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Screening and characterisation of antagonistic *Pseudomonas aeruginosa* FARP72 as a potential probiotic agent

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ABSTRACT

A search for antagonistic bacteria from the skin mucus of Indian major carps and freshwater catfish was attempted. A total of 41 potent antagonistic bacterial strains were isolated, purified and identified from 25 fish samples, of which 82.93% (n=34) were Gram positive and 17.07% (n=7) were Gram-negative bacteria. Antagonistic activity of these bacteria were confirmed against pathogenic human and fish bacteria *viz*., *Aeromonas hydrophila*, *Bacillus subtilis*, *Bacillus amyloliquefaciens, Escherichia coli*, *Edwardsiella tarda*, *Klebsiella pneumoniae*, Enterohaemorrhagic *Escherichia coli* (EHEC), *Pseudomonas aeruginosa*, *Salmonella typhi*, beta haemolytic *Streptococcus, Staphylococcus aureus* ATCC12598 and *Vibrio cholerae*. Among all the bacterial strains tested, broad spectrum of inhibitory activity was elucidated by *Pseudomonas aeruginosa* FARP72, isolated from *Clarias batrachus.* Species identity of the antagonistic bacterial strain *P. aeruginosa* FARP72 was confirmed using molecular technique (16S rDNA analysis) and also by analysis of fatty acid methyl esters (FAME analysis). The antagonistic bacterium *P. aeruginosa* FARP72 has fulfilled majority of the pre-requisite features for a bacterial strain to be used as probiotic. The strain was also proved to be non-pathogenic. Results suggest that *P. aeruginosa* FARP72 can be a substitute to the commercially available and indiscriminately used common antibiotics used in aquaculture.

Keywords: Antagonistic, Pathogenic bacteria, Probiotic, *Pseudomonas aeruginosa*, Skin mucus

Introduction

Diseases especially of bacterial etiology are a major problem in aquaculture. Indiscriminate use of antimicrobials and commercial antibiotics in disease prevention can bring about the emergence of drug resistant microorganisms, leave antibiotic residues in the body and in the environment and may also inhibit the beneficial normal microflora in the digestive tract of the treated animals. Therefore, alternative strategies to prevent bacterial infections in aquaculture are essential. The use of probiotic bacteria to control potential pathogens is an alternative method and is gaining acceptance (Gomez Gil, 2000; Robertson *et al*., 2000). Keeping this in view, the present study was taken up with the objectives to screen and characterise a potent antagonistic bacterium from skin mucus of freshwater fish, to assess its inhibitory activity against selected human and fish bacterial pathogens *in vitro*, and to assess its potential to be used as probiotic agent to inhibit fish bacterial pathogens.

Materials and methods

Apparently healthy specimens of Indian major carps *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala,* the exotic carp *Cyprinus carpio* as well as the fresh water catfish *Clarias batrachus* were collected from the farm sites of Chakgaria (22°48'N; 88°38' E), Garia (22°46'N; 88°37' E), Gariahat (22°51'N; 88°36' E) and Mukundapur (22°49'N; 88°40'E), were used for isolation of bacteria. The fishes on reaching the laboratory, were disinfected with 5 ppm potassium permanganate ($KMnO_4$) solution and acclimatised for 10 days in circular fibre glass reinforced plastic (FRP) tanks of 500 l capacity, holding borewell water with continuous aeration. Rohu (*Labeo rohita*) fingerlings (mean weight 21.21±5.01g) procured from commercial fish breeders were used for bacterial challenge tests. All fish were fed a balanced pellet diet at 2% of their body weight and maintained under optimal conditions.

Selection of indicator organism

Fish-borne *Brevibacillus brevis* S23 was selected as indicator bacterium based on its sensitivity and zone of inhibition against the producer strains. The bacterium was characterised by Gram reaction, morphology, catalase, sporulation and other biochemical characters (Collins *et al*., 1989; Guerra-Cantera and Raymundo, 2005).

Enumeration, isolation and identification of antagonistic bacteria

Antagonistic bacteria were screened from all the five fish species *viz*., *C. catla*, *L. rohita, C. mrigala, C. carpio*

and *C. batrachus*. Mucus from fish skin covering an area of 10 cm^2 was collected using a sterile swab, placed in 100 ml sterile physiological saline, mixed thoroughly for 15 min and then serially diluted. The counts of antagonistic bacteria were determined by agar overlay method (Spelhaug and Harlander, 1989) using *B. brevis* S23 as an indicator organism. Colonies with clear zone of inhibition, considered to be of antagonistic bacteria were counted, aseptically picked and purified repeatedly on tryptone soya agar (TSA). The purified cultures were transferred to TSA slants and maintained at 30±2°C. The purified antagonistic bacterial strains were characterised on the basis of their biochemical characters (MacFaddin, 1980; Collins *et al.,* 1989) and identified as per LeChevallier *et al.* (1980) and Holt *et al*. (1994).

Characterisation of P. aeruginosa FARP72 as a potential probiotic

In vitro inhibitory activity

In vitro inhibitory activity of the antagonistic bacteria *P. aeruginosa* FARP72 isolated from fish mucus was tested against eighteen opportunistic human and fish bacterial pathogens by modified cross streak technique, as described in Lemos *et al.* (1985) and Austin *et al*. (1992). The severity of inhibition was determined by parallel streaking method (Nakamura *et al*., 1999). Inhibitory activity of the antagonistic strains was determined on the basis of growth inhibition of the test strain and the severity of inhibition was rated as "++" for total inhibition, "+" for feeble/thin growth and "–" for no inhibition, as compared with control plates.

Qualitative biofilm formation on glass surface

The method consisted of inoculating 9 ml tryptone soya broth (TSB) in tubes with a loopful of potential antagonistic bacterium *P. aeruginosa* FARP72 from TSA plate culture and incubating the broth culture overnight (18 h) at 30±2°C. The culture tubes were emptied of their contents and stained with 0.1% safranin (Merck) and 2% crystal violet (Hucker's). Biofilm production was visible as in the form of a film occurred on tube walls and quantified the biofilm production as absent (score 0), weak (score 1), moderate (score 2), strong (score 3) or very strong (score 4), as described in Christensen *et al.* (1983).

