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An inhibitory compound produced by *Pseudomonas* with effectiveness on *Vibrio harveyi*

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(Correction added on 14 January 2010, after first online publication: The fifth author's name was corrected from Natamal S Jayaprakash to Naatamai S Jayaprakash.)

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Abstract

Persistence of the antivibrio property of the potential antagonistic probiotics, *Pseudomonas* MCCB 102 and 103, at different temperatures, pH and in organic solvents was studied. The antivibrio compound was extracted, purified and characterized using thin-layer chromatography, high-pressure liquid chromatography, liquid chromatography-mass spectroscopy, UV–Vis and nuclear magnetic resonance spectroscopy and identified as *N*-methyl-1-hydroxyphenazine, a phenazine antibiotic. The toxicity of the compound was tested in *Penaeus monodon* haemocyte culture and the IC_{50} value was found to be 1.4 ± 0.31 mg L^{-1}. The compound was found to be bacteriostatic at 0.5 mg L^{-1}. Its stability to varying temperature, pH, organic solvents, prolonged shelf-life and vibriostatic nature point to its suitability for prophylatic aquaculture application.

Keywords: antivibrio compound, *N*-methyl-1-hydroxyphenazine, liquid chromatography-mass spectroscopy, nuclear magnetic resonance spectroscopy, haemocyte culture, aquaculture

Introduction

Members of the genus *Pseudomonas* are common inhabitants of soil, fresh water and marine environments and are known to produce secondary metabolites (Raaijmakers, David & Thomasshow 1997; Norman, Moeller, McDonald & Pamela Morris 2004; Kumar, Ayyadurai, Pandiaraja, Reddy, Venkateswarlu, Prakash & Sakthivel 2005) inhibiting a wide range of pathogenic bacteria. *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* have been used widely in agriculture as microbial control agents, alternative to synthetic chemicals for combating plant diseases (Raaijmakers *et al.* 1997; Kumar *et al.* 2005). These organisms produce an array of secondary metabolites, including pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol and hydrogen cyanide, which inhibit plant pathogens (Brodhagen, Henkels & Loper 2004). Meanwhile, Torrento and Torres (1996) reported five strains of *Pseudomonas*, which showed inhibitory activity against *Vibrio harveyi*, the causative agent of luminescent vibriosis in *Penaeus monodon*. Subsequently, *P. fluorescens* was reported to inhibit *Saprolegnia* sp. in finfish culture and *Vibrio anguillarum* in rainbow trout (Bly, Quiniou, Lawson & Clem 1997; Gram, Melchiorsen, Spanggaard, Huber & Nielsen 1999). Later, it was reported that *Pseudomonas* 1–2 antagonized shrimp pathogenic *V. harveyi*, *Vibrio fluvialis*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Photobacterium damselae* (Chythanya, Karunasagar & Karunasagar 2002).

Following these leads, *Pseudomonas* MCCB (MCCB: Microbial Culture Collection of Bacteria of National

Centre for Aquatic Animal Health) 102 and 103 (Jayaprakash 2005; Vijayan, Bright Singh, Jayaprakash, Alavandi, Somnath Pai & Preetha 2006) were isolated from brackishwater and foregut of shrimp in a grow-out system, respectively, both antagonistic to a range of vibrios such as *V. harveyi*, *Vibrio alginolyticus*, *V. anguillarum*, *Vibrio proteolyticus*, *V. fluvialis*, *V. parahaemolyticus*, *Vibrio nereis*, *V. vulnificus*, *Vibrio mediterranei*, *Vibrio cholerae* and *Aeromonas* spp. in shrimp and prawn culture systems. Considering the requirement of novel drugs in aquaculture in lieu of antibiotics, the inhibitory compounds produced by these isolates were identified, and the shelf-life, IC_{50} in shrimp haemocyte culture and the growth inhibition imposed by the compound in *V. harveyi* were determined.

Materials and methods

Organisms and their culture

Pseudomonas MCCB 102 and *Pseudomonas* MCCB 103, previously described by Vijayan *et al.* (2006) and Jayaprakash (2005) and with confirmation of their identity based on 16S rRNA gene sequence analysis (GenBank accession no. EF062514 and EF053508), were used to produce the antivibrio molecule. *Pseudomonas* MCCB 102 was isolated on ZoBell's Marine Agar 2216 E from routine water samples collected from the Muttukkadu brackish water lagoon situated about 35 km south of Chennai, India (Vijayan *et al.* 2006), and Pseudomonas MCCB 103 from the foregut of shrimp in a grow-out system off Cochin, Kerala (Jayaprakash 2005). These isolates formed part of the culture collection of the National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology (CUSAT), India. Organisms were grown in ZoBell's marine broth 2216 E (peptone: 10 g L^{-1}, yeast extract: 1 g L^{-1}, sodium chloride: 12.9 g L^{-1}, pH 6.5 and incubation temperature: 25 °C for *Pseudomonas* MCCB 102 and peptone: 10 g L^{-1}, yeast extract: 1 g L^{-1}, sodium chloride: 5 g L^{-1}, pH 7.0 and incubation temperature: 25 °C for *Pseudomonas* MCCB 103). The cultures (3–5 days old) were centrifuged at 10 000 *g* at 4 °C for 20 min, the supernatant was filtered through a series of filters including a glass microfibre filter (GF/C, Whatman, Kent, UK), a cellulose acetate membrane (0.45 μm; Millipore, Bedford, MA, USA) and a PVDF membrane (0.22 μm; Millipore) and used for further analysis.

