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Original Article

Pseudomonas fluorescens is an Effective Probiotic against Fish-Pathogenic *Vibrio* sp

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Abstract

To study the possible use of probiotics in fish farming, we evaluated the *in vitro* and *in vivo* antagonism of *Pseudomonas fluorescens* against the fish-pathogenic *Vibrio* sp, isolated from infected fish. As iron is important in virulence and bacterial interactions, the effect of *P. fluorescens* was studied under iron-rich and iron-limited conditions. Sterile-filtered culture supernatants from iron-limited *P. fluorescens* inhibited the growth of fish-pathogenic *Vibrio* sp, whereas sterile-filtered supernatants from iron-replete cultures of *P. fluorescens* did not. *P. fluorescens* inhibited the growth of fish-pathogenic *Vibrio* sp during coculture, independently of the iron concentration, when the initial count of the antagonist was 100 to 1,000 times greater that of the fish pathogen. These in vitro results were successfully repeated in vivo. A probiotic effect *in vivo* was tested by feed probiotic i.e, the fish feed mixed with *P. fluorescens* at a density of 10^5 CFU/ml for 5 days before a challenge with fish-pathogenic *Vibrio* sp at 10^2 to 10^5 CFU/ml for 1 hour exposure.
Keywords: Probiotic, Antagonism, *Pseudomonas fluorescens*, *Vibrio* sp

Introduction

Aquaculture is one of the fastest developing growth sectors in the world and Asia presently contributes about 90% to the global production. However, disease outbreaks are constraint to aquaculture production thereby affects both economic development of the country and socio-economic status of the local people in many countries of Asia. Disease control in aquaculture industry has been achieved by following different methods using traditional ways, synthetic chemicals and antibiotics. However, the use of such expensive chemotherapeutants for controlling diseases has been widely criticized for their negative impacts like accumulation of residues, development of drug resistance, immunosuppressant's and reduced consumer preference for aqua products treated with antibiotics and traditional methods are ineffective against controlling new diseases in large aquaculture systems. Therefore, alternative methods need to be developed to maintain a healthy microbial environment in the aquaculture systems there by to maintain the health of the cultured organisms. Use of probiotics is one of such method that is gaining importance in controlling potential pathogens (Balcazar, 2003).

Fluorescent pseudomonads have been used as biocontrol agents in several rhizosphere studies (O'Sullivan and O'Gara,1992), where their inhibitory activity has been attributed to a number of factors, such as the production of antibiotics (Lemos *et al*.,1985), or iron-chelating siderophores (Kloepper *et al*.,1980). Pseudomonads constitute a large part of the microflora of the gills, skin, and intestinal tracts of live fish (Buyer,1994; Shanahan *et al*.,1992) and are only rarely reported as pathogens of fish (Hatai and Willoughby,1988). Aquatic pseudomonads are often antagonistic against other microorganisms (Gram,1993), including fish-pathogenic bacteria (Smith and Davey, 1993) and fish-pathogenic fungi (Hatai and Willoughby, 1988). One study demonstrated that bathing Atlantic salmon presmolts in a strain of *Pseudomonas fluorescens* reduced subsequent mortality from stress induced furunculosis (Smith and Davey,1993).When

tested in vitro, iron limitation has been found to facilitate the antibacterial activity of fluorescent pseudomonads (Gram, 1993).

Thus, inhibition may be due to the production of siderophores, which deprive the fish pathogen of iron. Production of siderophores is a virulence factor in many microorganisms, such as members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Vibrio anguillarum* (Crosa,1980), as reviewed by Wooldridge and Williams (Wooldridge and Williams,1993). An efficient salmonfurunculosis vaccine elicits antibodies against iron-repressible outer membrane proteins of *Aeromonas salmonicida* (Hirst and Ellis, 1994). Iron is available in the serum of fish, as in mammals (Griffith,1986), is crucial for infection, and fish with iron overload are more prone to attack by *V. anguillarum* than are fish with low serum iron sconcentrations (Nakai *et al*.,1987). To further study the potential of pseudomonads as biocontrol agents in fish farming, we investigated the inhibitory activity of a *P. fluorescens* against the fish-pathogenic *Vibrio* sp, *in vitro* and *in vivo*. The influence of iron on the inhibitory activity was assessed because the availability of iron is important in virulence and disease.

