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Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain

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Abstract

A marine bacterial strain, *Pseudomonas* I-2, produced inhibitory compounds against shrimp pathogenic vibrios including *Vibrio harveyi*, *V. fluvialis*, *V. parahaemolyticus*, *V. damsela* and *V. vulnificus*. The inhibitory substance was found to be a low molecular weight compound, heat stable, soluble in chloroform and resistant to proteolytic enzymes. The chloroform extract brought down *V. harveyi* levels in water by over a log unit when applied at 20 μg/ml while the extract did not affect shrimp larvae even at 50 μg/ml level. This marine *Pseudomonas* I-2 has potential applications for control of shrimp pathogenic vibrios in aquaculture systems. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Pseudomonas*; Pyocyanine; *Vibrio*; Probiotics

1. Introduction

Vibrios are among the most important bacterial pathogens of cultured shrimp responsible for a number of diseases, and mortalities up to 100% have been reported due to vibriosis (Lightner, 1983). Use of antibiotics to control these agents has led to problems of drug resistance (Karunasagar et al., 1994). The development and use of probiotics is being recognised as a useful strategy to combat fish and shrimp pathogens. Application of probiotics has a long standing history in the poultry industry and was based on the competitive exclusion of *Salmonella* (Stavric et al., 1987). In aquaculture, the use of probiotics is not as popular as in livestock and poultry but some studies have

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revealed their potential as an effective strategy in combating diseases in aquaculture (Gatesoupe, 1999; Gram et al., 1999). Nogami and Maeda (1992) and Maeda and Liao (1992) noted that a bacterium, *Thalassobacter utilis*, increased the survival of shrimp (*Penaeus monodon*) and crab (*Portunus tritubuculatus*) larvae and suppressed the pathogens *Vibrio anguillarum* and *Haliphthoros* sp. Use of lactic acid bacteria in larval rearing of *Scophthalmus maximus* has been shown to bring about increased resistance of larvae to pathogenic vibrios (Gatesoupe, 1994). The use of a probiotic strain of *V. alginolyticus* conferred greater resistance in *Salmo salar* to challenge with *Aeromonas salmonicida*, *V. anguillarum* and *V. ordalii* (Austin et al., 1995). Riquelme et al. (1997) have shown that a strain of *Vibrio* sp. was able to confer protection in scallop larvae against *Vibrio anguillarum* and related pathogens, when pretreated. Bacteria showing antagonistic activity have potential application as biocontrol agents. Sugita et al. (1998) isolated a strain of *Bacillus* spp. that was antagonistic to 63% of the bacterial isolates from fish intestine. Some bacteria have been observed to be antagonistic to even viruses (Kamei et al., 1987, 1988; Direkbusarakom et al., 1998) and such bacteria have potential use in biocontrol of viral diseases (Maeda et al., 1997). Pseudomonads are common inhabitants of the aquatic environment including shrimp culture ponds (Otta et al.,1999) and are commonly associated with gills, skin and intestinal tract of live fish (Cahill, 1990). Smith and Devey (1993) noted that bathing Atlantic salmon presmolts in a strain of *Pseudomonas fluorescens* reduced subsequent mortality from stress-induced furunculosis. The antagonistic activity of *Pseudomonas* against a number of pathogens has been reported in literature (Troller and Frazier, 1963; Daly et al., 1973). In the present communication, the antivibrio activity of a marine *Pseudomonas* I-2 strain is reported.

2. Materials and methods

The marine *Pseudomonas* designated I-2 used in this study was isolated on Zobell Marine Agar (HiMedia, Mumbai) from an estuarine water sample collected near Mangalore, India, and was maintained in tryptone soya agar supplemented with 1% (w/v) sodium chloride (TSAS) overlaid with sterile liquid paraffin. This organism was identified as *P. aeruginosa* based on the characteristics described in the *Bergey's Manual of Systematic Bacteriology* which included its ability to grow at 42 °C, sugar fermentation reactions and production of pyocyanine pigment (Krieg and Holt, 1984). Before each experiment, the culture was activated in tryptone soya broth (Himedia, Mumbai) supplemented with 1% sodium chloride (TSBS) and then grown on TSBS for 18–24 h at 30 °C. The shrimp pathogen *Vibrio harveyi* used for all the experiments was isolated on luminescent agar (West and Colwell, 1984) from moribund *P. monodon* larvae. The isolate was characterized by biochemical reactions as described earlier (Karunasagar et al., 1994). Other vibrios such as *V. vulnificus*, *V. parahaemolyticus*, *V. damsela* and *V. fluvialis* isolated from moribund shrimps on Thiosulphate citrate bile salt sucrose (TCBS) agar (Himedia, Mumbai) and identified in our laboratory using biochemical reactions (Farmer and Hickman-Brenner, 1992) were also used in the study. All the cultures were maintained in TSAS butt overlaid with sterile liquid paraffin.

