Purification and characterization of antibacterial compounds of *Pseudoalteromonas flavipulchra* JG1

Min Yu,† Junfeng Wang,‡ Kaihao Tang, Xiaochong Shi, Shushan Wang, Wei-Ming Zhu and Xiao-Hua Zhang

1College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China
2Key Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, China

*Pseudoalteromonas flavipulchra* JG1 produces a protein PfaP and a range of small-molecule compounds with inhibitory activities against *Vibrio anguillarum*. The PfaP protein was purified from the extracellular products of JG1 by electroelution, and antibacterial activity was observed by an in-gel antibacterial assay. The complete amino acid sequence (694 aa) of PfaP was determined by *de novo* peptide sequencing and subsequent alignment with the proteome sequence of strain JG1. The calculated molecular mass of PfaP was 77.0 kDa. PfaP was 58 % identical to L-lysine oxidase AlpP of *Pseudoalteromonas tunicata* D2, and 54 % identical to the marinocine antimiicrobial protein of *Marinomonas mediterranea* MMB-1. Five small molecules (compounds 1–5) with antibacterial activity, which were identified as *p*-hydroxybenzoic acid (1), *trans*-cinnamic acid (2), 6-bromoindolyl-3-acetic acid (3), *N*-hydroxybenzisoxazolone (4) and 2′-deoxyadenosine (5), were purified by sequential column chromatography over silica gel, Sephadex LH-20 and RP-18 from ethyl acetate extract of strain JG1, and their structures were determined by NMR and MS. Brown compound 3, the only brominated compound, showed antibacterial activity against both Gram-positive and Gram-negative bacteria.

INTRODUCTION

A continuously widening range of micro-organisms, including *Vibrio* spp., cause diseases of aquacultured organisms. *Vibrio anguillarum* is the pathogen of vibriosis, one of the oldest fish diseases, causing a major constraint to mariculture (Austin & Austin, 2007). Antibiotics have been extensively used in controlling bacterial infections in fish culture. Unfortunately, the abuse of antibiotics has been associated with diverse problems in the health of fish, terrestrial animals and humans and for the environment (Alcaide et al., 2005). As alternatives to antibiotics, more and more research has been focused on the application of marine antagonistic strains isolated from either aquatic animals or aquaculture environments. Various bacteria have been confirmed to produce antimicrobial substances, with activities ranging from broad-spectrum to species-specific, including bacterial pathogens (Anand et al., 2006). An increasing number of antibacterial compounds have recently been identified in *Pseudoalteromonas* spp. (Dopazo et al., 1988). In particular, *Pseudoalteromonas tunicata* D2 (Holmström et al., 1998) has been found to produce more than five types of inhibitory substances, including a toxic antibiotic protein and a yellow pigment (Franks et al., 2005; James et al., 1996). An isolate related to *Pseudoalteromonas piscicida*, designated X153, was confirmed to produce an unstable tetrameric 280 kDa vibriostatic protein with a broad-spectrum inhibitory activity against marine bacteria (Longeon et al., 2004). Another *P. piscicida* isolate, NJ6-3-1, also showed a wide antimicrobial spectrum and produced norharman (a β-carboline alkaloid) with inhibitory activity against *Staphylococcus aureus* (Zheng et al., 2005). However, there are gaps in the knowledge of possible synergistic effects between the antibacterial protein and the small-molecule compounds of *Pseudoalteromonas* spp. regarding antibacterial activity.

*Pseudoalteromonas flavipulchra* was isolated originally by Breittmayer & Gauthier (1979) from seawater off Nice, France, and was reclassified by Ivanova et al. (2002). Recently, antibacterial activity was observed in this species. In our previous work, we isolated *P. flavipulchra* JG1 from rearing water of healthy turbot (*Scophthalmus maximus*) in Qingdao, China. The isolate exhibited inhibitory activity against *V. anguillarum*, *Vibrio alginolyticus*, *Vibrio campbellii*, *Vibrio piscicida*, and *V. parahaemolyticus* (Holmström et al., 2010). As alternatives to antibiotics, more and more research has been focused on the application of marine antagonistic strains isolated from either aquatic animals or aquaculture environments.
**METHODS**

