Antibacterial marine bacterium deter luminous vibriosis in shrimp larvae

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Abstract

Inhibitory activity of a marine pigmented bacterium - *Alteromonas* sp. - isolated from *Penaeus monodon* Fabricius larva against pathogenic and environmental isolates of *Vibrio harveyi* was studied. All the isolates were inhibited to varying degrees by *Alteromonas* sp. *in vitro*. The antibacterial substance produced by the *Alteromonas* sp. was soluble in organic solvent and closely bound to the external surface of bacterial cells. The antibacterial *Alteromonas* sp., when allowed to colonize on shrimp larvae, suppressed the activity of *V. harveyi* M3 and reduced mortality of *P. monodon* larvae *in vivo*.

Introduction

The sea is an immense and practically unexploited source of new potentially useful biologically active substances. Given the fact that the oceans cover more than 70 percent of the earth's surface, the oceans are a promising source of novel pharmacologically active compounds. Although macro organisms of the ocean have proved to be good sources of novel bioactive metabolites, large-scale productions of these bioactive metabolites has been difficult (Bernan et al. 1997). Marine microorganisms such as bacteria and fungi have been reported to produce antibacterial (Rosenfeld and Zobell 1947), antifungal, antiviral and antitumor substances (Bernan et al. 1997). Several earlier studies have suggested that such marine bacteria can be used to combat epizootics in aquaculture systems (Maeda and Liao 1992; Maeda 1994; Douillet and Langdon 1994; Abraham at al. 2001). This paper reports on the extraction and characterization of an antibacterial substance from a pigmented marine bacterium and the control of *Vibrio harveyi* infection in *Penaeus monodon* larvae.

Materials and Methods

Bacterial strains

The antibacterial marine pigmented bacterium (*Alteromonas* sp. P7) was isolated from hatchery raised mysis 2 larva of *P. monodon* on seawater (75%), yeast extract (0.3% w/v) peptone (0.5% w/v) - SYEP - agar supplemented with glycerol (0.3% v/v), and characterized following Baumann et al. (1984). The luminous *Vibrio harveyi* isolates (n = 80) were from hatchery raised diseased *P. monodon* and *P. indicus* larvae and their environment (Abraham et al. 1999). All the isolates were routinely maintained and propagated on SYEP agar. An antibiotic resistant pathogenic luminous *V. harveyi* strain M3 isolated from diseased *P. indicus* mysis larva was used for further *in vitro* and *in vivo* experimentation with *Alteromonas* sp. The minimal inhibitory concentration (MIC) of antibiotics was determined by the agar dilution method on Muller Hinton agar supplemented with 1% w/v sodium chloride (Abraham et al. 1997).

Vibriostatic activity of *Alteromonas* sp. P7

In vitro vibriostatic activity of *Alteromonas* sp. P7 was tested against *V. harveyi* isolates (n = 80) by cross streak technique as described in Lemos et al. (1985) on SYEP agar. Vibriostatic activity was also tested by double agar layer method as described by Dopazo et al. (1988) with slight modification. Macro colonies of *Alteromonas* sp. P7 were developed on SYEP medium containing 1.2% agar by inoculating 3 µl of 18 h old SYEP broth culture. The colonies were killed after 48 hours by exposure to chloroform vapour for 20 minutes. The plates with chloroform-killed colonies were overlaid with 8 ml of soft agar (SYEP broth + 0.7% agar) seeded with 8 µl of *V. harveyi* strain M3 grown for 18 hours in SYEP broth. The plates were incubated for another 24 hours at 30±1ºC and observed for zone of inhibition around the macro colonies. Control plates without the macro colonies of *Alteromonas* sp. P7 were also used to evaluate the possible effect of chloroform on the growth of *V. harveyi* M3.

Extraction of antibacterial substance

The methods described in McCarthy et al. (1994) were followed by ammonium sulphate precipitation. Overnight culture of *Alteromonas* sp. was inoculated at 1.0% level into 300 ml each of SYEP broth and incubated at 30±1ºC for 24 hours. The cells were removed by centrifugation at 10 000 rpm for 15 minutes at 4ºC. The supernatant fractions were subsequently filtered through a 0.22 µ membrane filter to remove cellular debris. About 100 ml of cell free supernatant was concentrated up to 70% saturation by slow addition of ammonium sulphate, held overnight at 4ºC and centrifuged at 10 000 rpm for 15 minutes at 4ºC. The pellets were collected

and resuspended in 10 mM phosphate buffer containing 0.1 mM EDTA, pH 7.0. The concentrated precipitate was exhaustively dialyzed against the same buffer for 24 hours at 4ºC.

The ethyl acetate extraction as described in Wratten et al. (1977) was followed with modification. *Alteromonas* sp. was inoculated on to SYEP agar (1.2% agar) plates and incubated for 48 hours at 30±1ºC. The cells were then gently scraped off and washed with sterile seawater (17 ppt) by centrifugation at 10 000 rpm for 15 minutes at 4ºC. The cell pellet was suspended in 20 ml of ethyl acetate for 3 hours to extract the antibacterial substance. The cells were removed by centrifugation and the ethyl acetate extract was evaporated at 40-50ºC to get a concentrated crude extract. In addition, the spent agar medium was cut into small pieces, homogenized with ethyl acetate (50 ml) and allowed to stand for 3 hours without agitation at 30±1ºC. The solvent was decanted and evaporated at 40-50ºC to get a concentrated crude extract of the antibacterial substance.