2.1. Study of inhibition by well diffusion, disc diffusion and cross-streak methods

A lawn culture of *Vibrio harveyi* was prepared by pouring 2 ml of a young culture (16–18 h in TSBS) over the TSAS medium, draining the liquid and air drying the plate in the incubator (30 °C) for 15 min. Three-millimeter-diameter wells were punched in the plates using a sterile gel puncher. Thirty microliters of an 18-h culture of *Pseudomonas* I-2 in TSBS was pipetted into the wells and plates were incubated for 24 h. Zone of inhibition around the wells and presence of luminescence was recorded. For study of inhibition by disc diffusion method, the cell free supernatant of a 24-h culture of *Pseudomonas* I-2 in TSBS was prepared by centrifuging at $10{,}000 \times g$ for 10 min and filtering through 0.22 μm Millipore membrane (Millipore, USA). 6.25 mm discs punched from Whatman filter paper No. 1 and sterilized in the hot air oven at 140 °C for 1 h were dipped in the cell free supernatant and dried for 15 min in the incubator at 37 °C. The impregnated discs were placed on a lawn of *V. harveyi* culture prepared on TSAS plates and incubation continued for 24 h at 30 °C to record the zone of inhibition and luminescence around the discs.

For the study of inhibition by cross-streak method, an 18-h culture of *Pseudomonas* in TSBS was streaked as a 2-cm-thick band, across the diameter of the TSAS plate. After incubation for 24 h at 30 °C, the growth was scraped with a sterile slide. The remaining bacteria were killed by exposure to 5 ml chloroform poured on the glass lid and left for 15 min by keeping the medium inverted over the lid. The plates were then air dried for about 10 min to remove any residual chloroform and five different *Vibrio* cultures viz. *V. harveyi*, *V. parahaemolyticus*, *V. damsela*, *V. fluvialis* and *V. vulnificus* were streaked perpendicular to *Pseudomonas* I-2 band using sterile glass rod dipped in an 18-h-old culture. The plates were incubated for 24 h at 30 °C. The linear zone of inhibition was recorded in each case.

2.2. Effect of cell free supernatant of Pseudomonas I-2 on washed cells of V. harveyi in sterile physiological saline

The cell free supernatant of *Pseudomonas* was prepared as described above. Four 250-ml flasks containing 100 ml of physiological saline were sterilised by autoclaving at 121 °C for 20 min and designated I, II, III and IV. Washed cell suspension of *V. harveyi* was added to all the beakers to get a cell density of approximately 10^7 cells/ml. 10 ml, 5 ml and 1 ml of the *Pseudomonas* I-2 cell free supernatant were added to beakers I, II and III, respectively, while beaker IV without any cell supernatant added served as control. *V. harveyi* cells were enumerated at 0, 12 and 24 h on TSAS by standard spread plate method.

2.3. Pathogenicity of Pseudomonas I-2 to shrimp larvae

An 18-h culture of *Pseudomonas* I-2 in TSBS was centrifuged, cells washed in saline and the pellet resuspended to the original volume in saline. The cell numbers in the suspension was determined by plating serial 10-fold dilution in TSAS by spread plate

method. The cell density was observed to be around 10^8/ml. A suitable number of cells were added to four 1-l beaker containing 500 ml filter (0.45 μm) sterilised sea water to obtain 10^6, 10^5, 10^4 and 10^3 cells/ml. The control beaker did not have any bacterial inoculum. To each of the five beakers, 26 postlarvae (PL-18) of *P. monodon* were introduced and the larvae were monitored for any signs of disease or mortality up to 96 h.

2.4. Preliminary characterisation of the antibacterial component in cell free supernatant of Pseudomonas I-2

Ultrafiltration of the cell free supernatant of *Pseudomonas* I-2 was carried out using a low protein binding regenerated cellulose membrane (molecular weight cut off 10,000, UF P2, NIKON, Millipore, Japan) and the residue was washed by passing 1 ml of phosphate buffered saline. The residue was resuspended in the same volume of saline and tested for antibacterial activity by well diffusion method.