Antagonism assay of cell-free supernatant

The cell-free supernatant after each treatment was used for an antagonism assay towards pathogenic *V. harveyi* MCCB 111, the target bacterial culture obtained from the culture collection of NCAAH, CUSAT. The identity of the pathogen was confirmed following Oliver (1982) and based on 16S rRNA gene sequence analysis (GenBank accession no. EU404191). The culture grown on ZoBell's Marine Agar slants (prepared in seawater of salinity 20 ppt) was harvested in saline (2% sodium chloride, w/v) and optical density was adjusted to 1.5 at A_{600}, and 500 μL was inoculated onto ZoBell's Marine agar 2216 E plates with the same composition. The optical density of the cell suspension used for seeding the plates was maintained constant throughout the experiment. Aliquots of the supernatant (20 μL) from the *Pseudomonas* culture after each treatment were spotted onto sterile filter paper discs of 1 mm thickness and 5 mm diameter, were placed on ZoBell's Marine 2216 E plates seeded with *V. harveyi* MCCB 111 and the activity was measured after 18 h of incubation (at 28 °C) by measuring the inhibition zone (Jayaprakash, Pai, Anas, Preetha, Philip & Singh 2005).

Heat and pH stability of the inhibitory compound in the cell-free supernatants

Heat sensitivity of the inhibitory substance was tested by heating the cell-free supernatant of *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 in a water bath for 30 min at 60, 80 and 100 °C, and autoclaving for 15 min at 121 °C. Each of the treated and untreated supernatants was tested for antagonistic activity against *V. harveyi* MCCB 111 using the disc diffusion method as described earlier. pH stability was tested by adjusting the pH of the filter-sterilized supernatant to 2, 4, 6, 8 and 10 using 1 mol L^{-1} HCl and 1 mol L^{-1} NaOH, with the help of a pH meter, and incubating for 2 h. The pH was subsequently readjusted to 7.0 and the activity was tested against *V. harveyi* MCCB 111 as described above. The control consisted of a cell-free supernatant with its pH adjusted to 7.0.

Shelf-life of cell-free supernatants

Cell-free supernatants were stored at room temperature (28 ± 0.5), 4 and − 20 °C for 12 months and tested for antagonistic activity against *V. harveyi* MCCB 111 at regular intervals using the disc diffusion assay.

Identification of the inhibitory compound against *V. harveyi*

Solvent extraction was performed using 1-butanol, ethyl acetate, dichloromethane, chloroform and hex-

ane with the cell-free supernatant of *Pseudomonas* MCCB 102 and MCCB 103 at a 1:1 ratio for 10 min. The solvent layer pipetted out was impregnated onto sterile filter paper discs to obtain 40 μL per disc and dried in a vacuum desiccator at room temperature for 24 h, and the inhibitory activity was tested on *V. harveyi* with discs held with solvents alone as the control. Halo zones were measured after 18–24 h of incubation as described above. The culture supernatant left over after solvent extraction was also checked for inhibitory activity. With chloroform extraction, the fraction with blue colouration alone exhibited an antivibrio property, and based on previous reports, we assumed that the blue pigments of the culture under study were phenazine derivatives (Fernandez & Pizarro 1997). To extract the phenazine compound, 5 mL of culture supernatant was treated with 2 mL chloroform acidified with 1 mL 0.1 N HCl (Chang & Blackwood 1969). The phenazine compound was quantified by measuring absorbance at 520 nm, multiplying with 17.072 and expressed in $\mu g\ mL^{-1}$ (Essar, Eberly, Hadero & Crawford 1990).

For thin-layer chromatographic separation of the inhibitory compound, the filter-sterilized supernatant was concentrated by vacuum rotary evaporation and run on TLC plates (silica gel G, 0.2 mm thickness) with different solvent systems such as: (a) 4,1-butanol:1 acetic acid:1 water (Wright 1998), (b) 1 chloroform:1 methanol (Knight, Hartman, Hartman & Young 1979) and (c) 3 ethyl acetate:2 acetic acid:1 water (Knight *et al.* 1979). Visible spots from the TLC plates were scraped off and dissolved in double-distilled water and centrifuged at 10 000 *g* for 15 min at 4 °C and inhibitory activity was tested by disc diffusion, and the corresponding R_f values were determined. Parallel TLC plates were run and were observed under a UV transilluminator, sprayed with reagents such as ninhydrin (Knight *et al.* 1979), phenolite and iron reagent (Cox & Graham 1979) and kept in iodine vapour (Austin & Billaud 1990). Subsequently, on determining the active fraction, preparative TLC plates (0.5 mm thickness), with silica gel G as the sorbent, were used for generating sufficient quantity of the inhibitory compound for further analysis. The solubility of the bioactive compound was tested in water, methanol, ethanol, 1-propanol and acetone, and the inhibitory activity was examined following the disc diffusion method.

