Biocontrol of Fish Bacterial Pathogens by the Antagonistic Bacteria Isolated from the Coastal Waters of Gulf of Mannar, India

K. Jayanth, G. Jeyasekaran* and R. Jeya Shakila

Fisheries College & Research Institute, Tamilnadu Veterinary and Animal Sciences University, Tuticorin 628008, Tamilnadu, India

Abstract
Marine bacteria were isolated from seawater, sediment, seaweeds, bivalves and seaweeds, bivalves and submerged substrates of Tuticorin Bay of Gulf of Mannar, India. Marine pigmented bacteria were screened for antagonistic activity. Of the 166 pigmented bacterial strains isolated, 62 showed antibacterial activity against the Gram-positive indicator organisms viz., *Micrococcus*, *Lactobacillus* and *Arthrobacter*. *Alteromonas* was found to be the dominant antagonistic marine bacteria and it exhibited a wide antibacterial spectrum against various fish/shrimp bacterial pathogens viz. *Aeromonas hydrophila*, *A. sobria*, *Vibrio alginolyticus*, *V. harveyi* and *V. fischeri*. The other dominant antagonistic marine bacteria was *Flavobacterium*, which exhibited greater inhibitory activity against *A. sobria*. One strain of *Alteromonas* (A8) showed greater antagonism against fish and shrimp bacterial pathogens when compared to other strains, and it could be used as biocontrol agent in the aquaculture industry.

Introduction
Marine bacteria are known to produce inhibitory substances in the marine environment, even if they are not specifically antibiotic producers. Marine bacteria showing antibacterial activities have been isolated from various biotopes such as surface or deep sediments, seaweeds and substrates. The bacteria showing antagonistic activity mostly belong to *Bacillus*, *Micrococcus*, *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Alcaligenes*, *Xanthomonas* and *Achromobacter* (Gauthier *et al*., 1975, Austin, 1989, Bernan *et al*., 1997). It has been suggested that antagonistic marine bacteria could be employed to combat epizootics in aquaculture systems (Lodeiros *et al*., 1988) as probiotics or biocontrol agents at prophylactic or curative doses. As there are no reports on the incidence of antagonistic bacteria from the Indian coastal waters of Gulf of Mannar, the present investigation was undertaken to isolate antagonistic bacteria present in the marine environment of Tuticorin Bay of Gulf of Mannar, India as well as to examine their inhibitory action against selected fish/shrimp bacterial pathogens.

Materials and Methods
Sample collection
A total of 101 samples from seawater (24 Nos.), sediment (27 Nos.), seaweeds (17 Nos.) (*Gracilaria*, *Zostrea*), bivalves (15 Nos.) (*Donax*, *Paphea*) and swabs from the submerged substrates such as rock, boat, wooden poles and oyster shells (18 Nos.) were collected from five different stations viz. Therespuram, Fish-
ing Harbour, Roche Park, Thermal Beach and Hare Island of Tuticorin Bay of Gulf of Mannar, India. Water samples were collected in sterile glass bottles, sediment using sterile plastic core sampler and seaweeds by hand picking and then placing in sterile polyethylene bags. All the samples were brought to the laboratory within one hour of collection.

Isolation of marine bacteria
To isolate marine bacteria, seawater (10 ml), sediment (10 g) were placed directly into 90 ml of sterile half strength (HS) seawater. Seaweed (10 g) and bivalve (10 g) samples were homogenized in 90 ml of sterile HS seawater using a pestle and mortar. Swabs were taken from submerged substrates and placed in 100 ml of sterile HS seawater. From the above dilution, the samples were then ten-fold serially diluted in sterile HS seawater. The total marine bacterial and pigmented bacterial counts were determined on Seawater Yeast Extract Peptone (SYEP) medium (Hi-Media, Bombay, India) following spread plate technique (Schneider and Rheinheimer, 1988). The bacterial counts were expressed as cfu/g of sediment / seaweed / bivalve; cfu/ml of seawater; cfu/cm² of submerged substrates. Pigmented colonies were picked at random from each plate and streaked onto SYEP agar to obtain pure cultures and then to test for antagonism.

Selection of indicator organisms
For the screening of antagonistic marine bacteria, indicator organisms were selected from the predominant marine bacteria isolated from the marine environment, based on their sensitivity to antibiotics. Antibiogram was done by agar disc diffusion assay on Mueller Hinton agar (Hi-Media, Bombay, India) supplemented with 1.5% (w/v) sodium chloride (Bauer et al., 1966). The bacteria showing larger zones of inhibition were selected as indicator organisms and they were identified up to generic level following the standard taxonomic scheme of LeChavelier et al. (1980).