The sensitivity of the antibacterial principle to proteolytic enzymes was tested using pepsin (HiMedia, Mumbai) and trypsin (Central Drug House, Mumbai) at a level of 50 and 100 mg/ml, respectively. The mixture of culture supernatant and enzyme was incubated for 1 h at 30 °C and tested for antivibrio activity against *V. harveyi* by well diffusion method.

The thermotolerance of the antibacterial component was tested by heating the cell free supernatant in a boiling water bath for 5 min, 15 min, 30 min and 1 h. Antibacterial activity was measured by well diffusion method.

2.5. Chloroform extraction of the cell free supernatant

One liter cell free supernatant of *Pseudomonas* I-2 was extracted with 300 ml chloroform, which was then flash evaporated at 65 °C to concentrate the material. The residue was dissolved in 10 ml buffered physiological saline. The amount of residue was calculated by separately evaporating 5 ml chloroform extract in cup made of aluminium foil that was weighed before and after evaporation. The minimal inhibitory concentration (MIC) of chloroform extract to *V. harveyi* was determined as follows: eight test tubes each containing 5 ml of sterile TSBS were taken and chloroform extract was added to the tubes to get a concentration ranging from 0 to 80 μg/ml. To these tubes, 10 μl of an overnight culture of *V. harveyi* was added and incubated for 18 h at 30 °C and the tubes were observed visually for turbidity.

2.6. Effect of chloroform extract on V. harveyi cells in sterile sea water

Six conical flasks each containing 50 ml of sea water were taken and designated A, B, C, D, E and F. Washed cell suspension of *V. harveyi* was then added to each of the flasks to get a cell density of approximately 10^6 cells/ml. Chloroform extract was added to give a concentration of 5, 10, 20, 40, 60 and 80 μg/ml of residue to the flasks A, B, C, D and E, respectively, and flask F served as control. *V. harveyi* cells were enumerated at 0, 6 and 12 h on TSAS plates by spread plate method.

2.7. Toxicity of chloroform extract to shrimp postlarvae

500 ml filter-sterilised sea water was taken in each of four beakers and 10 postlarvae of *P. monodon* (PL-18) were placed in each beaker. In two beakers, chloroform extract of *Pseudomonas* I-2 was added at 50 ppm level while the other two served as control. Larvae were observed for mortality for 96 h.

2.8. Influence of growth conditions on production of inhibitor

The influence of pH, salt, temperature and time on the antivibrio activity of *Pseudomonas* I-2 was studied. Sterilised TSBS adjusted to pH 4, 5, 6, 7, 8 and 9 was inoculated with 0.1 ml of an 18-h old culture of *Pseudomonas* I-2 and incubated for 24 h at 30 °C. Similarly, TSBS with 0%, 1%, 2%, 3% and 4% sodium chloride concentration was prepared and *Pseudomonas* I-2 inoculated and incubated as described above. Four TSBS flasks inoculated with *Pseudomonas* I-2 were incubated at 10, 20, 30 and 37 °C for 24 h and cell free supernatant was obtained by centrifugation followed by filtration through 0.22 μm membrane to study the antivibrio activity. From the culture incubated at 30 °C, samples were drawn at 6, 12, 24, 30, 36, 48 and 72 h to study the relation between the growth phase and production of inhibitor.

3. Results

Pseudomonas I-2 showed good inhibition of *V. harveyi* both in well and disc diffusion assays. In well diffusion assay, the diameter of zone of inhibition was 17 mm whereas by disc diffusion assay, the zone of inhibition measured 9 mm. *Pseudomonas* I-2 inhibited all the five pathogenic *Vibrios* tested by cross-streak method. *V. vulnificus* was inhibited to the maximum extent (50 mm) followed by *V. harveyi* (45 mm), *V. damsela* (42 mm), *V. fluvialis* (40 mm) and *V. parahaemolyticus* (30 mm).

The effect of cell free supernatant of *Pseudomonas* I-2 on washed cells of *V. harveyi* in sterile saline is given in Table 1. Addition of 10 ml of cell free supernatant of *Pseudomonas* I-2 to *V. harveyi* resulted in complete suppression of *V. harveyi* within 12 h. Lower volumes of cell free supernatant added to *V. harveyi* had no inhibitory effect. As shown in Table 2, the survival of shrimp post larvae was not affected by the presence of *Pseudomonas* I-2 in sea water.