Haemolytic assay

Haemolytic assay was carried out as per Joseph *et al.* (1982) by spot inoculating young culture of *P. aeruginosa* FARP72 from TSA plate on to a blood agar plate (HiMedia, Mumbai) and incubated at 30±2°C for 24 h. Lightened (yellow) and transparent zone around and under the colony indicated complete lysis of red cells in the media, referred to as beta haemolysis. Incomplete haemolysis is identified as alpha haemolysis and non-haemolytic organisms as gamma haemolytic (Ray *et al.*, 2004).

Enzyme activity

Proteinase, amylase, lipase (Mourney and Kilbertus, 1976) and phospholipase (Haberman and Hardt, 1972) activities of *P. aeruginosa* FARP72 were determined on gelatin agar, starch agar, tributyrin agar and egg yolk emulsion agar, respectively. Young culture of *P. aeruginosa* FARP72 was spot inoculated on to respective agar plates and was incubated at 30±2°C for 24 h. After the incubation period, gelatin agar plate was flooded with saturated ammonium sulphate solution and starch agar plate with Lugol's iodine and were allowed to stand for 2 min. The solution was decanted and observed for presence of clear zone around the colony for positive result. A clear zone around the growth on tributyrin agar and egg yolk emulsion agar respectively after incubation at 30±2°C for 24 h was considered positive for lipase and phospholipase activity respectively.

Growth at different temperatures, pH and 6.5% salt concentration

A loopful of 24 h culture of *P. aeruginosa* FARP72 was transferred into each of the five test tubes containing TSB and incubated at different temperatures *viz*., 4, 22, 32, 45 and 65°C. Growth of *P. aeruginosa* FARP72 at different pH levels was tested by inoculating 24 h old culture in nutrient broth with pH values *viz*., 2.8, 3.6, 4.4, 5.2, 6.0, 6.8, 7.6, 8.4, 9.2 and 10, prepared using 0.2 M dipotassium hydrogen phosphate (K_2HPO_4) and 0.1 M citric acid (Salle, 1969). Nutrient broth with 6.5% sodium chloride was prepared and inoculated with a loopful of 24 h broth culture of the bacterium. Turbidity was measured after 48 h of incubation at 30°C to ascertain the growth of the bacterium at different temperatures, pH values and salt concentration.

Bile tolerance

Young culture of *P. aeruginosa* FARP72 was spot inoculated on to MacConkey agar containing 5% bile salts and incubated at 30°C for 24 h. Growth on the agar was considered positive for bile tolerance.

Antibiogram

Bacterial lawn prepared using young culture (grown at 30±2°C for 10 - 12 h) of *P. aeruginosa* FARP72 was tested for sensitivity to six antibiotics *viz*., oxytetracycline, O (30 µg), chloramphenicol, C (30 µg), gentamicin, G (10 µg), nitrofurantoin, N (300 µg), ciprofloxacin, F (5 µg) and co-trimoxazole, T (25 µg). Antibiotic impregnated discs were placed aseptically on to the inoculated agar

plates.The plates were then incubated for 24 h at 30±2°C and the diameter of zone of inhibition was measured in mm. Interpretation of sensitivity was based on the zone size interpretation chart, provided by the manufacturer of the antibiotic impregnated discs.

Pathogenicity evaluation in L. rohita

P. aeruginosa FARP72, maintained on TSA slant, was streaked on to TSA plate and incubated at 30±2°C for 24 h to get young discrete colonies. One or two young colonies were aseptically picked, transferred to 10 ml TSB and incubated at 30±2°C for 24 h. This 24 h old culture was then transferred to 300 ml TSB and reincubated at 30±2°C for 48 h. The cells were harvested by centrifugation at 7500 rpm for 20 min at 25°C in a cooling centrifuge (Remi, Mumbai). The cell pellets were washed twice by centrifugation with sterile physiological saline and finally resuspended in 30 ml sterile physiological saline. A portion of the cell suspension was suitably diluted up to 10^{-9} in sterile saline and the number of cells ml^{-1} of suspension was determined by drop count method (Miles *et al*., 1938) on TSA after incubation at 30±2°C for 48 h.

P. aeruginosa FARP72 was tested for pathogenicity in rohu (*L. rohita*) fingerlings by experimental challenge through intramuscular injection (Iqbal *et al.,* 1999) at levels of 5.65 x 10^{10}, 5.65 x 10^{9}, 5.65 x 10^{8} and 5.65 x 10^{7} cells $fish^{-1}$. Twelve glass aquaria (60 x 45 x 30 cm) of 50 l capacity were used for the experiment. The glass aquaria were cleaned with chlorinated water (200 ppm), flushed thoroughly with freshwater and dried for 5 days before use and arranged in three tier iron stands. The glass aquaria were divided in to six groups, *viz*., A, B, C, D, E and F with two aquaria in each group. All glass aquaria were filled with clean water to a volume of 30 l each and were conditioned for three days and then stocked with experimental fish at the rate of 10 fish per tank. The aquaria were labelled and covered with nylon netting and the fishes were acclimatised in the aquaria for three days. After acclimatisation in the glass aquaria, each fish group was injected 0.1ml of bacterial suspension at different concentrations as required. Fishes in control group E received 0.1ml of 0.85% physiological saline instead of bacterial suspension and control group F received no injection. The challenged fishes in each group were maintained in the respective aquaria for 20 days. Mortality, external signs of infection and behavioural abnormalities were recorded daily. The median lethal dose (LD_{50}) was calculated, according to the method of Reed and Muench (1938).