**Bacterial strains and culture conditions.** Twenty-nine Gram-negative and Gram-positive bacterial strains were used for evaluating the antibacterial activity of marine bacterium JG1 and its metabolites: *Vibrio metchnikovii* ATCC 7708, *Vibrio parahaemolyticus* LMG 3772, *Vibrio pelagia* LMG 3897, *V. harveyi* LMG 4044, *VIB 86* and *VIB 351*, *V. anguillarum* LMG 4437, *Vibrio cincinnatiensis* LMG 7891, *Vibrio diazotrophicus* LMG 7893, *Vibrio fluvialis* LMG 7894, *Vibrio natriegens* LMG 7896, *Vibrio orientalis* LMG 7897, *Vibrio aestuarinarum* LMG 7909, *Vibrio fumrettii* LMG 7910, *Vibrio mediterranei* LMG 11258, *Vibrio nereis* LMG 13543, *Vibrio chagassii* LMG 13239, *Vibrio logei* LMG 14011, *Vibrio corallilyticus* LMG 20984, *Vibrio xanii* LMG 21346, *Bacillus pumilus* FYB01, *Bacillus cereus* FYB02, *Bacillus flexus* FYB04, *Bacillus simplex* FYB18, *Bacillus firmus* FYB19, *Bacillus subitlis*, *Photobacterium damselae* subsp. *damselae*, *Marinomonas hydrophila* AHK1 and *S. aureus*. All the strains were obtained from the Marine Microbiology Laboratory of Ocean University of China. JG1, *Ph. damselae* subsp. *damselae*, *A. hydrophila* and all *Vibrio* cultures were routinely grown on marine agar 2216 (MA; Difco) at 28°C for 48 h, the lawn was scraped off and suspended in sterile PBS (0.01 M, pH 7.2). The cells were removed by centrifugation at 13 000 g for 30 min at 4°C, and the supernatant was filtered through a 0.22 μm porosity filter before further use.

**SDS-PAGE and in-gel antibacterial assay.** ECPs of JG1 were mixed (4:1) with sample buffer (0.25 M Tris/HCl, pH 6.8, 10% SDS, 5% β-mercaptoethanol, 50% glycerol and 0.5% bromophenol blue). The resultant mixture was loaded into the gel with or without heating and separated by SDS-PAGE (Laemmli, 1970). Duplicate lanes loaded with identical samples were treated separately after gel electrophoresis. One lane slice was stained with Coomassie brilliant blue. The other lane slice was washed three times in distilled water and then placed on a MA plate, after which an overnight broth culture of *V. anguillarum* inoculated into semi-solid MA medium was spread over the gel surfaces in order to examine the antibacterial activity of proteins (Chen et al., 2010). The plate was incubated at 28°C for 24 h to observe the inhibition zone.

**Purification of the antibacterial protein from JG1.** The antibacterial protein was purified by electroelution using a model 422 Electro-Eluter Module (Bio-Rad). The protein band with antibacterial activity was excised from the gel and placed into a tube connected with membrane caps (molecular mass cut-off 12–15 kDa). Electroelution was performed at 10 mA constant current per glass tube for 5 h in elution buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). After elution, the remaining liquid in the membrane cap was collected. SDS-PAGE and in-gel antibacterial assays were repeated as above to determine the homogeneity and activity of the antibacterial protein.

**Mass spectrometry identification of the antibacterial protein.**

**Mass spectrometry identification of the antibacterial protein.** The gel band containing the antibacterial protein was sliced, washed and digested following the method described by Chen et al. (2010). The posiled peptide extracts were marked with 4-sulphophenyl isothiocyanate (SPITC), desalted with a C18 Zip-Tip (Millipore) and analysed by a MALDI TOF/TOF mass spectrometer (Bruker, Ultraflex). After the peptide mass fingerprinting (PMF) screening, two peptides with higher intensity were further fragmented in the second TOF, and the peptide files were visualized via DataAnalysis software. De novo peptide sequencing was performed on these two peptides manually.