Inhibitory activity of the crude extracts *in vitro*

The supernatant fraction of SYEP broth and crude extract of the antibacterial substance were tested for their inhibitory activity against *V. harveyi* strain M3 by well diffusion assay (Tagg and McGiven 1971). Wells of 6 mm diameter were made on hardened SYEP agar (1.2% agar) plates. The bottom of the wells was sealed with molten SYEP soft agar (0.7% agar). The wells were filled with 50 µl each of crude extract in ethyl acetate, dialyzed ammonium sulphate precipitate and cell free culture supernatants, and allowed to diffuse for 1-2 hours. The plates were overlaid with 8 ml of SYEP soft agar seeded with 8 µl of overnight-grown *V. harveyi* M3. The overlaid plates were incubated at 30±1ºC for 24 hours and examined for definite zone of inhibition (clearance) around the wells.

Inhibition of *V. harveyi in vivo*

Hatchery raised *P. monodon* larvae of 100 numbers each at zoea-2 stage were introduced into a series of troughs -3 sets in duplicate - filled with 5 l sand-filtered aged seawater (salinity: 35 ppt). The larvae were acclimatized for 24 hours and fed with *Skeletonema* sp. The set 1 was inoculated with *Alteromonas* sp. P7 at a level of 10^6 cells/ml of rearing medium to facilitate attachment/colonization on the larvae. *Vibrio harveyi* M3 was then inoculated into the troughs of set 1 after 3 hours of addition of *Alteromonas* sp. P7 and also in set 2 at a level of 10^4 cells/ml of rearing medium to serve as positive control. The set 3 received no bacterial inoculums and served as negative control. Mortality was recorded till the larvae reached post-larvae 1 stage and no water exchange was done during that period.

Results and Discussion

The antibacterial bacterium from *P. monodon* larva was a Gram negative, motile, cytochrome oxidase positive rod, which produced an orange pigment on seawater-based medium. Loss in pigmentation was observed when grown on non-seawater based medium. It was capable of growth up to 6% sodium chloride (NaCl). It failed to grow on thiosulphate citrate bile salt sucrose agar, MacConkey agar and medium without NaCl. This bacterium was negative for luminescence, amylase, gelatinase, catalase, urease, iodole production and nitrate reduction, and positive for citrate utilization. Carbohydrates such as arabinose, dextrose, galactose, lactose, mannitol, sorbitol, and sucrose were not utilized. These characteristics and a comparison with Baumann et al. (1984) place this bacterium under the genus *Alteromonas* and designated as *Altreomonas* sp. P7. As seen in Table 1, all the pathogenic and environmental *V. harveyi* isolates were sensitive to *Altreomonas* sp. P7, although with varying degrees of inhibition. Antibacterial activity among marine bacteria is a well-known phenomenon and demonstrated in a number of studies (Rosenfeld and Zobell 1947; Anderson et al. 1974; Lemos et al. 1985; Dopazo et al. 1988; McCarthy et al. 1994).

The shrimp larval pathogen *V. harveyi* M3 was resistant to antibiotics such as chloramphenicol, erythromycin, furazolidone, neomycin, prefuran, streptomycin, sulphadiazine and trimethoprim, exhibiting high MIC values (Table 2). The antibiotic resistant *V. harveyi* M3 exhibited a zone of inhibition of 4.00±0.25 and 7.25±0.83 mm by cross streak and double agar layer methods, respectively (Table 3). The cell free supernatants and the dialyzed ammonium sulphate precipitate did not display any

Table 1. *In-vitro* inhibitory activity of *Alteromonas* sp. against luminous *Vibrio harveyi* by cross streak technique.

Source	Numbers tested / Numbers inhibited (n=80)	Percent isolates exhibiting zone	
		1-10 mm	>10 mm
Diseased shrimp	39/39	51.28	48.72
Seawater	41/41	43.90	56.10

Table 2. Minimal inhibitory concentration (MIC) of antibiotics against *Vibrio harveyi* M3.

Antibiotics	MIC µg/ml
Chloramphenicol	75.00
Ciprofloxacin	0.39
Erythromycin	75.00
Furazolidone	100
Gentamycin	12.50
Nalidixic acid	6.25
Neomycin	50.00
Oxytetracycline	6.25
Prefuran	50.00
Polymyxin B	25.00
Streptomycin	100
Sulphadiazine	>100
Trimethoprim	>100

inhibitory activity against *V. harveyi* M3. No loss in antibacterial activity was observed on treatment with proteolytic enzyme, trypsin 2 000 units/g by modified spot-on-the-lawn method (Okereke and Montville 1991) on SYEP agar (Table 3). This indicated that the antibacterial substance might not be proteinaceous in nature although certain strains of *Alteromonas* have been reported to produce proteinaceous antibacterial substances (McCarthy et al. 1994). In an earlier study by Rosenfeld and Zobell (1947), the inhibitory activity was not expressed when cell free preparations were used, as the inhibitory compounds did not diffuse into the aqueous environment.