Materials and Methods

Bacterial strains

Two probiotic strains *Pseudomonas fluorescens and Vibrio* sp used in this study. *Pseudomonas fluorescens* was isolated from fresh water fish (Catla catla), Pathogenic *Vibrio* sp, isolated from infected fish. *Pseudomonas fluorescens* cultured on *Pseudomonas* Agar F (Composition per liter: Agar 15.0g, Glycerol 10.0g, peptone 10.0g, 10.0g, K_2HPO 41.5g, $MgSO_4 \cdot 7H2O$ 1.5g, pH 7.0), *Vibrio* sp was routinely cultured on LB medium (Balcazar,2003) with a total of 2% NaCl. *Bacillus* sp was routinely cultured on LB medium. *Pseudomonas* Medium (ATCC Medium 775) Composition perliter:NH_4Cl 5.0g, K_2HPO_4 1.5g, L-Tryptophan 1.0g, KH_2PO_4 0.5g, Yeast extract0.5g$MgSO_4$. 0.2g

Agar antagonism assay

Initial screening of antagonism by *P. fluorescens* was done in a plate assay. Fish-pathogenic *Vibrio* sp (100 ml precultured in LB medium for 5 days at 37°C) was spread on Muller Hinton agar plates with and without additional iron. Wells 3 mm in diameter were punched into the solidified agar, and 10 ml of a 24-h culture of *P. fluorescens* was added. The plates were incubated at 15°C, and zones of inhibition around the wells were measured after 2 to 3 days.

Effect of P. fluorescens supernatants

P. fluorescens was precultured in LB broth with and without NaCl and with or without iron (four combinations) and then used to inoculate 50 ml of LB in the same four combinations at an initial cell density of 10^3 to 10^4 CFU/ml. The flasks were incubated at 12 to 13°Cwith agitation (150rpm), and samples were withdrawn daily. One milliliter was used for serial dilutions and estimation of colony counts on, *Pseudomonas* Agar F plates and 2ml was sterile-filtered (0.2-mm pore size; Sartorius no. 16534). The possible inhibitory activity of the sterile-filtered supernatant was tested by adding 100 ml of supernatant to 100ml of fresh medium in micro titer wells and inoculating it with 10ml of a dilution of fish-pathogenic *Vibrio* sp yielding approximately 10^4 CFU/ml. Controls were done by inoculating fish-pathogenic *Vibrio* sp in 200 ml of LB. Each combination was tested in triplicate, and the growth of the fish pathogen was monitored by recording the optical density at 600 nm with a microtiter plate reader.

Coculture experiments

P. fluorescens and fish-pathogenic *Vibrio* sp were precultured separately in LB broth with 1%NaCl at 13°C with aeration for 3 to 5 days. Appropriate dilutions were prepared in physiological saline, and fish-pathogenic *Vibrio* sp was inoculated in LB broth with 1%NaCl at an initial cell density of approximately 10^3 CFU/ml, whereas the initial levels of *P. fluorescens* were 10^4, 10^5, 10^6, 10^7, and 10^8 CFU/ml. All combinations were done in duplicate. The flasks were incubated at12 to 13°C with aeration, and samples were withdrawn daily for determination of bacterial cell densities. Numbers of *Vibrio* sp were estimated by preparing 10-fold serial dilutions using 1 ml from each dilution to inoculate tubes with 5 ml of H&L medium (23). The tubes were covered with paraffin and incubated at 25°C. The fermentative growth of *Vibrio* sp caused a change in the pH indicator of the

medium. The highest dilution still showing growth was used to calculate the number of *Vibrio* sp present. This procedure was chosen because low numbers of *Vibrio* sp had to be estimated in a high background of *P. fluorescens*, which did not grow in the anaerobic H&L tubes. Inoculation of H&L medium with 10^9 *P. fluorescens* bacteria did not affect the color of the medium.