**Amino acid sequence analysis.** The sequences of the two peptide fragments and the peptide files of the JG1 proteome, transformed from the complete genome sequence (authors’ unpublished data) were aligned to obtain a complete protein sequence. Homology searches were performed using BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple amino acid sequences were aligned using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The analysis of the amino-acid sequence was performed using the Signal P server [version 3.0; Center for Biological Sequence Analysis, Technical University of Denmark (http://www.cbs.dtu.dk)] (Bendtsen et al., 2004) and
Secretome P server [version 2.0; Center for Biological Sequence Analysis, Technical University of Denmark (http://www.cbs.dtu.dk/services/SecretomeP)] (Bendtsen et al., 2005).

**Antibacterial activity against *V. anguillarum* during the growth of JG1.** With the cellophane overlay method described above, the ECPs of JG1 were collected after culture at 28 °C for 24 h, 48 h, 72 h and 96 h. The total protein concentrations in the ECPs and the amount of biomass in suspensions of JG1 were measured by UV absorbance at 280 nm and optical density at 600 nm, respectively. The antibacterial activities of ECPs at different growth stages were determined by the disc diffusion method after adjusting the total protein in the ECPs to the same amount.

**Effects of temperature and pH on antibacterial activity.** The effect of temperature on antibacterial activity was evaluated by treating the antibacterial protein at 60, 80 and 100 °C in a water bath for 10, 30, 45 and 60 min. The effect of pH was evaluated by adjusting the antibacterial protein to various pH values (Na$_2$HPO$_4$/citric acid buffer, pH 4.0–8.0, at intervals of 1.0 pH unit) at 28 °C for 2 h. The pH value of each sample was readjusted to pH 7.0 before evaluation. The antibacterial activity was measured by disc diffusion. Untreated protein was used as a positive control.

**Purification and structure elucidation of the small-molecule compounds with antibacterial activity.** JG1 was inoculated into 200 ml MB in flasks and cultured at 125 r.p.m. for 24 h at 28 °C. The filtered fractions from 150 l fermentation broth were spun-dried and extracted with EtOAc three times at 35 °C, yielding 23 g crude extract, which was directly chromatographed over a silica gel column eluted with a CHCl₃/MeOH gradient to yield 12 fractions. The results of antibacterial experiments indicated that fractions I, IV and X were bioactive. Fraction I was subjected to column chromatography on silica gel eluted with petroleum ether/EtOAc gradients to give subfractions. Subfraction I-D (60 : 40, v/v) was purified on semi-preparative reverse-phase HPLC with MeOH/H₂O (90 : 10, v/v) to yield compound 1 (1.6 mg). After repeated column chromatography over silicone gel and Sephadex LH-20, fraction IV yielded compounds 2 (9.7 mg), 3 (12.5 mg) and 4 (14.3 mg). Fraction X was subjected to Sephadex LH-20 to give three subfractions. Compound 5 (9.1 mg) was obtained from fraction X-C by semi-preparative reverse-phase HPLC with MeOH/H₂O (15 : 85 v/v). The purities of compounds 1–5 ranged from 90% to 95%, and their structures were identified by NMR and MS.

**TLC bioautography overlay assay.** The antimicrobial activities of the small-molecule compounds were measured by TLC bioautography overlay assay (Zheng et al., 2005). Briefly, each fraction was developed using dichloromethane/MeOH (15 : 1, v/v) on TLC silica gel plates, followed by detection at 254 and 375 nm. The developed TLC plates were sterilized by UV for 30 min before covering with a layer of MA (45 °C) containing *V. anguillarum*. After 24 h diffusion at 4 °C, the plates were incubated for 24 h at 28 °C and then the upper agar was sprayed with 5 mg methylthiazoletetrazolium (MTT) ml⁻¹ which was converted to a formazan dye by the test microorganism. Inhibition zones were observed as clear spots against a purple background.

**MIC assay.** The MICs of the small-molecule compounds against *V. anguillarum* were determined by a broth microdilution method. Briefly, an overnight broth culture of *V. anguillarum* was inoculated into 5 ml Mueller–Hinton broth and adjusted to achieve a final concentration of 2 × 10⁷ c.f.u. ml⁻¹. Samples (10 μl) of compounds 1–5, dissolved in DMSO, and 190 μl of the bacterial solutions were dispensed into wells of a 96-well plate. The final concentrations of the small-molecule compounds were 0.05, 0.1, 0.125, 0.25, 0.5, 1, 1.25, 2.5, 5 and 10 mg ml⁻¹. A 10 μl volume of DMSO was used as a negative control. After incubating at 28 °C for 24 h, the lowest concentration in a dilution series that completely inhibited the growth of the test strain was taken as the MIC of the compound. The experiment was repeated three times.