The silica gel G-purified fraction was subjected to high-pressure liquid chromatography (HPLC) analysis according to Fernandez and Pizarro (1997) on a Merck-Hitachi L6200 (Darmstradt, Germany) instrument fitted with a 250 × 4.6 mm ODS column and a UV detector tuned to 280 nm with the help of Multi SHM manager software. A gradient method was used for eluting samples using solvent systems A and B. Solvent A was water–trifluroacetic acid (100:0.04, v/v) and solvent B was acetonitrile–water–trifluoroacetic acid (90:10:0.04, v/v/v). Authentic *N*-methyl-1-hydroxyphenazine (Colour Your Enzymes, Kingston, ON, Canada), a phenazine compound produced by *Pseudomonas*, was used as the standard because the activity of the chloroform-extracted compound was at a neutral pH and the active spots that appeared were blue on thin-layer chromatography plates, both being the characteristic properties of *N*-methyl-1-hydroxyphenazine (Chang & Blackwood 1969; Knight *et al.* 1979).

The same fraction was subjected to gas chromatography/mass spectrometric analysis along with the authentic sample of *N*-methyl-1-hydroxyphenazine on a Varion 1200 gas chromatography/quadrupole mass spectrometer. Liquid chromatography-mass spectroscopy (LC-MS) analysis of these samples was carried out on an Esquire 3000 plus ESI (Bruker Daltonics, Billerica, MA, USA) with Agilent 1100 series HPLC (Wilmington, DE, USA).

Subsequently, the UV–Vis–NIR spectra of the purified compound in different solvents such as chloroform, methanol and dichloromethane were generated on a Varian Cary 500 UV–Vis–NIR spectrophotometer (Mulgrave, Australia) (Fernandez & Pizarro 1997). Afterwards, the nuclear magnetic resonance (^{1}H NMR) spectra of the inhibitory compound were recorded on a Bruker AMX 400 High-resolution multinuclear FT-NMR spectrometer (Fallender, Switzerland) operating at 400 MHz. $CDCl_3$ was used as the solvent and tetramethylsilane was used as the internal standard.

Demonstration of the antivibrio property *in vitro*

An inhibitory compound was added to 18-h-old *V. harveyi* culture (10^3 cells mL^{-1}) in a 96-well microplate to obtain final concentrations of 0, 0.05, 0.1, 0.5, 1.0, 2.0 and 3.0 $mg\ L^{-1}$. Quadruplicates were obtained for each concentration. The growth of *V. harveyi* was measured from absorbance at 600 nm in a microplate reader (Tecan Infinite Tm, A-5082 Grödig/Salzburg, Austria) for 0–24 h at an interval of 6 h. After 24 h, 100 μL of the culture from each concentration, in duplicate, was plated onto ZoBell's agar (Himedia, Mumbai, India), incubated at 28 °C for 48 h and the total plate count was determined.

Determination of IC_{50} of the antivibrio compound in *P. monodon* haemocyte culture

Specific pathogen-free for the white spot syndrome virus *P. monodon* shrimps maintained in the organic recirculation system weighing 8–12 g were ice killed, surface sterilized with sodium hypochlorite solution (800 mg L^{-1}) and 70% ethanol and washed in autoclaved seawater of salinity 15 ppt. The haemolymph was withdrawn aseptically from the rostral sinus with a capillary tube containing an anticoagulant (Tris HCl 0.01 M, sucrose 0.25 M, trisodium citrate 0.1 M; pH 7.6) and transferred to culture medium, without separating haemocytes. The medium used was Leibovitz-15 at double strength (Sigma, Aldrich, St Louis, MO, USA) with 2% glucose, MEM vitamins (1 ×), tryptose phosphate broth (2.95 g L^{-1}), *N*-phenylthiourea (0.2 mM), 20% FBS, 0.06 μg mL^{-1} chloramphenicol, 100 μg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin. The haemolymph was diluted to obtain 5×10^5 cells mL^{-1} in medium and 200 μL was dispensed into each well of 96-well plates and incubated at 25 °C for 12 h. The antivibrio compound, extracted from cell-free supernatant with chloroform, was vacuum dried, dissolved in 2 × L-15 medium and different concentrations ranging from 0 to 14 mg L^{-1} were added to the wells with replicates for each concentration. Cells in growth medium without the addition of the inhibitory compound were kept as negative control. After 14 h of incubation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay) was performed. The assay is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt, MTT, with the mitochondria of metabolically active cells. The reduction of the tetrazolium salt by nicotinamide adeninie dinucleotide dehydrgenase and nicotinamide adeninie dinucleotide phosphate dehydrgenase within the cells produces insoluble purple formazan crystals, which are later solubilized, yielding a purple-coloured solution (Mosmann 1983). For the assay, the medium was replaced and 50 μL of MTT (Sigma) solution (5 mg mL^{-1} in PBS; 720 mOsm) was added to each well and incubated for 5 h in the dark. Control for the assay consisted of medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 200 μL dimethylsulphoxide. Absorbance was recorded immediately at 570 nm in a microplate reader (Tecan Infinite Tm). The percentage of inhibited haemocytes at each concentration of the antivibrio compound was calculated to plot the graph. This calculation was performed based on the formula: percentage inhibition of haemocytes = [100 − ((average absorbance (MTT assay) of haemocytes at a particular concentration of the compound/average absorbance of control haemocytes without the compound) × 100)]. Probit analysis of the data was performed.