Table 1
Effect of cell free supernatant of *Pseudomonas* I-2 on washed cells of *V. harveyi* in sterile saline

Saline no.	Volume of crude extract (ml)	*V. harveyi* counts/ml at		
		0 h	12 h	24 h
I	10	1.62×10^7	$< 1.00 \times 10^1$	$< 1.00 \times 10^1$
II	5	1.69×10^7	2.70×10^6	1.55×10^8
III	1	2.50×10^7	3.60×10^7	6.10×10^7
IV	0	2.99×10^7	6.50×10^7	2.10×10^6

Table 2
Effect of *Pseudomonas* I-2 culture on survival of shrimp larvae

Pseudomonas cells/ml	No. of larvae dead/no. tested				
	12 h	24 h	48 h	72 h	96 h
10^6	0/26	5/26	5/26	7/26	10/26
Nil	0/26	3/26	6/26	8/26	10/26

When the cell free supernatant was ultrafiltered using cut off at 10 kD, the filtrate did not have any effect thus indicating that the inhibitory component has a molecular weight of less than 10 kD.

The proteolytic enzymes trypsin and pepsin used at 100 and 50 mg/ml, respectively, did not affect the activity of the antibacterial component in the cell free supernatant and the zone of inhibition observed was equal to the untreated cell free supernatant that served as control. Study of the heat stability of the antivibrio principle showed that even when heated in a water bath at 100 °C for an hour, there was no loss of activity. The inhibitory activity of cell free supernatant of *Pseudomonas* I-2 to *V. harveyi* was observed in both nutrient broth and sea water. In nutrient broth, MIC of chloroform extract for *V. harveyi* was 50 μg/ml. From the results in Table 3, it can be seen that the chloroform extract at 10 μg/ml brought down *V. harveyi* count in sea water by 1 log unit within 12 h. At higher concentration of 40 μg/ml and onwards, *V. harveyi* counts were reduced by 2 log units in 12 h.

The effect of pH on the growth of *Pseudomonas* I-2 is presented in Table 4. Growth was observed at pH 6, 7, 8 and 9 but in varying degrees. pH 7 was found to be optimum followed by pH 8. Very slight turbidity was observed in pH 6 and pH 9, but in pH 4 and pH 5 there was no growth at all. pH 7 which showed maximum growth also showed maximum zone of inhibition of 16 mm against *V. harveyi*. Inhibition zone observed at pH 6 and pH 9 was 12 and 8 mm, respectively.

Table 5 shows the effect of temperature on the production of antibacterial component by *Pseudomonas* I-2. There was no growth at 10 °C and minimal growth at 20 °C. Maximum turbidity was observed at 30 °C and 37 °C and production of antivibrio factor as indicated by the zones of inhibition which correlated well with turbidity.

Effect of NaCl concentration of the growth media on the production of antibacterial component is presented in Table 5. TSB with 1% NaCl was optimum for growth followed

Table 3
Effect of chloroform extract on *V. harveyi* in sea water

Time (h)	Nil	Concentration of the chloroform extract (μg/ml)					
		5	10	20	40	60	80
0	1.13×10^6	1.13×10^6	1.13×10^6	1.13×10^6	1.13×10^6	1.13×10^6	1.13×10^6
6	3.19×10^6	1.13×10^6	6.64×10^5	6.50×10^4	5.50×10^4	1.65×10^4	1.24×10^4
12	1.19×10^6	6.12×10^5	6.15×10^5	5.12×10^4	2.16×10^4	2.15×10^4	2.10×10^4

Table 4
Effect of pH on the growth of *Pseudomonas* I-2 and production of antivibrio component

pH	Growth	Zone of inhibition (mm) at		
		12 h	24 h	48 h
4	–	0	0	0
5	–	0	0	0
6	+	0	0	12
7	+++	0	13	16
8	++	0	0	10
9	+	0	0	8

+: Visible growth; ++: moderate growth; +++: excellent growth.

Table 5
Effect of growth temperature and salt concentration on the growth of *Pseudomonas* I-2 and production of antivibrio component

Parameter	Growth	Zone of inhibition (mm) at	
		12 h	24 h
Temperature of incubation 0 °C			
10	–	Nil	Nil
20	+	10	12
30	+++	17	18
37	+++	17	18
Salt concentration in percentage			
0	+	Nil	Nil
1	+++	11	18
2	++	9	17
3	+	Nil	Nil
4	–	Nil	Nil

+: Visible growth; ++: moderate growth; +++: excellent growth.