Detection and identification of antagonistic bacteria
The cross streak method reported by Lemos et al. (1985) was used for assaying the inhibitory activity of pigmented marine bacteria. Young cultures (20 h old) of marine pigmented bacteria were streaked (4-6mm width) across the diameter of SYEP agar (1.2% agar) plates and cultures of indicator bacteria were streaked at right angles across them. Plates were incubated at 30±2°C for another 24 h. The inhibitory activity of the marine bacteria, which inhibited the growth of the indicator organism in the confluence area, are termed as antagonistic bacteria; and they were identified up to generic level following the taxonomic scheme of LeChavelier et al. (1980).

Antagonistic activity against fish bacterial pathogens
The double agar layer method of Dopazo et al. (1988) was used for the assay of antagonistic marine bacteria to examine their ability to inhibit the growth of various fish/shrimp bacterial pathogens. The fish/shrimp bacterial pathogens which were used as test organisms in this study are Aeromonas hydrophila, A. sobria, Vibrio fischeri, V. alginolyticus and V. harveyi. Macrocolonies of antagonistic marine bacteria were developed on SYEP agar (1.2% agar) plates by inoculating 18 h old SYEP
broth culture with a micropipette and incubated at 30±2°C for 40 h. The colonies grown were killed by exposure to the chloroform vapour for 15-20 min. All the test organisms were grown in Trypticase soy medium and incubated at 37°C for 18 h. Ten mL of the culture was suspended in 8 ml of soft SYEP agar (0.7% agar) maintained at 45-50°C and poured immediately over the macrocolonies of the antagonistic marine bacteria on the SYEP agar. The plates were incubated for 24 h at appropriate incubation temperature depending on the pathogenic bacteria. A clear zone around the macrocolony of the antagonistic marine bacteria was measured. A control plate without macrocolonies of the antagonistic marine bacteria was also used to examine the possible effect of chloroform on the growth of test organisms.

**Statistical analysis**

Simple correlation and two-way analysis of variance technique were used to test the significance by following the procedure of Snedecor and Cochran (1962).

**Results**

For the isolation of antagonistic marine bacteria, mainly pigmented marine bacteria were enumerated. The numbers of the pigmented bacterial population and the total marine bacterial population is depicted in Fig.1. The pigmented bacterial population was lower by about 2 log counts than the total bacterial population. The proportion of pigmented bacteria in the different samples of the marine environment showed wide variance and it ranged from <0.19% to 45.55%; <0.10% to 33.33%; <1.27% to 11.10%; £ 3.13% to 20.15%; £0.07% to 38.07% in seawater, sediment,
Of the 166 pigmented bacteria isolated, 62 (32.35%) were found to be antagonistic to bacteria (Table 1). The proportion of antagonistic marine bacteria was found to be high in seaweed samples (35.48%), followed by bivalves (30.63%). The majority of the antagonistic bacteria were Gram-negative and only one strain was Gram-positive. The bacteria that exhibited antagonistic activity were identified as Flavobacterium, Alteromonas, Pseudomonas/Alteromonas/Flavobacterium group and Micrococcus. The proportion of Flavobacterium was high (67.7%), followed by Alteromonas (29.0%) and other bacteria. Flavobacterium was found in all the samples of seaweeds and bivalves. The proportion of antagonistic marine bacteria in the samples collected from different stations was quite variable (Table 1). Presence of antagonistic marine bacteria was high in samples collected from Fishing Harbour, followed by Therespuram, Roche Park, Hare Island and Thermal Beach.

Three indicator organisms were selected from the predominant bacteria isolated from the marine environment and they were identified as Micrococcus (S1), Lactobacillus (S2) and Arthrobacter (S3). The antagonistic bacteria isolated from the marine environment exhibited variable inhibitory activity against the indicator organisms. Alteromonas was found to exhibit a wide spectrum of antibacterial activity, although Flavobacterium was recorded in highest numbers.

Antagonistic marine bacteria which were tested for their ability to inhibit the growth of test organisms showed that out of the 62 antagonistic marine bacteria, 24 had the ability to inhibit at least one of the test organisms at the varied levels (Table 2). Among the 24 isolates, which showed antagonistic effect, 5 isolates (F1 - F5) belonging to the genus Flavobacterium and 8 isolates belonging to the genus Alteromonas (A1 – A3, A5 – A9) had higher inhibitory activity. The antagonistic marine bacteria belonging to Pseudomonas / Alteromonas / Flavobacterium group isolated from seawater and submerged substrate was found to exhibit an inhibitory effect against only a few test organisms. The test organism, Aeromonas sobria, a fish pathogen, was invariably inhibited by nine isolates of Flavobacterium. However, the major shrimp pathogen, Vibrio sp. were not inhibited by the Flavobacterium strains.