Molecular characterisation of P. aeruginosa FARP72

The identity of the antagonistic bacterial strain *P. aeruginosa* FARP72 was further confirmed using molecular technique by 16S rDNA gene sequence analysis. Pure culture of *P. aeruginosa* FARP72 was used for DNA extraction. Few colonies of *P. aeruginosa* FARP72 on TSA were picked with a sterilised toothpick and suspended in 0.5 ml of sterile saline in 1.5 ml centrifuge tube. The contents of the tube were centrifuged at 10000 rpm for 10 min. After removal of the supernatant, pellet was suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA), incubated at 56ºC for 30 min and then heated at 100ºC for 10 min. After heating, the supernatant was used for polymerase chain reaction (PCR).

PCR was carried out using universal primers, *viz*., 27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′ (Lane *et al*., 1985). Amplification was done by initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing of primers at 55°C for 60 sec and extension at 72°C for 60 sec. The final extension was at 72°C for 10 min. The amplicons were analysed on a 1.5% agarose (HiMedia, India) gel containing 0.5 µg ml^{-1} ethidium bromide in 1x Tris-acetate-EDTA (TAE) buffer and purified using Miniprep DNA purification kit (Takara Biotech, Japan), prior to sequencing.

The edited sequence (1466 bp) was compared with the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) programme to identify the nearest neighbour of the amplified sequence (www.ncbi.nlm.gov/BLAST). Data analysis and multiple alignments were performed using Clustal X and MEGA5 softwares. The confidence values of the internal nodes were calculated by performing 1,000 bootstrap analyses. Phylogenetic tree was constructed, based on the alignment of 400-1541 bp long 16S rRNA gene sequences of 22 bacterial strains from NCBI GenBank database comprising *P. aeruginosa* (n=4) and *P. putida* (n=2) along with other Gram positive (n=10) and Gram negative (n=6) species. The evolutionary history was inferred, using the maximum likelihood method and distance calculations were done using the Kimura-2 correction for evolutionary rate.

Fatty acid methyl ester (FAME) analysis

The whole cellular fatty acid methyl esters (FAME) profile of the antagonistic bacterium, *P. aeruginosa* FARP72 was determined using the MIS Sherlock automatic identification system (MIDI, Inc., Newark, USA) at the Royal Life Sciences Private Limited, Secunderabad, India. The fatty acid methyl esters were extracted in accordance with the MIDI FAME™ protocol of the Microbial Identification System. The extract was analysed by gas chromatography, using a flame ionisation detector, after capillary column separation (Ultra 2, 25 m, 0.20 mm, 0.33 µm - phenyl methyl silicon fused silica). FAME profile of the strain was identified by MIS Sherlock software (ACTIN6 method). The similarity of the FAME profile of the isolate with the species in ACTIN1 was represented by similarity indices (IS). IS value higher

than 0.5, was considered to be positive. Aerobic library (RTSBA6 6.00) was referred for the analysis. The analysis was performed as per Sasser (2001).

Results

Screening and selection of indicator organism

On the basis of inhibition by 4 producer strains, *B. brevis* S23 was selected as the potent indicator organism for further studies related to the enumeration of antagonistic bacteria from fish skin mucus (Table 1).

Table 1. Screening and selection of indicator organism

Producer strains	Zone of inhibition in mm and indicator strains		
	Lactobacillus sp. S12	*Brevibacillus brevis* S23	*Corynebacterium* sp. S31
Bacillus sp. P39	2.00	7.00	2.00
Lactobacillus sp. P32	8.00	5.00	2.00
Enterobacter sp. P36	4.00	6.00	3.00
Pseudomonas fluorescens P37	3.00	7.50	3.00
Micrococcus sp. P52	1.00	9.00	2.50

Identification of antagonistic bacterial strains

A total of 41 antagonistic bacterial strains were isolated, out of which 82.93% (n=34) were Gram positive and 17.07% (n=7) were Gram negative. Among the Gram positive bacteria isolated from fish skin mucus, Gram positive rods were the dominant group (Table 2). The antagonistic bacteria isolated from *C. catla* include *Bacillus* spp. (n=3), *Corynebacterium* spp. (n=2), *Pseudomonas* spp. (n=2), *Lactobacillus* spp. (n=1) and *Enterobacter* spp. (n=1). Gram positive cocci such as *Staphylococcus* spp. (n=5), *Micrococcus* spp. (n=3) were the dominant groups isolated from *L. rohita,*. Other antagonistic strains identified were *Pseudomonas* spp. (n=2), *Corynebacterium* spp. (n=1), *Bacillus* spp. (n=1) and *Enterobacter intermedius* (n=1). All the antagonistic bacteria isolated from *C. mrigala* were Gram positive belonging to the genus *Bacillus* (n=4), *Arthrobacter* (n=2) and *Lactobacillus* (n=1). Likewise, the antagonistic bacterial strains isolated from *C. carpio* include *Arthrobacter* spp. (n=2), *Bacillus* spp. (n=1) and *Lactobacillus* spp. (n=1). Eight antagonistic bacterial strains, comprising *Bacillus* spp. (n=2), *Lactobacillus* spp. (n=2), *Staphylococcus* spp. (n=2), *Corynebacterium* spp. (n=1) and *Pseudomonas* spp. (n=1), were isolated from *C. batrachus*.