Results

The cell-free supernatants of *Pseudomonas* MCCB 102 MCCB 103 did not show any significant difference ($P > 0.05$) in inhibitory activity to *V. harveyi* MCCB 111 on exposing to temperatures of 60, 80 and 100 °C (Table 1). However, a significant reduction ($P < 0.05$) was observed after autoclaving for 15 min

Table 1 Effect of temperature, pH and solvent extraction on the antivibrio property of the cell-free supernatants of *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103

	Antagonistic cultures	
Treatments	***Pseudomonas* MCCB 102 (diameter of the halozone in mm)**	***Pseudomonas* MCCB 103 (diameter of the halozone in mm)**
Exposure of the cell-free supernatant to varying temperature		
60 °C (30 min)	15 ± 0.82	13.75 ± 1.23
80 °C (30 min)	15 ± 0.5	13.25 ± 0.96
100 °C (30 min)	14.5 ± 0.58	12.5 ± 0.58
121 °C/15 lb (20 min)	13 ± 0.82*	10.75 ± 0.5*
Control	15.5 ± 0.58	13.75 ± 1.26
Exposure of the cell-free supernatant to varying pH		
2	12.25 ± 1.71	14.5 ± 1
4	11 ± 1.71	14.75 ± 0.5
6	12 ± 0	13.5 ± 1
8	11.5 ± 0.96	13.75 ± 0.5
10	11.5 ± 0.58	13.75 ± 1.26
Control	11.5 ± 0.58	13.75 ± 1.26
Extraction of the cell-free supernatant in different solvents		
Chloroform	11.5 ± 1**	9.25 ± 0.96**
Dichloromethane	10.75 ± 0.5	8 ± 0
N-butanol	10.75 ± 0.5	8.75 ± 0.96
Ethyl acetate	Nil	Nil
Hexane	Nil	Nil
Control (solvent alone)†	Nil	Nil

*Significantly ($P < 0.05$) lower in activity compared with that of the control.

**Significantly ($P < 0.05$) higher in activity compared with that of the control.

†Control was kept for each solvent shown above.

MCCB, Microbial Culture Collection of Bacteria of National Centre for Aquatic Animal Health.

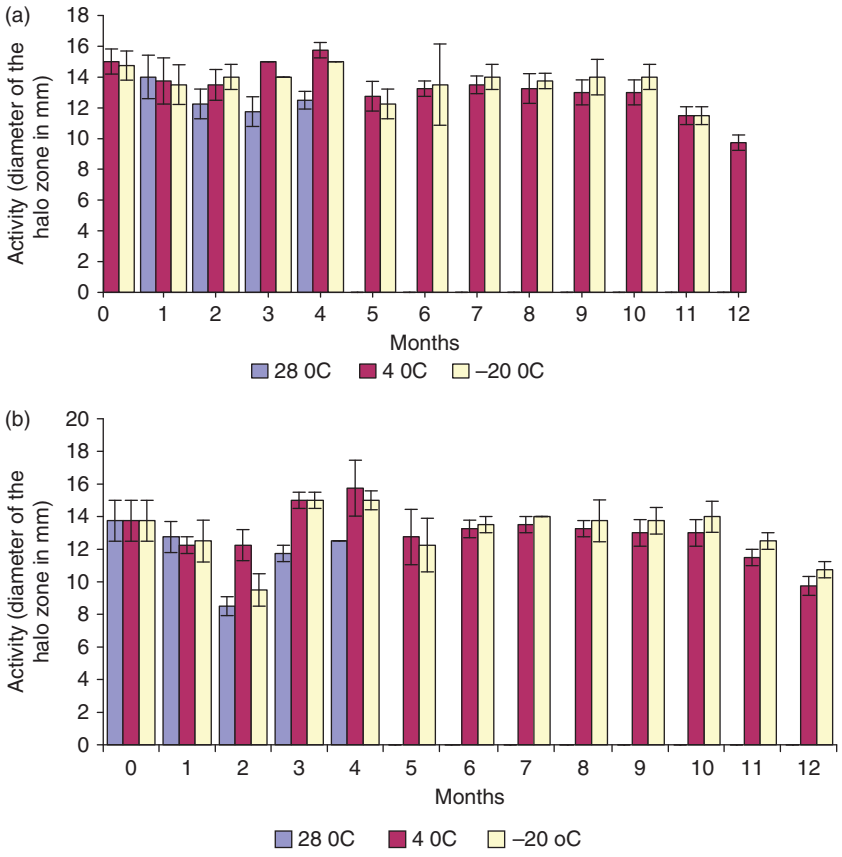

Figure 1 Shelf-life of cell-free supernatants of *Pseudomonas* MCCB 102 (a) and *Pseudomonas* MCCB 103 (b). MCCB, Microbial Culture Collection of Bacteria of National Centre for Aquatic Animal Health.

