ-Proteobacteria, and approximately 30 strains in this genus have been isolated from different marine environments, including the Pacific Ocean, the Arctic and the Antarctic. At present, among species of *Pseudoalteromonas*, only *Pseudoalteromonas byunsanensis* is reported to have an AHL lactonase that degrades AHLs (C$_4$-$C_{12}$) with or without 3-Oxo substitution [38]. The present study found that the cell content, but not the cell-free supernatants, of *P. flavi pulchra* JG1 had C$_{12}$-HSL-degrading...
activities, and the intracellular protein PfmA was confirmed to be responsible for the QQ activity. Besides, according to sequencing alignment, PfmA homologues are widespread in the genus *Pseudoalteromonas* but none of their AHL-degrading activities have been characterized.

Of the AHL acylases reported from various bacteria and metagenomic libraries, several have been described in detail. Based on phylogenetic analysis, AiiD [14], AhlM [16], PvdQ [39] and Aac [15] belong to the aculeacin A acylase family, while AmiE [20] is located in a different branch and belongs to the amidase family. AiiC [17], QuiP [21], HacB [19], PfmA and their homologues all belong to the penicillin G acylase family (Fig. 7). Amino acid sequence analysis showed that PfmA showed <30% identity to each of the known AHL acylases across the entire length. This suggests that PfmA may be considered as a novel marine AHL acylase. Most AHL acylases reported to date degrade long-chain AHLs more efficiently than short-chain AHLs. For instance, Aac [15], PvdQ [39], AhlM [16] and QuiP [21] were shown to be capable of degrading AHLs with acyl chains longer than eight carbons. In our study, purified PfmA degraded only AHLs with acyl chains longer than 10 carbons. These results suggest that although the sequence identity among different AHL acylases is low, they may exhibit similar AHL-degrading activities. However, the substrates that PfmA from *P. flavipulchia* JG1 could degrade are more specific in comparison to the AHL acylases reported to date.

Although PfmA and its homologues are distinct from the aculeacin A acylase and amidase families, all of these enzymes belonged to the Ntn hydrolase superfamily. A typical protein belonging to the Ntn hydrolase superfamily includes an α-subunit, a spacer sequence and a β-subunit, most having a signal peptide. Most AHL acylases were reported to be intracellular and may be secreted into the periplasm based on their signal sequence, and can degrade AHLLs that are diffused within the cell, while extracellular AHL acylase AhlM is an exception. The predicted PfmA polypeptide was cleaved into a two-subunit form. This polypeptide showed the significant features of post-translational processing typical of members of the Ntn hydrolase superfamily. Although the signal peptide was not predicted by the SignalP server, there was a putative transmembrane domain in the N-terminal region according to secondary structure analysis. The reduction of 20 amino acid residues from N-terminal sequences of PfmA corresponding to the signal peptides of other AHL acylases may enhance the expression of PfmA and not affect its AHL-degrading activity. Furthermore, PfmA also shares well-conserved serine, aspartic acid, asparagine, histidine and leucine residues that have been demonstrated to be of importance in both autoproteolytic processing and catalysis. It has been shown that the structure of a covalent ester intermediate identified Ser256 in PvdQ [23]. The Ser256 in PfmA, which
corresponds to Serβ1 in PvdQ, was predicted as the active site and was assumed to attack the carbonyl carbon of the scissile bond of the substrate. Site-directed mutagenesis assay also confirmed that this conserved Ser256 is essential for AHL-degrading activity.

Acylases as a group are capable of degrading an widely diverse group of compounds. The penicillin acylase family includes penicillin G acylases, aculeacin A acylases and cephalosporin, and these acylases are specific to certain substrates to a certain extent. Most AHL acylases, including HacB and AiiD, have no penicillin G-degrading activity. Exceptionally, AhlM of the aculeacin A acylase family exhibited a broad substrate specificity capable of catalysing the hydrolysis of penicillin G and releasing 6-APA. It was shown that PfmA could not degrade penicillin G, but ampicillin which is the analogue of penicillin G was hydrolysed by PfmA. Although PfmA showed an AHL-degrading activity similar to known AHL acylases, the substrates of different AHL acylases belonging to the penicillin G family showed high diversity. Further experiments should be carried out to determine the substrate extent of PfmA and its physiological function in the marine bacterium P. flavipulchra JG1.

QQ is potentially a new antivirulence therapy, and has attracted considerable attention. It was reported that heterologous expression of QQ enzymes in certain pathogenic bacteria, or the administration of purified QQ enzyme, significantly decreased virulence and protected animals in an experimental setting. For instance, overexpression of the pa0305 gene in P. aeruginosa PAO1 reduced elastolytic activity and pyocyanin production [19]. The addition of purified AhlM to the growth medium eliminated 3-Oxo-C12-HSL production and delayed and reduced the accumulation of C4-HSL in P. aeruginosa PAO1. Similarly, the production of virulence factors was also mly decreased by the presence of AhlM [16]. In our study, PfmA reduced protease production in V. anguillarum VIB72 and P. aeruginosa PAO1, reduced pyocyanin production in P. aeruginosa PAO1 and protected Artemia from killing by strain PAO1, which suggests that enzymatic attenuation of bacterial infection may be developed as a highly attractive quorum-quenching agent.

**Funding information**

This study was supported by projects from the National Natural Science Foundation of China (No. 31502171 and No. 41476112) and a General Financial Grant from the China Postdoctoral Science Foundation (No. 2015M570611).

**Acknowledgements**

We thank Robert J. C. McLean (Texas State University, USA) for biosensors C. violaceum CV026 and A. tumefaciens A136, and Tomohiro Morohoshi (Utusnomiya University, Japan) for C. violaceum VIR24.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

This article does not contain any studies with human participants performed by any of the authors.

**References**


Edited by: S. P. Diggle and M. Whiteley

Liu et al., *Microbiology* 2017;163:1389–1398