Screening and selection of potential antagonistic bacterium

In vitro inhibitory activity of antagonistic bacteria against test bacterial pathogens

Eighteen opportunistic human and fish pathogenic bacteria belonging to the genera *Aeromonas, Bacillus, Edwardsiella, Escherichia, Klebsiella, Pseudomonas, Salmonella, Staphylococcus, Streptococcus* and *Vibrio* were inhibited *in vitro* at varying levels by the potential Gram positive antagonistic strains such as *Lactobacillus* spp. (P32 and P81), *Bacillus* spp. (P33, P35, P39, P55, P91, P92, P101, P134, P136, P201 and P251), *Micrococcus* spp. (P52 and P61), *Staphylococcus* spp. (P65 and P66), *Arthrobacter* spp. (P131, P161and P181) and also by Gram negative antagonistic strains such as *Pseudomonas* spp. (P37, P38 and P72) and *Enterobacter* spp. (P36). The inhibitory activity of the producer strains was observed by the growth inhibition of test organisms. *P. aeruginosa* FARP72 effected the maximum inhibition against the test strains and the inhibitory zone ranged from 2 to 37 mm (Table 3). Inhibitory zone ranging from 1 to 35 mm were exhibited by *Arthrobacter* spp. P131 against the test strains. Based on the wide spectrum of inhibitory activity exhibited by *P. aeruginosa* FARP72 and *Arthrobacter* sp. P131, they were considered to be the most potent antagonistic strains. Further, *P. aeruginosa* FARP72 produced inhibition zone >10 mm against 9 out of 18 pathogenic test strains *viz*., *P. aeruginosa* CNMC isolated from patient suffering from acute pneumonia; *E. coli* CNMC from human stool; *E. tarda* SDDL isolated from diseased fish in Chennai; *S. aureus* ATCC 12598 FCRI; *V. cholerae* FCRI; Enterohaemorrhagic *E. coli* FCRI; *Salmonella typhi* FCRI; *Aeromonas* sp. AAH and *S. aureus* FPT (Table 4). *Arthrobacter* sp. P131 was effective against only seven pathogenic test strains, which showed inhibitation zone >10 mm. *Bacillus* sp. P35 produced zone ranging from 1 to 37 mm against the test strains and among them only nine test strains produced zone >5 mm. Likewise, *Bacillus* sp. P39 produced inhibitation zone ranging from 2 to 35 mm against the test strains and among them only six test strains produced inhibitation zone >5 mm. Similarly, *Bacillus* sp. P55, *Bacillus* sp. P92, *Bacillus* sp. P101 and *Bacillus* sp. P251 exhibited inhibition zone varying from 1 to 35 mm, 2 to 29 mm, 2.5 to 7.5 mm respectively against the test strains. Inhibition zone produced by *Arthrobacter* sp. P161 and P181 against the test strains were in the range of 1 - 8 mm.

Table 2. Antagonistic bacteria isolated from fish skin mucus

Source (Fish species)	Antagonistic bacteria		
	Gram positive bacteria		Gram negative bacteria
	Rods	Cocci	Rods
Catla catla	*Corynebacterium* sp. P31 *Lactobacillus* sp. P32 *Bacillus* sp. P33 *Corynebacterium* sp. P34 *Bacillus* sp. P35 *Bacillus* sp. P39		*Enterobacter* sp. P36 *Pseudomonas fluorescens* P37 *Pseudomonas putida* P38
Labeo rohita	*Corynebacterium* sp. P44 *Bacillus* sp. P54	*Micrococcus* sp. P41 *Staphylococcus* sp. P43 *Micrococcus* sp. P52 *Micrococcus* sp. P61 *Staphylococcus* sp. P62 *Staphylococcus* sp. P63 *Staphylococcus* sp. P64 *Staphylococcus* sp. P65	*Pseudomonas* sp. P42 *Pseudomonas* sp. P51 *Enterobacter* sp. P53
Cirrhinus mrigala	*Bacillus subtilis* P67 *Arthrobacter* sp. P131 *Bacillus polymyxa* P101 *Bacillus subtilis* P133 *Bacillus subtilis* P134 *Arthrobacter* sp. P135 *Lactobacillus* sp. P141		
Cyprinus carpio	*Arthrobacter* sp. P161 *Bacillus* spp. P136 *Lactobacillus* sp. P201 *Arthrobacter* sp. P221		
Clarias batrachus	*Corynebacterium* sp. P71 *Lactobacillus* sp. P81 *Bacillus* sp. P91 *Bacillus* sp. P92 *Lactobacillus* sp. P93	*Staphylococcus* sp. P82 *Staphylococcus* sp. P66	*Pseudomonas aeruginosa* FARP72

Antibacterial spectrum of P. aeruginosa FARP72 against human and fish pathogens

The potent antagonistic bacterial strain, *P. aeruginosa* FARP72 was chosen, among the 41 antagonistic bacterial strains, on the basis of the broad spectrum of activity and zone of inhibition (>5 mm) produced against the 18 test strains by cross streaking method (Table 3 and 4). In parallel streaking method, it was observed that the growth of *P. aeruginosa* CNMC; *E. coli* CNMC, *E. tarda* SDDL; *S. aureus* ATCC 12598 *V. cholerae* FCRI; Enterohaemorrhagic *E. coli* FCRI; *Salmonella typhi* FCRI; *Aeromonas* sp. AAH and *Pseudomonas* sp. FPT; *Bacillus pumilus* FPT; *B. amyloliquefaciens* FPT, *S. aureus* S2, FPT; *E. coli* FPT; β-haemolytic *Streptococcus* sp. AAH; β-haemolytic *A. hydrophila* AAH were totally inhibited, whereas *K. pneumoniae* CNMC and *S. aureus* CNMC were only slightly inhibited (Table 4).

Characteristics of potential antagonistic bacterium P. aeruginosa FARP72 as a probiotic

The characteristics exhibited by *P. aeruginosa* FARP72 as potential probiotic agent are presented in Table 5.