**RESULTS**

**Antibacterial spectrum of JG1**

*P. flavipulchra* JG1 was originally isolated from rearing water of healthy turbot (*Scophthalmus maximus*) in Qingdao, China, and the bacterial culture had excellent inhibitory effects against bacterial pathogens of aquaculture including species of *Vibrio* and *Aeromonas* (Jin et al., 2010). In this study, JG1 was found to inhibit another 17 of the 19 *Vibrio* strains tested, the exceptions being *V. proteolyticus* LMG 3772 and *V. xuii* LMG 21346, and five *Bacillus* strains. *V. anguillarum* LMG 4437 was the most sensitive strain to JG1 among the test strains. However, *V. xuii* LMG 21346 was inhibited by all three antibiotics tested and *V. proteolyticus* LMG 3772 was inhibited by ampicillin and kanamycin. JG1 and kanamycin showed equivalent inhibitory effects against all the test strains, while the antibacterial activities of ampicillin and tetracycline were much weaker. All the antibiogram assays were repeated three times with similar results.

![Fig. 1. In-gel antibacterial assay and SDS-PAGE for the antibacterial protein PfaP produced by JG1.](http://mic.sgmjournals.org)

Duplicate lanes loaded with the antibacterial protein were analysed by SDS-PAGE: (A) blank control without any antibacterial protein; (B) non-stained lane placed on an MA plate containing *V. anguillarum*; (C) Coomassie-brilliant-blue-stained lane; (M) molecular mass ladder (*Fermentas* SMO431). The arrows indicate the PfaP protein band and the corresponding zone of inhibition.
Antibacterial components of JG1

After extraction with petroleum ether, EtOAc and n-BuOH, the supernatant of JG1 was separated into four fractions, petroleum ether, EtOAc, n-BuOH and water extracts. The antibacterial activities against *V. anguillarum* were observed in the extracts with petroleum ether, EtOAc and water, indicating that the antibacterial substances of JG1 may include small-molecule compounds and macromolecules such as antibacterial proteins.

Purification of the antibacterial protein

The ECP sample without heat treatment maintained its antibacterial activity. A single band of antibacterial activity, which corresponded to a protein band visualized by SDS-PAGE and Coomassie brilliant blue staining, was observed by the in-gel antibacterial assay (results not shown). After electroelution of the gel band with antibacterial activity, the protein was purified to apparent homogeneity and the antibacterial activity of the single band was also observed (Fig. 1). From comparison with molecular mass standards, the antibacterial protein band on SDS-PAGE was determined to be approximately 70 kDa in size. In addition, the ECP sample was treated for 5 min at 95 °C and the protein band disappeared, which suggested that the protein was sensitive to heat treatment.

Sequence analysis of the antibacterial protein (PfaP)

Sequences of two peptide fragments of the antibacterial protein, named PfaP, were obtained via peptide sequencing: Ser-Asp-Thr-Met-Phe-Asp-Val-Ala-Val-Arg and Tyr-Tyr-Gln-Asp-Ile-Glu-Pro-Ile-Ile-Gln-Arg. After sequence alignment between the two peptide fragments and the proteome sequence of JG1, a complete protein sequence and an ORF were obtained. The ORF, named *pfaP*, was 2085 bases in

![Fig. 2. Alignment of the deduced amino acid sequence of the *P. flavipulchra* JG1 antibacterial protein PfaP with those of related proteins. PfaP, antibacterial protein from *P. flavipulchra* JG1; Nhal, hypothetical protein from *N. halophilus* Nc4; mari, an antimicrobial protein from *M. mediterranea* MMB-1; AlpP, autolytic and antibacterial protein from *P. tunicata* D2. Symbols: *, identical residues in all sequences; :, conserved substitutions; , semiconserved substitutions; –, gaps introduced during the alignment process. Numbers on the right refer to the amino acid residue at the end of each line.](https://www.microbiologyresearch.org/microbiology/158/838)