Table 6
Effect of duration of incubation on the production of antivibrio component

Duration of incubation (h)	Cfu/ml	Zone of inhibition (mm)
0	1.26×10^6	0
6	7.45×10^7	0
12	1.97×10^9	14
24	2.40×10^9	17
30	2.48×10^9	18
36	2.14×10^9	18
48	1.42×10^9	18
72	4.50×10^8	18

by TSB containing 2% NaCl and the zone of inhibition was 18 and 17 mm, respectively. Minimal growth was observed in TSB containing 3% NaCl with no antivibrio activity. TSB with 4% NaCl did not permit growth of the organism. Results in the Table 6 show that the culture reached a stationary phase at 12 h but maximum antivibrio activity was observed only at 30 h.

4. Discussion

Pseudomonads are commonly associated with seawater and even *Pseudomonas aeruginosa* may be recovered from water, soil and plants (Pellet et al., 1983). The *P. aeruginosa* strain I-2 isolated in this study inhibited all the pathogenic vibrios tested. The results of well diffusion and cross-streak methods show that it is not the bacterial cell but an extracellular product that is likely to be responsible for inhibition. Antagonistic activity of *Pseudomonas* against a number of pathogens such as *Salmonella*, *Staphylococcus aureus* and *V. parahaemolyticus* has been reported in the literature (Seminiano and Frazier, 1960; Troller and Frazier, 1963; Goatcher and Westhoff, 1975; Daly et al., 1973; Oblinger and Kreft, 1990). Champomier-Verges and Richard (1994) noted that a *Pseudomonas* strain of fish origin inhibited a number of *Pseudomonas* strains involved in meat spoilage. The results in our study show that *Pseudomonas* I-2 isolated from the marine environment produced a wide zone of inhibition against several *Vibrio* sp. tested. Addition of cell free supernatant of *Pseudomonas* I-2 to *V. harveyi* suspended in sterile saline completely killed the latter in 12 h (Table 1). It can be suggested that *Pseudomonas* I-2 produces some extracellular anti-vibrio component. These results are very significant since *V. harveyi* is a great problem in shrimp hatcheries. One of the most important criteria for a candidate to be used in biocontrol is that the organism should be nonpathogenic to the host. Results in this study (Table 2) have clearly shown that *Pseudomonas* I-2 is nonpathogenic to shrimp larvae even at very high levels of 10^6 cells/ml. Characterisation of the inhibitory component in the cell free extract of *Pseudomonas* I-2 suggests that it is a low molecular weight, nonproteinaceous heat stable compound.

The inhibitory effect of chloroform extract of *Pseudomonas* I-2 cell free supernatant clearly indicates that the inhibition of vibrios is due to a chloroform soluble substance. Kumar et al. (1997) have reported that the inhibitory activity of *P. aeruginosa* against *S. aureus* could be due to pyocyanine. Pyocyanine is a chloroform soluble substance and the results in this study (Table 3) also indicate that pyocyanine could be the antibacterial principle responsible for the inhibition of vibrios. The chloroform extract did not exhibit any toxicity to shrimp larvae even at 50 μg/ml while a concentration of 20 μg/ml was adequate to bring down the level of *V. harveyi* by nearly 2 log units in seawater. Therefore, it can be suggested that the chloroform extract of *Pseudomonas* I-2 could be used to treat water whenever *V. harveyi* counts rise to undesirable levels in sea water systems. This would find application in shrimp hatcheries where vibriosis of larvae is a common problem.

The maximum production of antivibrio factor was observed at pH 7.0 (Table 4) and at temperature of 30–37 °C (Table 5) which was also the optimum pH and temperature for the growth of the organism. This suggests a close relation between growth of *Pseu-*

domonas I-2 and production of antivibrio factor. This is further confirmed by results in Table 5, which show that production of antivibrio factor was maximum at a NaCl concentration of 1% which was optimal for growth of the organism. The appearance of the antivibrio activity in the medium when the cells reach stationary phase of growth and maximum activity at late stationary phase (Table 6) suggests that the antivibrio factor is a secondary metabolite.

In conclusion, it can be stated that the *Pseudomonas* I-2 strain has the properties of a biocontrol agent for use in shrimp hatcheries and farms. There is also a potential to use the chloroform extract of this strain for the control of luminous *Vibrio* spp. in aquaculture systems.

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