Pathogenicity in L. rohita

The first mortality was observed at 24 h post-injection in the tank containing fish challenged with 5.65 x 10^{10} cells $fish^{-1}$ of *P. aeruginosa* FARP72. The groups injected with *P. aeruginosa* FARP72 at levels of 5.65 x 10^{9}, 5.65x10^{8} and 5.6 5x 10^{7} cells $fish^{-1}$ remained active with no serious abnormalities and mortalities except for the primary inflammatory response at the place of injection, which was recovered in 3-4 days post-injection. After 15 days, 30% fish mortalities were observed in the stock inoculated with 5.65x10^{10} cells $fish^{-1}$. The LD_{50}

Table 3. In vitro antagonistic activity of producer strains against fish and human bacterial pathogens by cross streak method

Producer strains	Zone of inhibition (mm)																	
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16	T17	T18
Lactobacillus sp.P32	1.00	1.00	1.00	2.00	2.00	2.00	1.50	1.50	1.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	2.00
Bacillus sp.P33	1.00	2.00	1.50	1.00	1.00	2.00	1.00	1.00	1.50	1.00	1.00	2.00	1.00	2.00	1.00	1.00	2.00	1.00
Bacillus sp.P35	3.50	11.00	3.50	3.00	7.50	1.50	5.50	2.00	3.00	3.00	3.00	14.00	5.00	6.00	14.50	37.00	16.00	2.50
Enterobacter sp.P36	10.00	6.50	3.00	2.50	6.50	4.50	2.00	2.00	2.50	1.00	2.50	1.00	3.00	3.50	2.00	19.00	3.00	1.00
Pseudomonas fluorescens P37	1.00	2.00	1.00	1.50	1.00	1.00	1.50	1.50	2.00	1.50	1.50	1.50	1.50	1.00	1.00	2.00	3.50	2.00
Pseudomonas putida P38	1.00	1.00	1.00	2.00	1.00	1.00	2.00	1.00	1.00	1.50	1.00	2.50	1.00	2.50	2.00	1.00	1.00	1.00
Bacillus sp.P39	2.00	8.00	3.50	2.00	2.50	19.00	5.00	3.00	2.50	11.00	2.50	2.00	5.00	3.00	35.00	4.50	2.50	1.50
Pseudomonas sp.P42	1.50	1.00	2.00	2.50	2.00	1.50	2.00	2.50	1.00	1.00	2.50	2.00	2.00	2.00	1.00	1.00	1.00	2.50
Micrococcus sp.P52	1.00	1.50	2.00	2.50	2.00	3.50	4.00	2.00	6.00	1.00	1.00	1.00	2.00	1.00	2.00	2.00	2.00	3.50
Bacillus sp.P55	1.50	10.00	3.50	3.50	3.50	3.00	1.00	6.00	8.00	6.00	3.50	8.50	4.50	4.50	2.00	9.00	35.00	15.00
Micrococcus sp.P61	12.00	6.50	1.00	1.00	2.00	2.50	2.00	5.00	4.00	6.00	1.00	1.00	1.00	2.00	2.00	2.50	1.00	7.50
Staphylococcus sp.P65	5.50	2.50	3.00	4.50	2.00	1.00	1.50	1.00	1.00	6.00	6.00	6.50	1.00	1.00	1.00	1.00	2.50	2.00
Staphylococcus sp.P66	1.00	2.00	3.00	1.00	2.00	2.00	1.00	4.00	2.00	2.00	1.00	1.00	1.00	2.00	3.50	3.00	2.00	1.00
Pseudomons aeruginosa FARP72	2.50	8.50	2.00	13.00	14.50	22.00	17.00	23.00	25.00	31.00	6.00	6.50	2.00	4.00	37.00	4.00	8.50	11.00
Lactobacillus sp P81	1.00	1.00	1.00	1.00	2.00	2.50	2.00	3.00	3.50	2.00	2.00	1.00	2.00	2.50	1.50	1.50	1.50	1.50
Bacillus P91	1.00	2.00	2.50	2.00	2.00	2.00	2.00	1.00	1.00	1.00	10.00	30.00	1.50	1.50	3.00	3.00	16.00	2.00
Bacillus sp. P92	2.00	19.00	3.00	3.00	3.00	3.00	2.50	3.50	12.00	2.50	10.00	1.50	1.50	10.00	19.00	29.00	20.00	4.50
Bacillus sp. P101	7.00	7.00	7.50	7.00	2.00	1.00	4.50	5.00	5.00	2.50	2.00	2.00	7.00	7.50	8.00	2.00	1.50	1.50
Arthrobacter sp. P131	31.00	1.00	30.00	32.00	35.00	1.00	5.00	2.50	5.00	1.00	35.00	1.00	1.50	13.00	1.00	32.00	1.50	1.00
Bacillus sp. P134	7.00	5.50	5.00	5.00	6.00	6.50	4.00	2.00	2.00	4.00	4.00	6.50	6.00	11.00	15.50	6.50	6.00	6.50
Bacillus sp. P136	4.50	6.00	2.50	2.50	2.00	5.00	4.00	7.00	1.00	2.00	3.00	6.00	5.50	1.00	1.00	1.50	2.50	2.00
Arthrobacter sp. P161	8.00	3.00	4.00	2.00	6.00	5.00	7.00	4.50	4.50	2.00	2.00	2.00	2.00	4.00	4.00	7.00	2.00	1.00
Arthrobacter sp. P181	8.50	4.50	4.50	3.00	8.00	7.00	1.00	1.00	6.00	3.50	2.00	4.50	4.50	3.00	7.00	8.00	1.00	4.00
Bacillus sp. P201	6.00	2.50	7.50	6.00	4.50	3.00	7.50	5.00	2.50	5.00	6.50	1.00	1.50	2.00	2.50	3.50	5.00	7.00
Bacillus sp. P251	8.00	1.00	4.50	6.00	4.50	7.00	5.50	6.00	1.00	1.00	2.00	3.00	3.00	5.00	4.50	4.50	2.00	1.00