M. Yu and others
length. Proximal to the 5' end of ORF were a number of sequences resembling Shine–Dalgarno (SD), −10 and −35 promoter sequences. The SD sequence, AAGGA, was located 7 bp upstream of the initial codon of pfaP. The consensus sequences of the putative −35 region and −10 region were TTGTGC (DNA bases −35 to −30) and TATATC (DNA bases −20 to −15). The deduced translation product of pfaP was a protein of 694 aa with a predicted molecular mass of 77.0 kDa and a theoretical pI of 4.63. The PfaP protein was 58% identical to L-lysine oxidase AlpP of P. tunicata D2 (GenBank AAP73876.1), 56% identical to hypothetical protein Nhal_1391 of Nitrosococcus halophilus Nc4 (GenBank YP_003526929.1) and 54% identical to the marinocine antimicrobial protein of Marinomonas mediterranea MMB-1 (GenBank AAY33849.1) (Fig. 2). No putative signal peptide was detected with either the Signal P server or the Secretome P server.

Characteristics of the antibacterial protein

The concentrations of the total proteins in the ECPs after 24, 48, 72 and 96 h growth were 216, 488, 423 and 390 μg ml−1, respectively. The biomass of JG1 was also highest after 48 h growth, as was the diameter of the inhibition zone formed by ECPs. This suggested that the antibacterial activity was positively correlated with the total secreted proteins and the biomass of JG1. This protein was very sensitive to heat treatment, and its antibacterial activity continuously decreased with time and temperature increase. The protein totally lost its activity after being held at 60 °C for 60 min, 80 °C for 30 min or 100 °C for 10 min. It was resistant to acid–alkali treatment: its antibacterial activity was only slightly decreased after holding at pH 4.0 and remained unaltered after treatment at pH 5.0–8.0 for 2 h.

DISCUSSION

Several marine bacteria have been found to have antimicrobial activities, including species of Bacillus (Jaruchokaweechai et al., 2000), Pseudoalteromonas (Egan et al., 2001; Gómez et al., 2008), Pseudomonas (Gram et al., 1999), Vibrio (Sugita et al., 1997), Aeromonas (Messi et al., 2003), Alteromonas (Zheng et al., 2004), Marinomonas (Anand et al., 2006), Photobacterium (Wan et al., 2006),
Table 1. Antibacterial activity of the small-molecule compounds isolated from JG1 against the test organisms indicated
+ , Antibacterial activity observed by TLC bioautography overlay assay; −, no inhibition zone detected.

<table>
<thead>
<tr>
<th>Compound</th>
<th>V. anguillarum</th>
<th>V. harveyi VIB 286</th>
<th>Ph. damselae subsp. damselae</th>
<th>A. hydrophila</th>
<th>S. aureus</th>
<th>B. subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. p-Hydroxybenzoin acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>2. trans-Cinnamic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3. 6-Bromoinodolyl-3-acetic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4. N-Hydroxybenzoisoxazolone</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>5. 2′-Deoxyadenosine</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Roseobacter (Planas et al., 2006) and Carnobacterium (Yamazaki et al., 2003). The genus Pseudoalteromonas was described by Gauthier et al. (1995), and can be divided clearly into pigmented and non-pigmented species clades. Pigmented Pseudoalteromonas species have been reported to secrete a range of extracellular compounds, including pigment compounds with bioactivity against various pathogens (Bowman, 2007). P. flavipulchra JG1, which has a golden yellow colour, has excellent antibacterial activities, especially against pathogens in marine aquaculture. Since JG1 is an indigenous bacterium in aquatic water which is harmless to aquatic animals (Jin et al., 2010), and its antibacterial activity is similar to that of some antibiotics, JG1 and/or its products have the potential to be used as probiotics or antibiotics in aquaculture.

It has been reported that several marine bacterial strains belonging to the Gammaproteobacteria can synthesize antibacterial proteins, including the l-lysine oxidase AlpP of P. tunicata D2 (James et al., 1996), marinocine of Marinomonas mediterranea (Lucas-Elio et al., 2005) and an antibacterial protein of P. flavipulchra C2 (Chen et al., 2010). The molecular masses and antibacterial activities of these proteins were different from each other. AlpP was approximately 190 kDa in size, while the marinocine and the antibacterial protein of P. flavipulchra C2 had apparent molecular masses of 140–170 kDa and 60 kDa, respectively. The Pfap protein from this study had a calculated molecular mass of 77.0 kDa. Furthermore, the AlpP protein was an autotoxic protein and showed distinct inhibitory activity against P. tunicata D2, while Pfap could not inhibit the growth of P. flavipulchra JG1 (results not shown). In particular, the antibacterial protein of P. flavipulchra C2 could inhibit the growth of S. aureus, while Pfap exhibited strong antibacterial activities against Vibrio species but not against S. aureus (results not shown). This indicated that different P. flavipulchra strains could produce different antibacterial substances.