The Alteromonas strains exhibited a wide antibacterial spectrum. They were able to inhibit
all the test organisms viz., *Aeromonas hydrophila*, *A.sobria*, *Vibrio alginolyticus*, *V.fischeri* and *V.harveyi*. Only one strain of *Alteromonas* (A8) was found to exhibit a wide and marked spectrum of antibacterial activity against the test organisms when compared to other strains. The antagonistic effect of *Alteromonas* strains against *A. hydrophila* and *A. sobria*, the causative agents of fish diseases, was very pronounced.

**Discussion**

Pigmented marine bacteria were selected and tested for antagonistic activity, because earlier studies have demonstrated that most of the antibiotic producing marine bacteria were pigmented (Lemos et al., 1985). A significant positive correlation (P<0.5) existed among the pigmented and total bacterial populations. Nair and Simidu (1987) have recorded the proportion of pigmented bacteria as 30%. However, in the present study, the proportion of pigmented bacteria was only 6% in the total number of samples analysed. About 37% of the selected marine pigmented bacteria exhibited antagonism. The occurrence of antagonistic marine bacteria was high in seaweeds and bivalves. It was reported that a beneficial relationship exists between the antagonistic bacteria and seaweeds/algae (Lemos et al., 1985) and animals that harbour them (Bernan et al., 1997). However, the occurrence of antagonistic marine bacteria to the total bacterial population among the different marine samples did not show any significant correlation (P<0.05). The number of antagonistic marine bacteria recorded in the Fishing Harbour was high, which might be due to the unfavourable growth condition that exists in the area. Nair and Simidu (1987) have also reported that the absence of or reduction in the proportion of pigmented bacteria as 30%.
in number of bacteria with antibacterial activity in Tokyo bay is due to its eutrophic nature, which may tend to moderate the production of antibacterial compounds.

The indicator organisms selected in the present study were identified as *Micrococcus*, *Lactobacillus* and *Arthrobacter*, which belong to Gram-positive bacteria. It is relevant to note that Gram-positive bacteria are more susceptible than Gram-negative bacteria to the antibiotics derived from the marine microorganisms (Gauthier and Flatau, 1976).

Most of the antagonistic marine bacteria isolated in this study were found to belong to the genera *Alteromonas* and *Flavobacterium*. Several workers have also recorded higher incidence of *Flavobacterium* and *Alteromonas* and their inhibitory activity against many bacteria (Gauthier et al., 1975, Nair and Simidu, 1987, Austin 1989). Nair and Simidu (1987) reported that constant nutritional inputs from external sources assuage the necessity for bacterial populations to produce antibacterial compounds to survive competition. However, in this study, the incidence of antagonistic marine bacteria did not show any significant relationship (P<0.05) with the different sampling stations.

Of the antagonistic marine bacteria isolated, 38.7% exhibited inhibitory action against at least one of the test organisms at varied levels. *Alteromonas* strains isolated from seaweeds and bivalves exhibited antagonism towards all the test organisms, while *Flavobacterium* showed inhibition against only few test organisms. However, the *Pseudomonas/Alteromonas/Flavobacterium* group and *Micrococcus* did not show any antagonism towards any of the test organisms. Lemos et al. (1985) have reported that the epiphytic bacteria belonging to the *Pseudomonas/Alteromonas* group have exhibited inhibitory activity against *Aeromonas sobria* and *A. hydrophila*. The inhibitory effect of all the *Alteromonas* and *Flavobacterium* strains against the fish pathogen, *A. sobria* was similar, but the *Alteromonas* also inhibited all other pathogens.

Among the various strains of *Alteromonas* tested, only one strain (A8) exhibited broad spectrum of antibacterial activity against bacterial pathogens. Its effect was very high on the bacterial pathogens of fish viz., *Aeromonas hydrophila* and *A. sobria*. Dopazo et al. (1988) have reported that the *Alteromonas/Pseudomonas* group exhibited antagonistic effect against *Aeromonas*. Their inhibitory activity against *Vibrio alginolyticus*, a potential pathogen of shrimp disease was also high when compared to its activity against the other shrimp pathogens viz., *Vibrio fischeri* and *V. HARVEYI*. The present study clearly indicated that the *Alteromonas* strain had a wide inhibitory activity against *Vibrio* sp., which was similar to the earlier observations.

The wide antibacterial activity exhibited by the *Alteromonas* strains isolated from the marine environment against the test organisms, which included fish/shrimp bacterial pathogens, suggests that these strains could be used as probiotic and biocontrol agents in the aquaculture industry to prevent bacterial diseases. For future studies, the antibacterial substances present in the *Alteromonas* strains have to be extracted and identified for their use in aquaculture systems.
Acknowledgements
The authors like to thank the Dean, Fisheries College and Research Institute, Tuticorin, India for providing the facilities to carry out this work along with constant support and encouragement. This work was supported by the Tamil Nadu State Council for Science and Technology, Govt. of Tamil Nadu, India.

References