Table 2 R_f value of antivibrio compound in different solvent systems

	R_f value of antivibrio compound in different solvent systems		
Antagonistic cultures	**Solvent system A [1-butanol–acetic acid–water (4:1:1, v/v/v]**	**Solvent system B [chloroform–methanol (1:1, v/v)]**	**Solvent system C [ethyl acetate–acetic acid–water (3:2:1, v/v/v)]**
Pseudomonas MCCB 102	0.35 ± 0.06	0.57 ± 0.04	0.25 ± 0.02
Pseudomonas MCCB 103	0.37 ± 0.005	0.61 ± 0.05	0.25 ± 0.01

MCCB, Microbial Culture Collection of Bacteria of National Centre for Aquatic Animal Health.

at 121 °C. On exposing the culture supernatant to pH 2, 4, 6, 8 and 10, the antagonistic activity remained unaltered compared with that of the control (Table 1).

On storage at − 20 and 4 °C, the inhibitory activity of the culture supernatant could be retained for 12 months, after which a slow decrease was observed. Meanwhile, at room temperature, the activity was found to be considerably declined after 4 months (Fig. 1).

The inhibitory compound could be extracted to 1-butanol, dichloromethane and chloroform having the highest activity recorded in chloroform (Table 1). The inhibitory property was detected in chloroform only when extracted at a neutral pH. The supernatant at neutral pH after chloroform extraction did not show any inhibitory action.

On TLC separation of the inhibitory compound, the blue-coloured spots (dark pink in an acidic condition) alone showed an inhibitory property, with R_f values as shown in Table 2.

In HPLC, the retention time (RT value) of the inhibitory compound of *Pseudomonas* MCCB 102 was 31.6 min and that of *Pseudomonas* MCCB 103 was 31.4 min (Fig. 2), respectively, identical (within acceptable limits) to the RT of the *N*-methyl-1-hydroxyphenazine standard (30.8 min).

GC-MS analysis of the active compounds did not yield dependable data. However, LC-MS of the active

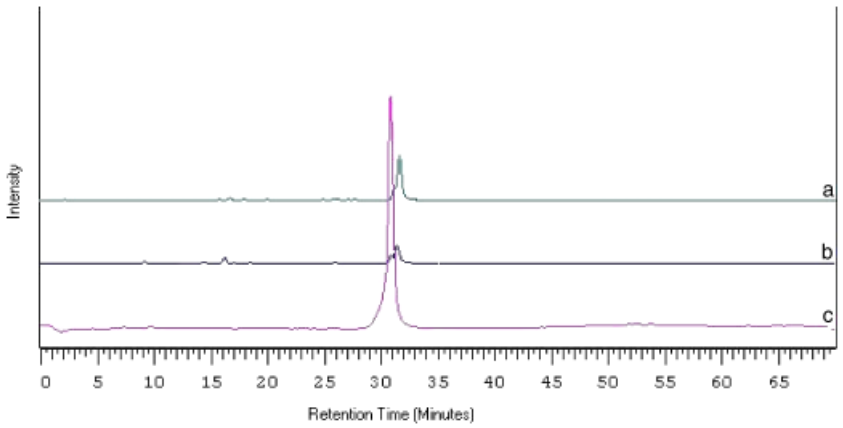

Figure 2 High-pressure liquid chromatography analysis of antivibrio compounds from *Pseudomonas* MCCB 102 (a), *Pseudomonas* MCCB 103 (b) and *N*-methyl-1- hydroxyphenazine standard (c). MCCB, Microbial Culture Collection of Bacteria of National Centre for Aquatic Animal Health.