T1: *Klebsiella pneumoniae* CNMC; T2: *Pseudomonas aeruginosa* CNMC; T3: *Staphylococcus aureus* CNMC; T4: *Escherichia coli* CNMC; T5: *Edwardsiella tarda* SDDL; T6: *Staphylococcus aureus* ATCC 12598 FCRI; T7: *Vibrio cholerae* FCRI; T8: *Enterohaemorrhagic Escherichia coli* FCRI; T9: *Salmonella Typhi* FCRI; T10: *Aeromonas* sp. AAH; T11: *Staphylococcus aureus* FPT; T12: *Pseudomonas* sp. FPT; T13: *Bacillus subtilis* FPT; T14: *Bacillus amyloliquefaciens* FPT, T15: *Staphylococcus aureus* FPT; T16: *Escherichia coli* FPT; T17: β-haemolytic *Streptococcus* sp. AAH; T18: *Aeromonas hydrophila* HS3 AAH

CNMC-Calcutta National Medical College and Hospital, Kolkata; SDDL –Shrimp Disease Diagnostic Laboratory, Tamil Nadu Fisheries University, Madhavaram, Chennai; FCRI - Fisheries College and Research Institute, Thoothukudi; AAH - Department of Aquatic Animal Health, FFSc, Kolkata; FPT- Department of Fish Processing Technology, FFSc, Kolkata

value of *P. aeruginosa* FARP72 was calculated to be >5.65 $\times 10^{10}$ cells $fish^{-1}$.

Molecular and phylogenetic characterisation of P. aeruginosa FARP72

BLAST analysis of the sequence data and phylogenetic tree (Fig. 1) revealed that the strain *P. aeruginosa* FARP72 showed closest similarity with two strains of *P. aeruginosa* (Accession no. KC776528 and EU221384). The 16S rDNA sequences of the strain have been deposited in the National Center for Biotechnology Information (NCBI) GenBank database, USA with the accession no. KC570343 and designated as *P. aeruginosa* FARP72.

Fatty acid methyl esters (FAME) analysis

FAME analysis of *P. aeruginosa* FARP72 (Fig. 2) revealed a total of 26 fatty acids of unsaturated, mono-saturated and poly-saturated origin. The predominant fatty acids are C18:1 w7c or C18:1 w6c, C16:0 and 16:1 w 7c/16:1 w6c or 16:1 w6c/16:1. The other important fatty acids are the hydroxyl fatty acids; C10:0 3-OH, C12:0, C12:0 2OH and C12:0 3-OH. FAME analysis identified the strain FARP72 as *P. aeruginosa* with similarity index 0.700.

In the present study, Gram positive rods were the dominant (70.59%) bacteria isolated from fish skin mucus which corroborate the observations of Abraham and

Table 4. Antibacterial spectrum of *P. aeruginosa* FARP72 against human and fish pathogens by cross streak and parallel streak methods *in vitro*

Test strains and source	Cross streaking: Zone of inhibition (mm)	Parallel streaking: Severity of inhibition
Klebsiella pneumoniae CNMC	2.50	+
Pseudomonas aeruginosa CNMC	8.50	++
Staphylococcus aureus CNMC	2.00	+
Escherichia coli CNMC	13.00	++
Edwardsiella tarda SDDL	14.50	++
Staphylococcus aureus ATCC 12598 FCRI	22.00	++
Vibrio cholerae FCRI	17.00	++
Enterohaemorrhagic Escherichia coli FCRI	23.00	++
Salmonella Typhi FCRI	25.00	++
Aeromonas sp. AAH	31.00	++
Staphylococcus aureus FPT1	6.00	+
Pseudomonas sp. FPT	6.50	++
Bacillus pumilus FPT	2.00	++
Bacillus amyloliquefaciens FPT	4.00	++
Staphylococcus aureus FPT2	37.00	++
Escherichia coli FPT	4.00	++
β-haemolytic *Streptococcus* sp. AAH	8.50	++
Aeromonas hydrophila HS3 AAH	11.00	++
Aeromonas hydrophila N10P AAH	22.00	++

+: Feeble/Thin growth, ++: Total inhibition.
CNMC-Calcutta National Medical College and Hospital, Kolkata; SDDL-Shrimp Disease Diagnostic Laboratory, Tamil Nadu Fisheries University, Madhavaram, Chennai; FCRI - Fisheries College and Research Institute, Thoothukudi; AAH - Department of Aquatic Animal Health, FFSc, Kolkata; FPT- Department of Fish Processing Technology, FFSc, Kolkata

Table 5. Potential characteristics of *Pseudomonas aeruginosa* P72 to be used as probiotics

Characteristics	Reactions
Biofilm formation	4* (++++)
Haemolytic activity	γ
Enzymatic activity	
Proteinase	+
Amylase	-
Lipase	+
Phospholipase	+
Growth at (ºC):	
4	-
22	++
32	++
45	++
65	-
Growth at pH:	
2.8	-
3.6	-
4.4	+
5.2	++
6.0	++
6.8	++
7.6	++
8.4	++
9.2	++
10.0	++
Growth at 6.5% sodium chloride (NaCl)	+
Bile (5%) tolerance (Growth on MacConkey agar)	++
Antibiogram	
Chloramphenicol, 30 µg	S
Ciprofloxacin, 5 µg	S
Co-trimoxasole, 25 µg	R
Gentamycin, 10 µg	S
Nitrofurantoin, 300 µg	R
Oxytetracycline, 30 µg	R
Pathogenicity (LD50 in cfu/ fish)	>5.65×1010

*0: Absent, 1: Weak, 2: Moderate, 3: Strong, 4: Very strong
+: Feeble/Thin growth, ++: Dense/ Thick growth, - : No growth
R: Resistant; S: Sensitive; LD: Lethal dose; cfu: Colony forming unit

Banerjee (2007). Inhibition of the growth of pathogenic bacteria seen in the cross streak method may be attributed to antibiotics, antimicrobial peptides, bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide or organic acids produced by the producer strains (Verschuere *et al.,* 2000). Torrento and Torres (1996) reported *in vitro* inhibition of *Vibrio harveyi* by a *Pseudomonas* sp. isolated from the aquatic environment. As with their terrestrial counterparts, aquatic pseudomonads are often antagonistic to other microorganisms (Gram, 1993), including fish pathogenic bacteria (Smith and Davey, 1993) and fish pathogenic fungi (Bly *et al*., 1997). Chythanya *et al.* (2002) also reported inhibition of shrimp pathogenic vibrios by an estuarine strain of *Pseudomonas,* attributed to a low molecular weight, heat