The antibacterial substance produced by *Alteromonas* P7 was recovered from the cells and the spent agar medium using ethyl acetate as solvent (Table 4). Burkholder et al. (1966) observed better antibiotic production by a marine bacterium on solid media. Since the antibacterial substance could be recovered from both the cells and spent agar medium, it can be inferred that the antibacterial substance may be bound to the outer cell surface and secreted into the solid medium. This corroborates with earlier observations (Rosenfeld and Zobell 1947; Lemos et al. 1985) that the inhibitory compounds remain closely bound to the cell. The production of antibiotic substance by *Alteromonas* sp. was observed to be a growth-associated phenomenon in an earlier study and probably released as secondary metabolite into the surrounding environment (Abraham et al. 2001). The specific role of the cell wall in the secretion process is still unknown. However, according to Lemos et al. (1985), if the antibiotics remain bound to the cells, they can be excreted slowly and continually to the environment, preventing colonization of the adjacent space by competitors. A rapid release of the antibiotic substance by antibiotic bacteria probably would not provide them any competitive advantage because it would be immediately washed away by the seawater. The observations of the present study further indicated that the antibacterial compound of *Alteromonas* P7 could be an organic compound or polysaccharide as other antibacterial substances recovered from marine bacteria using ethyl acetate have turned out to be a pyrole (Burkholder et al. 1966) or quinolinol (Wratten et al. 1977) or tyrosol and isatin (Gil-Turnes and Fenical 1992) or a polysaccharide (Anderson et al. 1974).

Table 3. Inhibitory activity of *Alteromonas* sp. against *Vibrio harveyi* M3 on seawater yeast extract peptone agar.

Technique	Zone of inhibition (mm)
Cross streak method	4.00 ± 0.25
Double agar layer method*	7.25 ± 0.83
Spot-on-the-lawn method with or without trypsin 2000 units/g	5.75 ± 0.30

Values are mean ± standard deviation of four observations.
* No inhibition was observed on control plates without Alteromonas sp. macrocolonies.

Table 4. Inhibitory activity of the crude extracts of the antibacterial substance from *Alteromonas* sp. on *Vibrio harveyi* M3.

Treatment	Zone of inhibition in test wells (mm)
Cell free supernatant of SYEP broth	0
Crude extract of ammonium sulphate precipitate, dialyzed from SYEP broth	0
Ethyl acetate crude extract from whole bacterial cell	21.50 ± 1.12
Ethyl acetate crude extract from spent SYEP agar	1.00 ± 0.50

SYEP: Seawater yeast extract peptone broth. Control wells with 50 µl each of sterile SYEP broth and ethyl acetate showed no zone of inhibition. Values are mean ± standard deviation of four observations.

Table 5. Mortalities in *Penaeus monodon* larvae: *In-vivo* inhibition of *Vibrio harveyi* M3 by *Alteromonas* sp.

Treatment	Mortality (%)
Alteromonas sp. @ 10^6/ml plus *Vibrio harveyi* @10^4/ml	59.0 ± 3.0^{a,b}
Vibrio harveyi @10^4/ml	72.0 ± 3.0^{a,c}
Uninoculated control	47.0 ± 2.0^{b,c}

Values are mean ± standard deviation of two observations. Mean values sharing common superscripts differed significantly. a and c: $P<0.01$; b: $P<0.05$.

The data presented in Table 5 revealed that the antibacterial bacterium, *Alteromonas* sp. P7 was capable of reducing the luminous *V. harveyi* induced mortality significantly in *P. monodon* larvae *in vivo*. *Vibrio harveyi* M3 affected about 72±3% mortality in *P. monodon* larvae in 72 hours. The *V. harveyi* induced mortality was, however, reduced significantly ($P<0.01$) to 59±3% in larvae colonized prior with antibacterial bacterium, *Alteromonas* sp. P7. Similar effects have been observed with beneficial marine bacteria in protecting the embryos of *Homarus americanus* (Gil-Turnes and Fenical 1992), larval rearing of oyster (Douillet and Langdon 1994), penaeid shrimp and larvae (Maeda and Liao 1992; Maeda 1994; Abraham et al. 2001). Significant differences in the mortalities of shrimp larvae of *Alteromonas* and *V. harveyi* inoculated group and uninoculated control group were observed ($P<0.05$), probably due to the fouling of bacteria on gills and/or other appendages. The results of the present study, thus, evinced that the antibacterial bacterium isolated from shrimp larva inhibited shrimp larval pathogen *V. harveyi* both *in-vitro* and *in-vivo*. Therefore, the colonization/attachment of non-pathogenic antibacterial marine bacterium, not exceeding the threshold level, on shrimp larvae would help reduce larval mortalities in hatcheries by

preventing attachment and proliferation of bacterial pathogens on them. It appears to be an effective way of controlling luminous vibriosis in shrimp larvae in lieu of the negative impacts of antibiotics.

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