The antibacterial activities of Pfap were detected by non-denaturing SDS-PAGE after treatment for 5 min at 95 °C. The antibacterial protein might be blocked during electrophoresis without SDS. Moreover, the Pfap protein disappeared during SDS-PAGE after treatment for 5 min at 95 °C. Since the Pfap protein contained under non-denaturing SDS-PAGE.

Analysis of the amino acid sequence using Signal P and the Secretome P server showed that there was no signal peptide cleavage site in the Pfap protein. However, the Pfap protein appeared in the ECPs of JG1, which suggested that the protein could be secreted into extracellular matrix without a signal peptide. This coincides with the AlpP of P. tunicata D2 and the marinocine of M. mediterranea MMB-1, which were also predicted to lack signal peptides. Moreover, AlpP (James et al., 1996) and marinocine (Lucas-Elio et al., 2006) were confirmed to possess antimicrobial activity due to hydrogen peroxide generation. According to the sequence similarity and secretion feature, the Pfap protein may also generate hydrogen peroxide as a mechanism of microbial competition.

The small-molecule compounds produced by Pseudoalteromonas species can be divided into non-halogenated and halogenated compounds (Bowman, 2007); the halogenated compounds might be responsible for giving the bacteria their bright colour. Both the non-halogenated and the halogenated compounds showed inhibitory activity against different target organisms. For example, Pseudoalteromonas phenolica produced a brominated biphenyl compound (3,3′,5,5′-tetrabromo-2,2′-diphenyldiol) inhibitory to meticillin-resistant S. aureus strains (Egan et al., 2002; Isanayeto & Kamei, 2003), and the non-halogenated compound isatin (indole-2,3-dione) found in the ETOAc extracts of Pseudoalteromonas issachenkonii (Kalinovskaya et al., 2004)
showed an antifungal activity. Most of the bioactive compounds have a cyclic or benzene ring structure, implying that they may share some structural features. In this study, compound 3 isolated from JG1 was the only brominated compound, with a brown colour, while the other four compounds were non-halogenated and almost colourless. In addition, compound 3 exhibited a yellow colour when it was diluted, so it probably contributes to the yellow colonies of JG1 on MA plates. It has been reported that the pigmented species of *Pseudoalteromonas* could produce a range of bioactive substances (Bowman, 2007), and the pigments were correlated directly or indirectly with bioactivities such as antimicrobial, antifouling and algicidal activities (Egan et al., 2002). In the present study, compound 3 showed the greatest antibacterial activity, which indicated that this pigment could be associated with the antibacterial activity of JG1. The growth of *V. anguillarum* was inhibited more by the mixture of compounds than by each of the five compounds individually (results not shown), suggesting that the compounds may act synergistically. Although the structures of the five compounds described here have been reported previously (Niu et al., 2003; Yang et al., 2000; Rasmussen et al., 1993; Kuberski & Gebicki, 1992; Shi et al., 2009), they were first discovered to exhibit antibacterial activities in this work.

In conclusion, we have discovered an antibacterial protein and five small-molecule compounds from *P. flavipulchra* JG1. All of them showed inhibitory activity against the bacterial pathogen *V. anguillarum*. The antibacterial protein and the small-molecule compounds may have a synergistic antibacterial effect. Further studies are required to clarify the mechanism and the connection of these bioactive compounds.

**ACKNOWLEDGEMENTS**

This work was supported by grants from the National Natural Science Foundation of China (no. 40876067), the National High Technology R&D Program of China no. 2007AA09Z434), the National Basic Research Program of China (no. 2010CB833800) and the Special Fund for Marine Scientific Research in the Public Interest of China (no. 2010418022-3).

**REFERENCES**


Edited by: A. J. O’Neill