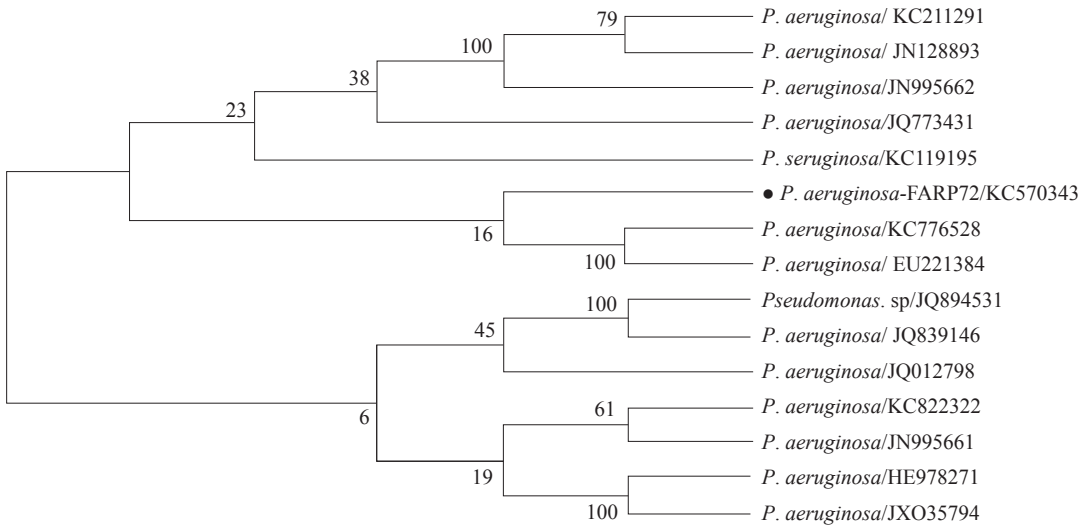

Fig. 1. Phylogenetic tree generated by neighbour-joining Kimura-2 parameter of the 16S rDNA sequence of *P. aeruginosa* FARP72 (●). Numbers at nodes indicate bootstrap confidence value (1000 replication).

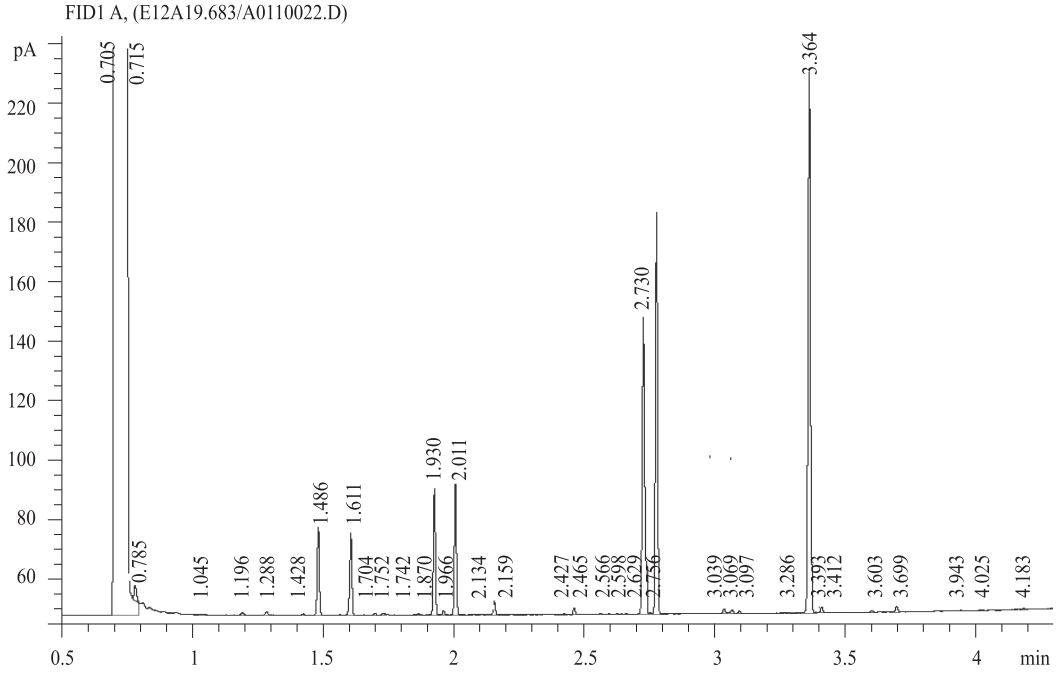

Fig. 2. Chromatogram of *P. aeruginosa* FARP72 showing the fatty acid peaks

stable non-protein antimicrobial substance. The mode of action of *Pseudomonas* strains have been attributed to the production of several metabolites from different groups such as indoles (Kang *et al*., 2006), phenasines (Kumar *et al*., 2005; Cazorla *et al*., 2006), pyocyanine, pyrrolnitrin (Ligon *et al*., 2000), pyolu-teorin, acetyl phloroglucinols (Raaijmakers *et al*.,1999; Guihen *et al.*, 2004), pseudotrienic acids A and B (Pohanka *et al.*, 2005), tensin and viscosinamide (Nielsen *et al*., 2000). Results of earlier studies reported the dominance of Gram positive antagonistic bacteria in fish, exhibiting broadspectrum action on Gram negative bacteria (Jack *et al.,* 2005; Abraham and Banerjee, 2007). However, in the present study, it has been demonstrated that both Gram-positive and Gram negative human as well as fish pathogens were inhibited by the Gram negative bacterium, *P. aeruginosa* FARP72 bacterium. The inhibitory results of *P. aeruginosa* FARP72 by parallel streak method on four strains of *S. aureus,* indicated variations from strain to strain. Herron-Olson *et al.* (2007) opined that specific gene

variations can play important roles in *in vivo* bacterial pathogenicity, colonisation and resistance. The observed variations in the sensitivity among the *S. aureus* strains may be due to the genetic variation of the species or due to the different sources of collection.