Water probiotic Treatment

P. fluorescens was grown for 6 days at 13°C (150 rpm) in LB medium and *Vibrio* was grown for 16 h in tryptone soy broth. A total of 150 *Catla catla* weighing approximately 20g were divided into eight 150-liter tanks,four tank considered as control and fish from four of the tanks were exposed for 5 days to *P. fluorescens* at a level of 10^5 CFU/ml (long-term treatment) by adding the bacteria to the water. After the probiotic treatment all the tanks were exposed to pathogenic *Vibrio* sp at a level of 10^4 to 10^5 CFU/ml.

Feed probiotic Treatment

P. fluorescens was grown for 2 days at 37ºC (200 rpm) in *Pseudomonas* Medium (ATCC Medium 775) the cells from the culture were separated by centrifugation. Around 50 ml of culture was taken in a sterile centrifuge tubes and were centrifuged at 10000 rpm for 15 minutes. The supernatant was transferred carefully in to another sterile storing vial and washed twice by saline then suspended in Phosphate-Buffer Saline and were stored in refrigeration condition. The total viable count of the washed bacterial cells suspension was used to probiotic treatment. That broth culture had a density of 10^{-9} cells per ml were mixed well for the culture evenly spread the feed. The feed was dried at room temperature for 5 to 6 hours. . A total of 300 *Catla catla* weighing approximately 20 g were divided into eight 300-liter tanks, four tank considered as control. The dried feed was given at a rate 10% of the body weight, twice a day. In control fish tank microbial free feed was given.

Results and Discussion

Agar antagonism assay

P. fluorescens caused a clearing zone with a radius of 3 to 5 mm in lawns of fish pathogenic *Vibrio* sp the absence of iron. No inhibition zones were observed when the medium was supplemented with iron.(Table 1).

Table- 1: Antagonism of probiotic bacteria

Sl. No	Resistant	Zone of inhibition (mm)
Pseudomonas fluorescens (without ions)	+	5
Pseudomonas fluorescens (with ions)	–	Nil

Effect of P. fluorescens supernatants

Strain *P. fluorescens* grew well in LB medium with and without 3% NaCl and Fe. Addition of iron caused more-rapid growth and a slightly higher maximum cell density, whereas 3% NaCl caused a prolonged lag phase. Sterile-filtered *P. fluorescens* supernatants were inhibitory to fish pathogenic *Vibrio* sp.

Coculture experiments.

The growth of fish pathogenic *Vibrio* sp was inhibited under iron-limited conditions by inoculated at an initial level of 10^6 to 10^7 CFU/ml. Lower concentrations of *P. fluorescens* (10^4 to 10^5 CFU/ml) allowed initial growth of *Vibrio* sp, but cell densities never reached the level of the control. High inoculum concentrations (10^5 to 10^7 CFU/ml) of *P. fluorescens* under iron-replete conditions allowed an initial increase of *Vibrio* sp followed by a decrease in the viable count. Growth of *P. fluorescens* was not affected by coculturing with *Vibrio* sp (data not shown).

Water and Feed probiotic Treatment

A total of 300 *Catla catla* weighing approximately 20g were divided into eight 300-liter tanks, four tanks considered as control .After the probiotic treatment all the tanks were exposed to pathogenic *Vibrio* sp after 30 days control tanks having more accumulated mortality(i.e. 50% of total count).In water probiotic treated tanks *P. fluorescens* caused a decrease in accumulated mortality, to 38 and 35%, respectively. In feed treatment was caused a further reduction in accumulated mortality to 32%. (Table -2 & 3).

