Isolation of Putative Probionts from the Intestines of Indian Major Carps

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Abstract

Probiotic strains were isolated from the intestines of 28 Indian major carps (*Labeo rohita*, *Catla catla*, *Cirrhinus mrigala*) cultured in sewage-fed ponds. Intestinal microflora were isolated, counted, and identified, and their *in vitro* antibacterial properties were determined. The double layer method revealed that four strains (SG1, SG2, SG3, SG4) elicited antagonism against the pathogen *Pseudomonas fluorescens*. Using cross streak and parallel streak methods, SG4 most inhibited the fish pathogens *Pseudomonas fluorescens*, *Aeromonas hydrophila*, and *Edwardsiella tarda*. Therefore, SG4 was chosen for further study as a probiont. SG4 was non-pathogenic to *C. mrigala* fingerlings, and adhered well to stainless steel, high density polyethylene, and glass plate substrates. Biochemical tests showed that SG4 was the endospore-forming, aerobic, motile, rod-shaped *Bacillus subtilis*. However, since *in vitro* experiments can only give an indication of *in vivo* success, future research should include large-scale *in vivo* experiments.

Introduction

Disease is a major problem in the fish farming industry. Although vaccines are being developed and marketed, they cannot be used as a universal disease control measure in aquaculture. The use of antibiotics to cure bacterial infection and prevent fish mortality in aquaculture is becoming limited as pathogens develop resistance to the drugs (Alderman and Hastings, 1998). Further, beneficial bacterial flora are killed or inhibited by orally administered antibiotics, leading to efforts to find alternative disease prevention methods such as the use of non-pathogenic bacteria as probiotic biocontrol agents.

Verschuere et al. (2000) defined probiotics as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host

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response towards disease, or by improving the quality of its ambient environment".

The use of commercial probiotics in fish is relatively ineffective as most commercial preparations are based on strains isolated from non-fish sources that are unable to survive or remain viable at high cell density in the intestinal environment of fish during the active growth phase of the fish (Moriarty, 1996). Hence, there is elegant logic in isolating putative probiotics from the host in which the probiotic is intended for use. Such strains should perform better because they have already adhered to the gut wall of the fish and, thus, are well-adapted to compete with pathogens for nutrients. Presumably, strains that develop dominant colonies in the fish intestine are good candidates for preventing the adhesion of pathogens on the gut wall. The present study was undertaken to isolate probiotic bacterial strains from fish intestines and screen them by *in vitro* testing of their antagonism to pathogens and attachment to substrates.

Materials and Methods

Isolation of bacterial strains. Fingerlings and juveniles of Indian major carps, *Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala*, were collected at regular intervals from the Nalban sewage-fed fisheries at Salt Lake City, Calcutta. They were brought to the laboratory alive and sacrificed. The ventral surfaces were sterilized using 70% ethanol and aseptically dissected to remove the intestines. The intestines were opened by a longitudinal incision and thoroughly flushed with sterilized chilled normal saline solution (NSS) to remove feed materials, dirt, and other impurities. Excess moisture was blotted with filter paper and the intestines were weighed, macerated with a sterile glass rod, and homogenized in sterile NSS (1:10; wt:vol) using a vortex mixer. Samples of the thoroughly macerated and homogenized intestines were serially diluted in NSS and aseptically plated by the spread plate technique on All Purpose Tween's Agar (APTA; Evans and Niven, 1951) to isolate putative probiotic bacteria and on Nutrient Agar (NA; Hi-Media) to determine total plate count. The inoculated agar plates were aerobically incubated at 35°C for 48 h and the total bacteria were counted.