The initial screening and selection of probiotics/ biocontrol agents include testing of the strain for the following important criteria: phenotype and genotype stability, carbohydrate and protein utilisation patterns; acid and bile tolerance; viability, survivality and growth; intestinal epithelial adhesion properties and colonisation; production of antimicrobial substances; antibiotic resistance patterns; ability to inhibit known pathogens and spoilage organisms or both, as well as immunogenicity (Harzallah and Belhadj, 2013). The ability to adhere to the intestinal mucosa is one of the important selection criteria for probiotics, because adhesion to the intestinal mucosa is considered to be a pre-requisite for colonisation so that the strain can proliferate and grow (Tuomola *et al.,* 2001). *P. aeruginosa* FARP72 produced a very strong visible biofilm on glass surface, which indicated the characteristics of the bacterium to have strong colonisation property. According to Montgomery and Pollak (1988), the caecal pH of fish gut doesn't vary greatly from day to night (7.1-7.0). When starved fish were fed, however, both cardiac and pyloric stomachs exhibited pH values of 5.7 and 6.2, respectively. In the present study, growth of *P. aeruginosa* FARP72 was observed in pH ranging from 4.4 to 10.0. This indicated that it can withstand the normal fish gut pH value of 5.7-6.2 (Montgomery and Pollak, 1988) and grow. The strain further proved itself to be bile tolerant, as it grew profusely on media containing 5% bile salt.

Harzallah and Belhadj (2013) opined that the *in vivo* pathogenicity test needed to be done, followed by *in vitro* assays. The antagonistic strain *P. aeruginosa* FARP72 was found to be non-haemolytic or gamma haemolytic (γ-haemolysis). The demonstration of proteinase, lipase and phospholipase activities of *P. aeruginosa* FARP72 confirm the nutrient utilisation patterns through various enzymatic activities, which is an important feature of any probiotic strain (Tuomola *et al.* 2001). Long shelf life is a pre-requisite for a bacterial strain to be used as probiotics. The bacterium can sustain in normal room temperature can withstand high temperatures up to 45ºC and can also grow in 6.5% sodium chloride concentration (Table 5), which indicated that it can withstand salinity fluctuations. An ideal probiotic need to be harmless to the host and there must be no local or general pathogenic, allergic or mutagenic/ carcinogenic reactions provoked by the microorganism itself, its byproducts or its cell components (Desai, 2008). Stocks of *L. rohita* when injected intramuscularly with *P. aeruginosa* FARP72 at levels of $5.65x10^9$, $5.65x10^8$ and $5.65x10^7$ cells $fish^{-1}$ remained active with no serious abnormalities/mortalities. These observations indicated that the *P. aeruginosa* FARP72 strain did not provoke any systemic pathologic reactions. Mittal *et al.* (1980) categorised bacterial strains which exhibited $LD_{50} > 10^8$ cfu ml^{-1} as non-virulent. The recovery of fish in 3-4 days post-injection period, the LD_{50} value of $>5.65\times10^{10}$ cells $fish^{-1}$ and the categorisation of Mittal *et al.* (1980) suggested that the antagonistic bacterial strain *P. aeruginosa* FARP72 is non-virulent and non-pathogenic.

The evaluation of the safety of probiotic strains or biocontrol agents also includes the exclusion of antibiotic resistance of clinical importance (Klein, 2011). *P. aeruginosa* FARP72 was resistant to three antibiotics of importance in human medicine, *viz*., co-trimoxasole, nitrofurantoin and oxytetracycline. Earlier studies demonstrated that development of resistance to co-trimoxasole, oxytetracycline and nitrofurantoin are plasmid mediated (Shotts *et al*., 1976; McCalla *et al*., 1978). It is possible that the resistance gene(s) can be identified and eliminated to support normal safety assessment of the antagonistic strain.

The 16S rDNA gene sequence of the isolate FARP72 identified and reconfirmed the phenotypically characterised bacterium as *P. aeruginosa.* Phylogenetic tree based on 16S rDNA gene sequence of *P. aeruginosa* (Acc. no. KC570343) with other 16S rDNA gene sequences, as shown in Fig. 1, revealed that the strain *P. aeruginosa* FARP72 showed closest similarity with two strains of *P. aeruginosa* (Acc. no. KC776528 and EU221384). *P. aeruginosa* (KC776528) represented the bacterial strain that biodegrade polycyclic aromatic hydrocarbon whereas, *P. aeruginosa* (EU221384) represented *Pseudomonas* population in the rhizosphere of wheat which promote plant growth and both of them are non-pathogenic and beneficial bacteria. This also implied that the present strain *P. aeruginosa* FARP72 could be a non-pathogenic strain, by virtue of its close similarity with the above non-pathogenic strains.

The fatty acids revealed from *P. aeruginosa* FARP72 are in conformity with the observations of Panda *et al.* (2013). Basically, the cellular fatty acid profiles of *Pseudomonas aeruginosa* contain four acids (3-OH 10:0, 12:0, 2-OH 12:0, 3-OH 12:0), which is absent in other species of *Pseudomonas* (Mitruka, 1975).

The potential antagonistic bacterium *P. aeruginosa* FARP72 from fish skin mucus has fulfilled majority of the pre-requisite features for a bacterial strain to be used as probiotic or biocontrol agent. Hence, it can be interpreted

that *P. aeruginosa* FARP72 could be an effective and potential candidate species for use as probiotic or biocontrol agent in aquaculture. Further studies on plasmid curing and genetic engineering aspects of this potent antagonistic bacterium are needed to ensure its safety before commercialisation.

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