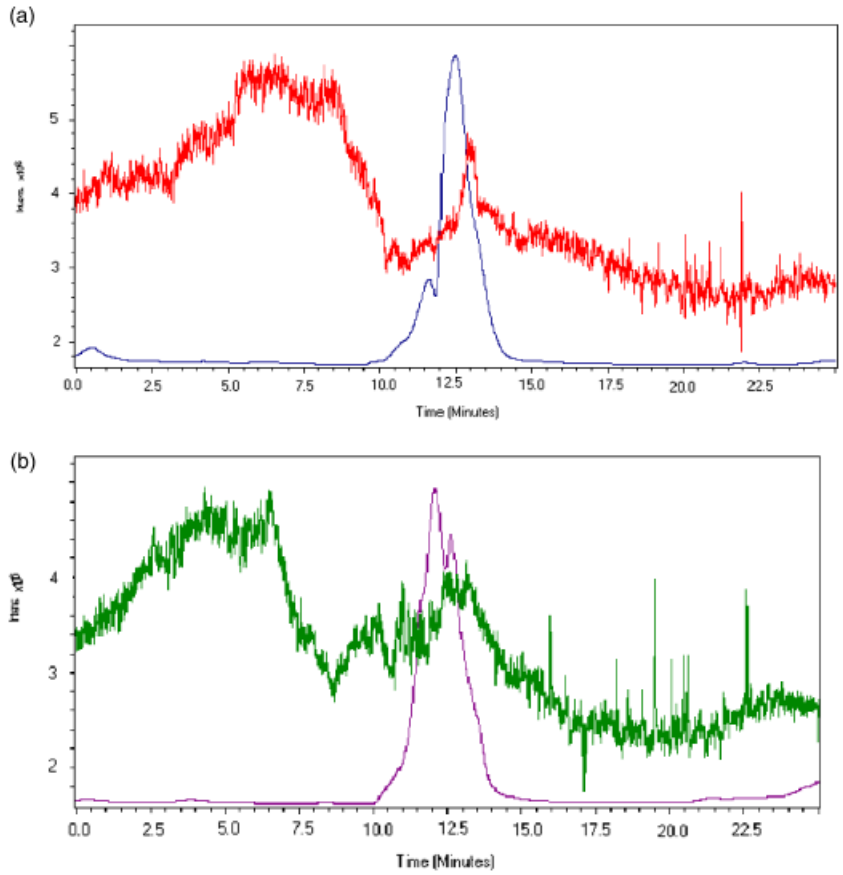

Figure 3 Liquid chromatograms of the antivibrio compounds from *Pseudomonas* MCCB 102 (a) and *Pseudomonas* MCCB 103 (b). MCCB, Microbial Culture Collection of Bacteria of National Centre for Aquatic Animal Health.

fraction and *N*-methyl-1-hydroxyphenazine standard exhibited an identical retention time (12.5 min) (Fig. 3). Further analysis of this peak by mass spectroscopy demonstrated a protonated molecular ion at *m*/*z* 211 (Fig. 4). Absorption maxima of the inhibitory compounds in solvents such as chloroform, 0.1 N HCl, methanol and dichloromethane (Table 3) confirmed the uniformity of the compounds in the supernatants

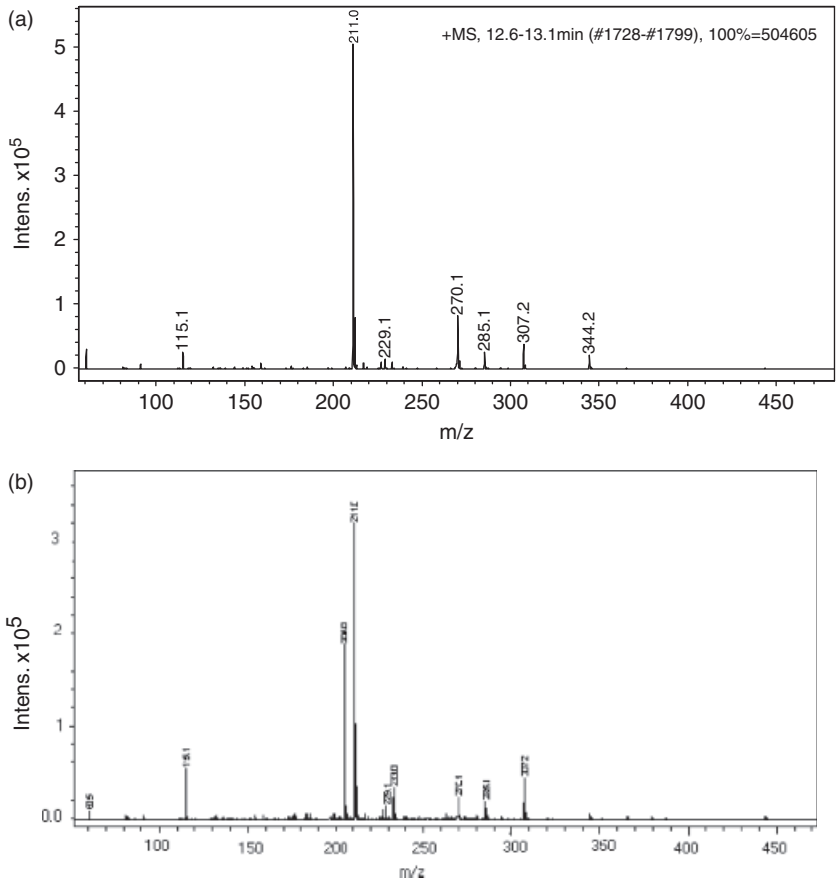

Figure 4 Mass Spectra of antivibrio compounds from *Pseudomonas* MCCB 102 (a) and *Pseudomonas* MCCB 103 (b). MCCB, Microbial Culture Collection of Bacteria of National Centre for Aquatic Animal Health.

of both the organisms. The absorption spectrum of *N*-methyl-1-hydroxyphenazine recorded in solvents such as chloroform and dichloromethane remained relatively unchanged. However, protonated *N*-methyl-1-hydroxyphenazine (recorded in 0.1 mol L^{-1} HCl) exhibited a large blue shift (170 nm). On the other hand, a substantial red shift (23 nm) was observed when the spectrum was recorded in a polar protic solvent such as methanol. These results were consistent with a zwitterionic structure in resonance with a semiquinoid structure for *N*-methyl-1-hydroxyphenazine. It appeared that the zwitterionic form was more predominant in polar protic solvents, where it was further stabilized by hydrogen bonding. The ^{1}H NMR spectrum is given in Fig. 5. The peaks observed in the aromatic region (δ 7–9) and the methyl protons, which appeared as a broad peak at δ 3.9, were identical to that of *N*-methyl-1-hydroxyphenazine itself (Fig. 6).