Assay for antibacterial activity. The double layer method reported by Dopazo et al. (1988) was used as an inhibition test to identify potential probionts against the fish pathogen *Pseudomonas fluorescens*. Bacterial strains that produced a clear inhibitory zone with a diameter at least 5 mm greater than that of the *P. fluorescens* colony were further examined for antagonistic properties against three fish pathogens, *P. fluorescens*, *Aeromonas hydrophila*, and *Edwardsiella tarda* by cross streak assay (Austin et al., 1992) and the parallel streak method (Nakamura et al., 1999). The pathogens were provided by the Central Institute of Fisheries Education in Mumbai, India. Their identities were confirmed following the procedures of Krieg and Holt (1984).

Determination of pathogenicity of the most effective probiotic strain. The pathogenicity of the most effective probiotic strain was determined by challenging *C. mrigala* fingerlings (avg wt 6.6±1.3 g, avg length 7.8±1.4 cm) in an immersion assay and observing their health status (Austin et al., 1995). Fingerlings were procured from commercial fish breeders in Calcutta and acclimatized in laboratory conditions for five days. The experiment was carried out in triplicate 500-l circular FRP tanks for ten days. Fingerlings were stocked at 30 per tank and maintained in uniform water conditions with aeration and filtration. The probiotic strain was grown for 24 h in tryptone soya broth (TSB; Hi-Media). Cells were harvested by centrifugation at 6000 rpm (2000 x *g*) for 20 min at 4°C in a cooling centrifuge (C-23, Remi, Mumbai, India). The cells were washed and resuspended in sterile saline. The number of cells was estimated by spread plating on tryptone soya agar (TSA; Hi-Media) after incubation at 35°C for 48 h. The remaining probiotic cells in the sterile saline were added to the fish tanks to a level of 10^7 cells/ml. Fish in control tanks received a sterile saline solution lacking bacterial inoculum. All fish were fed a basal diet (35% protein) at 5% of their body weight in two doses. At the end of the experiment, gross pathological features, abnormalities, and mortality rates, considered to be due to the inocu-

lation, were recorded.

Adherence or biofilm formation of the most effective probiotic strain on substrates. The ability of the most effective of the isolated probiotics to adhere to and/or form a biofilm on stainless steel, high density polyethylene (HDPE), and glass plates was tested as described by Karunasagar et al. (1996) and Venugopal et al. (1999).

Biochemical characterization and identification of the most effective probiotic strain. The most effective probiotic was characterized and identified based on Gram reaction; spore formation; cellular morphology; motility; growth in different salinities and pH; sugar utilization; amino acid decarboxylation; catalase and oxidase production; nitrate reduction; hydrogen sulfide production; starch, casein, and urea hydrolysis; gelatin liquefaction; and IMVIC tests. The biochemical tests were performed according to Collins et al. (1989) and Pacarynuk and Danyk (2004).

Results

Isolation of bacterial strains. The intestines of 28 fish were screened to isolate probiotic bacteria. The sampled fish ranged 11-27 cm (avg 17.5±4.8 cm) and 12-150 g (avg 57.88±49.03 g). The $\log_{10}$ values of total plate count (TPC) per gram intestine ranged 7.544-9.531 on NA (avg 8.605±0.525) and 5.114-6.978 on APTA (avg 6.019±0.602).

Assay for antibacterial activity. The double layer method showed that four strains, SG1 isolated from *C*. *mrigala* (23 cm; 100 g), SG2 isolated from *C*. *catla* (18 cm; 60 g), SG3 isolated from *C*. *catla* (27 cm; 140 g), and SG4 isolated from *C*. *mrigala* (23 cm; 115 g) produced a zone of inhibition with a diameter that was more than 5 mm greater than the diameter of the *P. fluorescens* colony.

The cross streak method revealed that the two strains from *C*. *catla* (SG2 and SG3) did not inhibit the pathogenic test strains. The two strains from *C*. *mrigala* (SG1 and SG4) had inhibition zones of 5.21±0.57 mm and 5.90± 3.37 mm against *P. fluorescens*, 4.05±1.73 mm and 3.16±0.64 mm against *A. hydrophila*, and 3.48±1.16 mm and 3.8±1.27 mm against *E. tarda*, respectively. The antibacterial activity of S1 and S4 was further confirmed using the parallel streak method against the same three pathogenic test strains. For S1, there was no noticeable reduction in the width of any pathogen streak on the test plates compared to that of the control. SG4, however, markedly reduced the width of all pathogen streaks when compared to the control.