Table -2: Feed Probiotic Treatment

Sl.No	Day intervels	Probiotic concentration in feed	Pathogen Concentration in water	Accumulated mortality
1	After 5 days	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	1%
2	After 10 days	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	4%
3	After 15 days	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	12%
4	After 20 days	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	20%
5	After 25 days	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	28%
6	After days	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	32%%

Table -3: Water probiotic treatment

Serial No	Day intervels	Probiotic concentration in water	Pathogen Concentration in water	Accumulated mortality
1	After 5 day	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	2%
2	After 10 day	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	8%
3	After 15 day	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	16%
4	After 20 day	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	23%
5	After 25 day	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	32%
6	After day	*P. fluorescens* at a level of 10^5 CFU/ml	*Vibrio* sp at a level of 10^4 to 10^5 CFU/ml	44%

Discussion

In recent years there has been considerable increase in the probiotic in aquaculture. The probiotics were defined us live microbial feed supplement that improve the health of man and terrestrial livestock .This study shows that experimental evidence is accumulating that the health and zoo technical performances of cultured aquatic species can be improved by the prophylactic use of probiotics. Progress has been made in the culture of live food, crustacea, mollusks, and fish. The use of probiotics as biological control agents should be considered to be a kind of risk insurance that may not provide any notable benefit when the culture is performing under optimal conditions and in the absence of (opportunistic) pathogens but that will be very helpful if infectious diseases break out.

A strain of *P. fluorescens* was successfully used to reduce the frequency of stress-induced infections by *A. salmonicida* (Smith and Davey,1993). Austin *et al*.,(1995) reported that exposure to a nonpathogenic *Vibrio alginolyticus* strain reduced subsequent mortality due to vibriosis. To our knowledge, our data are the first to demonstrate a reduction in vibriosis-caused mortality in trout by the use of a probiotic *P. fluorescens* strain.

In agreement with other studies (Gram *et al*., 1999; Voisard *et al*., 1989), we have seen that under iron-limited conditions, *P. fluorescens* is inhibitory to fish pathogenic *Vibrio* sp when

tested in vitro in a well diffusion assay or when sterile-filtered supernatants were tested. Similar to Smith and Davey (Smith and Davey,1993), we found that addition of iron to sterile-filtered supernatants of *P. fluorescens* eliminated the inhibitory activity. Due to its fast growth, *P. fluorescens* may compete for other nutrients, occupy colonization sites, or excrete antibacterial substances (Gram *et a*l., 1999).

However, such substances, if produced, were not present in sufficient concentrations to allow detection in vitro in supernatants from iron-enriched cultures. High levels of *P. fluorescens* were required before inhibition of *Vibrio* sp could be detected in coculture assays. In agreement with earlier studies of interactions between fish spoilage bacteria (Gram,1996), sterile-filtered supernatant from *P.fluorescens* did not inhibit the growth of *Vibrio* sp until an antagonist level of 10^8 CFU/ml was reached. Our studies also show that the antagonist must be present at significantly higher levels than the pathogen, and the degree of inhibition increases with the level of the antagonist. Thus, during coculture, 10^7 to 10^9 CFU/ml was required to inhibit the growth of the pathogen.Therefore, a potential probiotic culture must either be supplied on a regular basis or be able to colonize and multiply on or in the host.Rhizosphere studies often have great difficulties moving from in vitro to in vivo situations (Buyer, 1994).

In preliminary in vivo studies with *P. fluorescens* (data not shown), we found that short-term exposure, even to high numbers of the bacterium, had no effect on subsequent fish mortality. However, with more-constant exposure to *P. fluorescens*, a significant reduction in mortality was obtained. The fact that the reduction in mortality obtained by the two different treatments was additive indicated that further reduction in mortality may be obtained by optimizing the procedure. Probiotic treatment of fish thus offers a very promising alternative to the use of antibiotics and chemotherapy.

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