On testing the dose-dependent activity of the antivibrio compound in microwell plates, at 0.5 mg L^{-1},

Table 3 Absorption maxima of antivibrio compound from *Pseudomonas* MCCB 102 and *Pseudomonas* MCCB 103 in different solvents

	Antivibrio compounds	
Appearance	***Pseudomonas* MCCB 102 Blue in alkaline and pink/purple in acidic condition**	***Pseudomonas* MCCB 103 Blue in alkaline pink/purple in acidic condition**
Absorption maxima (nm)		
In chloroform	693, 327, 309, 265, 242	691, 327, 309, 264, 243
In hydrochloric acid (0.1 mol L^{-1})	520, 387, 278, 243, 205	522, 387, 278, 242, 203
In methanol	717, 321, 239, 209	717, 319, 239, 207
In dichloromethane	693, 327, 308, 260, 240	695, 326, 308, 238

MCCB, Microbial Culture Collection of Bacteria of National Centre for Aquatic Animal Health.

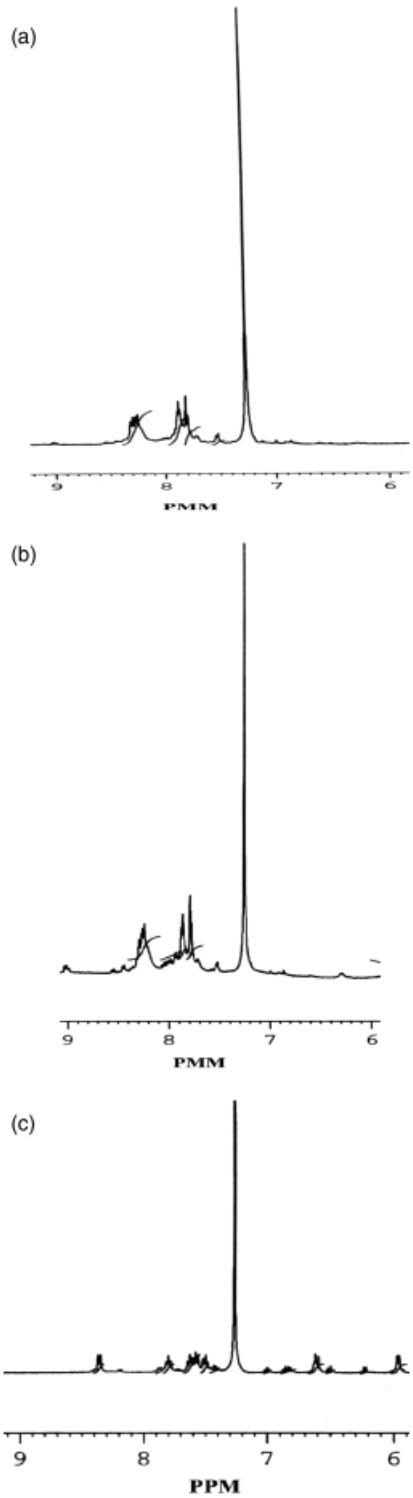

Figure 5 Nuclear Magnetic Resonance Spectra of the antivibrio compounds from MCCB 102 (a), MCCB 103 (b) and *N*-methyl-1- hydroxyphenazine standard (c). MCCB, Microbial Culture Collection of Bacteria of National Centre for Aquatic Animal Health.

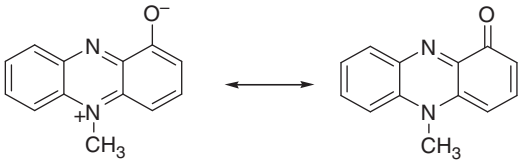

Figure 6 Structure of the antivibrio compound produced by *Pseudomonas* MCCB 102 and 103 derived based on UV–Vis, ^{1}H NMR and mass spectral data. MCCB, Microbial Culture Collection of Bacteria of National Centre for Aquatic Animal Health; ^{1}H NMR, nuclear magnetic resonance spectroscopy.

the growth of *V. harveyi* was inhibited by arresting the cell multiplication at 10^3 CFU mL^{-1} (Fig. 7). However, at 0.05 and 0.1 mg L^{-1}, the cell count increased to $> 10^9$ CFU mL^{-1}. On the contrary, at concentrations above 1 mg L^{-1}, the bacterial count reduced to $\leq$ 40 CFU mL^{-1}. The IC_{50} value of the compound in the primary haemocyte culture of *P. monodon* was found to be 1.4 $\pm$ 0.31 mg L^{-1} (Fig. 8).

Discussion

In vitro antagonism of *P. fluorescens* against *V. anguillarum* (Gram *et al.* 1999) and *Pseudomonas* I-2 against *V. harveyi* (Chythanya *et al.* 2002) and *Pseudomonas* MCCB 102 and 103 against a range of vibrios (Jayaprakash *et al.* 2005; Vijayan *et al.* 2006) points to the possibility of using those organisms and their products as viable alternatives to the conventional antibiotics in aquaculture. However, a detailed investigation of the nature of the inhibitory compounds produced by *Pseudomonas* was required. In general, the antibacterial property of the antagonistic bacteria is either due to the production of antibiotics (Raaijmakers *et al.* 1997), bacteriocins (Parret, Schoofs, Proost & De Mot 2003), siderophores (Gram *et al.* 1999), lysozymes, proteases, hydrogen peroxide and/or alteration in pH (Sugita, Shibuya, Shimooka & Deguchi 1996).