By virtue of its greater inhibition zone in the cross streak method and positive antibacterial activity in the parallel streak method, SG4 was further tested in the following experiments.

Determination of pathogenicity of SG4. The addition of SG4 cells to the rearing water of *C*. *mrigala* fingerlings produced no gross pathological symptoms, abnormalities, or mortality, confirming its non-pathogenicity.

Adherence or biofilm formation of SG4 on substrates. The adherence or biofilm formation of SG4 was highest on HDPE, followed by stainless steel and glass. The $\log_{10}$ counts on HDPE, steel, and glass were 3.316/cm^2, 2.592/cm^2, and 0.47/cm^2, respectively.

Biochemical characterization and identification of SG4. SG4 had a strong gram-positive reaction. Cellular morphology suggests that it is a thick unicellular rod-shaped bacteria, capable of forming round, wavy, convex, rough, and opaque colonies. Cells are aerobic, endospore-forming, and motile while spores are ellipsoidal and central in position. SG4 is capable of utilizing glucose, xylose, sucrose, mannitol, arabinose, and fructose as carbohydrate sources for growth and grew at pH levels of 5, 8, 9, and 11 and salinities of 3%, 6%, and 8%, but not 10%. It did not decarboxylate the amino acids lysine, ornithine, and arginine. SG4 was positive for the Vogues Proskaeur reaction and negative for the methyl red test. It can produce catalase, oxidase, and indole, but not hydrogen sulfide and can utilize citrate, reduce nitrate, liquefy gelatin, and hydrolyze starch and casein, but not urea.

Discussion

Isolation of bacterial strains. The mean $\log_{10}$ intestinal count on NA, 8.605±0.525/g, agrees with that of Galindo (2004), who reported a mean $\log_{10}$ intestinal count of 8.09±0.43/g in 17

species of carnivorous, herbivorous, and omnivorous freshwater fishes, and Ringo et al. (1995), who found that the digestive tract of finfish contains 10^8 cells/g, equivalent to 8/g when converted into a logarithmic scale with a base of 10. The mean $\log_{10}$ count of lactic acid bacteria on APTA, 6.019±0.602/g, was higher than that obtained by Galindo (2004) who obtained a mean $\log_{10}$ intestinal lactic acid bacteria (LAB) count of 4.72±1.4/g while working on 17 freshwater fish species. The fish intestine is a favorable ecological niche for microorganisms, which reach much higher numbers than in the surrounding water (Austin and Austin, 1987).

Assay for antibacterial activity. The double layer method showed that only four strains of all those isolated from the intestines of 28 fish had inhibition zones that exceeded the circumference of a colony of pathogenic *P. fluorescens* by at least 5 mm. A similar frequency of inhibitory bacteria was observed for isolates from halibut larvae (Bergh, 1995), rainbow trout (Brunt and Austin, 2005), turbot (Hjelm et al., 2004), and shrimp (Rengpipat et al., 1998). The inhibition was caused by the release of chemical substances with bactericidal or bacteriostatic effects on *P. fluorescens*.

The inhibition zones exhibited by SG1 and SG4 in the cross streak method were similar to those reported by Abraham (2004) for *Alteromonas* sp. P7 (4.0±0.25 mm) and by Rengpipat et al. (1998) for *Bacillus* strain BY-9 (3-6 mm) against the pathogenic *Vibriosis harveyi*. In another study, Galindo (2004) reported *Lactobacillus plantarum* 44a inhibition zones of 12.4±1.3 mm and 7.3±0.90 mm against *A. hydrophila* at pH levels of 3.7 and 4.5, respectively, and *Lactobacillus lactis* 18f inhibition zones of 11.2±1.16 mm, 2.0 mm, and 8.9±2.1 mm against *A. hydrophila*, *E. tarda*, and *Staphylococcus aureus*, respectively, at pH 6.0. The inhibition seen in the cross streak method resulted from interruption of pathogen growth caused by production of one or more of the following factors: antibiotics, antimicrobial peptides, bacteriocins, siderophores, lysosymes, proteases, hydrogen peroxide, and organic acids (Verschuere et al., 2000).

SG4, but not SG1, reduced the width of pathogen streaks, in agreement with Nakamura et al. (1999) who reported that only twelve of 51 bacterial isolates with suppressive activity had inhibitory effects on pathogen growth in the parallel streak method. The reduction of pathogen growth and cell density indicate that extracellular bacteriolytic products produced by the SG4 were responsible for this inhibition.

The *in vitro* production of compounds that inhibit known pathogens is often used in the selection of putative probiotic strains (Rengpipat et al., 1998). In the present *in vitro* study, SG4 inhibited all three pathogenic strains and hence was selected for further study as probiont.

Determination of pathogenicity of S4. There is no evidence that SG4 has any harmful effect on *C*. *mrigala* fingerlings since there was no mortality and a complete absence of any pathology or abnormality in the fingerlings. Austin et al. (1995) also found that isolated probiotic strains were non-pathogenic. The non-pathogenicity of SG4 ensures that it is safe for use as a probiotic.

Adherence or biofilm formation of S4 on substrates. The adherence or biofilm formation of SG4 on three substrates was similar to the findings of Faille et al. (2002), who observed a higher number of adhered cells on the hydrophobic materials HDPE and Teflon, but not much difference (less than 1 log unit) between these two materials. The ability to adhere to a substrate is an important criterion for selection of a probiont because adhesion to host surfaces is essential for effective colonization of the probiont in the host and interruption of adhesion of pathogens is essential for biocontrol of the pathogen (Chabrillon et al., 2005).

Biochemical characterization and identification of S4. The morphological and physiological characteristics of SG4 and comparison with Sneath (1986) and Priest (1993) place this bacterium in the *Bacillus* genus, species *subtilis*. *Bacillus* is distinguished from other endospore-forming bacteria by being a strict or facultative aerobe, rod-shaped, and catalase-positive. *Bacillus subtilis* is distinguished from other members of the genus by its biochemical reactions, unicellular rod-shaped morphology, and ellipsoidal spore-forming ability.

Bacilli are not autochthonous to the gas-

trointestinal tract but have been isolated from carps (Kumar et al., 2006), coastal fishes (Sugita et al., 1998), shrimps (Sharmila et al., 1996), bivalves (Sugita et al., 1981), shrimp culture ponds (Vaseeharan and Ramasamy, 2003), and shrimp larvae-rearing medium (Rengpipat et al., 1998). Most *Bacilli* produce antibiotics such as difficidin, oxydifficidin, bacitracin, polymyxin, subtilin, mycobacillin, bacillin, gramicidin, or bacillomycin B and are antagonistic to pathogenic bacteria in both *in vivo* and *in vitro* conditions (Korzybski et al., 1978; Zimmerman et al., 1987). *Bacilli* are not associated with aquatic organism pathology and are widely accepted and used as probionts (Moriarty, 1998).

In this study we isolated probionts from fish intestines and screened their potential to combat fish pathogens in aquaculture in *in vitro* experiments. The use of a probiont originating from the fish intestine will enable the probiont to proliferate rapidly once it reaches the fish intestine as a feed supplement. While *in vitro* experiments can indicate success *in vivo*, assumptions based on *in vitro* experiments may not be true for *in vivo* modes of action. Therefore, future research should include large scale *in vivo* experiments along with study of the mechanism of antagonistic action between the probiont and the pathogen.

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