On the basis of spectral and chromatographic data, we have now identified the bioactive compound produced by P*seudomonas* MCCB 102 and 103 as *N*-methyl-1-hydroxyphenazine, a secondary metabolite having a wide spectrum of antibacterial activity produced by *Pseudomonas* (Watson, Mac Dermot,Wilson, Cole & Taylor 1986; Vukomanovic, Zoutman, Stone, Marks, Brien & Nakatsu 1997; Norman *et al.* 2004) as the active ingredient. We ascribe the undependable data from GC-MS analysis to the low volatility/stabililty of the compound under the conditions used for the analysis. However, our findings are consistent

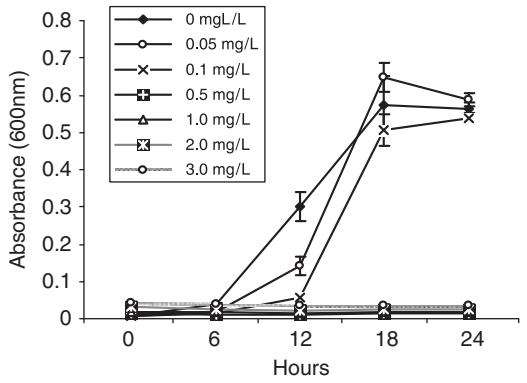

Figure 7 Growth of *Vibrio harveyi* at different concentrations of inhibitory compound.

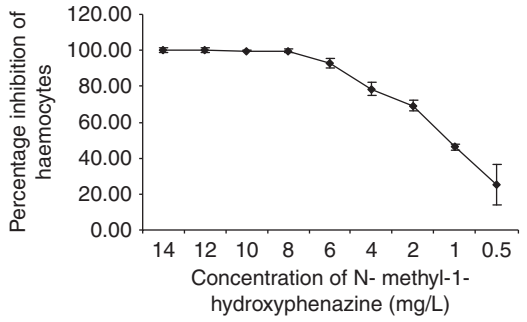

Figure 8 Cytotoxicity of the antivibrio compound in *Penaeus monodon* haemocyte culture.

with those reported by Watson *et al.* (1986). Mass spectra confirmed that *N*-methyl-1-hydroxyphenazine (MW = 210) was the active component isolated from *Pseudomonas* MCCB 102 and MCCB 103. The absorption spectral data observed by us were consistent with that of *N*-methyl-1-hydroxyphenazinee described by Fernandez and Pizarro (1997); the aromatic region (δ 7–9) exhibited a good correspondence with those reported in the literature (Rao & Sureshkumar 2000). The methyl protons appeared as a broad peak at δ 3.9. Based on these data, we concluded that the active ingredients present in the extracts from *Pseudomonas* MCCB 102 and MCCB 103 were *N*-methyl-1-hydroxyphenazine itself.

Many species of *Vibrio* are found to be pathogenic to shrimps/prawns and are reported to be resistant to antibiotics. Among them, *V. harveyi* has been reported as the most important aquaculture pathogen with multiple antibiotic resistance, causing mass mortalities in shrimp/prawn hatcheries (Tendencia & de la Pena 2001). However, *in vitro* tests suggested that *N*-methyl-1-hydroxyphenazine was a potential inhibitory compound and it could arrest the growth of *V. harveyi* at a low concentration (0.5 mg L^{-1}). The IC_{50} value of pyocyanin in *P. monodon* haemocyte culture was found to be 1.4 ± 0.31 mg L^{-1}. These data suggest that the compound can be applied as a prophylactic in the rearing water of cultured aquatic species, especially shrimp, at 0.5 mg L^{-1}. The antibacterial and antifungal property of phynazine antibiotic, *N*-methyl-1-hydroxyphenazine, has been described against human and plant pathogens (Baron & Rowe 1981; Kerr, Taylor, Rutman, Hoiby, Cole & Wilson 1999). This is the first report on its antivibrio property in aquaculture pathogens.

N-methyl-1-hydroxyphenazine as such in the cell-free supernatant can be stored at 4 °C for 1 year without losing its inhibitory property. The persistence of antivibrio property up to 100 °C, shelf-life at 4 °C, stability in different organic solvents and varying pH make the molecule ideal for aquaculture application. The significant reduction ($P < 0.05$) obtained after autoclaving for 15 min at 121 °C suggests the synergistic effect of temperature and pressure on the compound. This is unlikely to interfere with its application as the feed materials are seldom autoclaved. The activity of the compound was also found to be stable after treatments with proteases such as α-chymotrypsin, trypsin, proteinase K and pronase E (Jayaprakash 2005). Moreover, the bacteriostatic nature of the molecule at a low concentration of 0.5 mg L^{-1} makes it an ideal prophylactic. Currently, the organism has been made available as a commercial product named PS-1^{TM} for use in aquaculture ponds in the event of luminous vibriosis. The culture (300 mL) is brewed in 100 L pond water by supplementing with boiled spent feed 250 g and 100 g rice bran for 24 h and broadcasted over a 1 ha area with 70 cm water depth. As the next step, it is planned to undertake studies on its pharmacodynamics and pharmacokinetics as an aquaculture drug.

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