Introduction

The use of antimicrobial drugs for disease control in agriculture and aquaculture has led to the emergence of antibiotic resistant bacteria (Schwarz et al., 2001; Akinbowale et al., 2006). The problem of antibiotic resistance and its epidemiological consequences led to the exploration of several alternate approaches for disease management in aquaculture systems. Amongst them the most popular and practical approach was the use of probiotics as prophylactics. Verschuere et al. (2000) and Moriarty (1998) defined aquatic probiotics as live microorganisms that have a beneficial effect on the host, by modifying the microbial community associated with the host, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of ambient environment. This implies a much wider range of microorganisms being used as probiotics for aquaculture animals.

Enhancements of colonization resistance and/or direct inhibitory effect against pathogens are important factors where probiotics have reduced the incidence and duration of diseases. Probiotic strains have inhibited pathogenic bacteria both in vivo and in vitro through different mechanisms, which include protection creating a hostile environment for pathogens by the production of inhibitory compounds such as bacteriocins, siderophores, lysozymes, proteases, or hydrogen peroxide, formation of ammonia and diacetyl, alteration of pH value by the production of organic acids (Verschuere et al., 2000), and competition for essential nutrients and adhesion sites (Vine et al., 2004).

Antagonism toward pathogens is one of the properties of probiotic bacterial strains (Moriarty, 1999; Atlas and Bartha, 1997). The antagonistic property against bacteria is characterized by the production of antimicrobial substances such as bacteriocins, siderophores, and other bacteriocin like inhibitory substances. Because of the relative specificity of bacteriocins as compared with antibiotics, it can be anticipated that the identification of broader spectrum bacteriocins will be an active research endeavour (Diez-Gonzalez, 2002). The high density of fish in hatchery tanks and ponds is conducive to the spread of pathogens and the aquaculture industry has recognized that the use of siderophore producing bacteria to displace pathogenic bacteria through iron competition is a better remedy than the use of antibiotics (Gatesoupe, 1999; Havenaar et al., 1992). Adhesion of probiotic microorganisms to the intestinal mucosa is considered important for many of the observed probiotic health effects (Ouwehand et al. 1999). Colonization, followed by biofilm formation, starts by the adhesion of a single cell or cell aggregates at the surface. Bacterial aggregation ability between microorganisms of the same strain (autoaggregation) or between
genetically different strains (coaggregation) is, in most cases, related to cell adherence properties. Quorum sensing antagonists in aquaculture might constitute an alternative approach for the biocontrol of microbial infections (Dong et al., 2005).

In this study, potential probiotic bacterial strains, namely, MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4 from the natural flora of ornamental fish guppy were isolated and characterized.

Objectives

- Isolation, screening and identification of potential probiotic strains MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4 from the natural flora of guppy (Poecilia reticulata) based on the antagonistic activity tests of their cellular components against five indicator strains (Aeromonas hydrophila 1739, Vibrio cholerae 3906, Flavobacterium 2495, Acinetobacter 1271 and Alcaligenes 1424), the antibiotic sensitivity tests, plasmid profiles and in vitro growth characteristics like lag period and doubling time of these isolates.

- Characterization of bacteriocin like substances produced by potential probiotic strains MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4.

- Characterization of siderophores produced by potential probiotic strains MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4.

- Adhesion, biofilm formation and N-acyl homoserine (N-AHL) degradation abilities of potential probiotic strains MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4.

Materials and methods

Isolation and screening of potential probiotic bacterial strains

Four isolates MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4 were selected from the normal flora of guppy, Poecilia reticulata (obtained from Government Model Fish Farm, Kerala), based on their inhibitory spectrum against the five indicator strains. All the above four isolates were identified using bioMérieux VITEK 2 compact system and molecular characteristics (16S rDNA sequencing).

Antagonistic activity of cellular components and antibiotic sensitivity test

Four types of antimicrobial metabolites, namely, heat killed whole cell product (HKWCP), whole cell product (WCP), intra cellular product (ICP) and extra cellular product (ECP), were
prepared from MBTU_PB1, MBTU_PB2, MBTU_PB3, and MBTU_PB4 (Das et al., 2006). Antibiogram of the four selected strains was prepared using selected thirteen antibiotic discs.

Analysis of plasmid DNA

Small-scale preparation of plasmid DNA from the four selected strains was performed using rapid alkaline lysis procedure of Maniatis et al. (1982). The curing of plasmid was performed as described by Trevors (1986). To check the role of plasmids, the isolated strains were tested for inhibitory activity in well diffusion assay against the indicator strains after the successful curing of plasmid DNA.

Growth profiles

To obtain the growth profiles of the selected strains and the indicator strains, the nutrient broth was inoculated with 50μL of 24h culture and incubated for 36 h at 25°C during which the optical density (OD) was read every 1 hour at 640 nm. Each culture was inoculated in triplicate and the readings of the profiles were averaged.

Preliminary characterization of bacteriocin like inhibitory substances (BLIS)

The cell free supernatants of the four selected strains were collected and the influences of temperature, pH, proteolytic enzymes and organic solvents on the bacteriocin activity of the isolated strains were studied.

UV-Vis and FTIR analysis and molecular weight determination of BLIS

The lyophilised samples of the four selected strains were diluted in double distilled water and the UV-Vis absorbance measurements were recorded using UV–Vis Shimadzu spectrophotometer (220-360 nm). FTIR spectrums of the lyophilized samples were recorded by KBr pellet method in the region 4000-400 cm⁻¹ using Shimadzu FTIR spectrophotometer. Molecular mass of each of the partially purified BLIS from the four selected strains were estimated using Tricine-SDS-PAGE, MALDI-TOF MS and LC/MS techniques.

Siderophore production

The four strains MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4, were grown under iron limited condition to secrete siderophores (Payne, 1994). Also, the inhibitory activity of the four selected strains against indicator strains cultured with and without iron was compared. Production of siderophores by the isolates was determined using the CAS universal chemical assay (Schwyn and Neilands, 1987). The types of siderophores were determined by using specific assays: FeCl₃ test, tetrazolium test and ferric perchlorate assay for hydroxamates (Neilands, 1981; Snow, 1954; Atkin et al., 1970), FeCl₃ test and Arnow’s
test for catecholates (Neilands, 1981; Arnow, 1937) and spectrophotometric test for carboxylates (Shenker et al., 1992). The quantity of siderophores in the samples was extrapolated from the standard curve obtained with aqueous dilutions of deferoxamine mesylate (Sigma) and denoted in µg/mL.

**In vitro adhesion, autoaggregation, coaggregation assays and the cell surface hydrophobicity**

The adhesion (Vesterlund et al., 2005) and autoaggregation assays (Pan et al., 2008) and the determination of cell-surface hydrophobicity (Ouwehand et al., 1999) of the four selected strains and the five indicator strains were separately performed. The method of coaggregation experiments (Pan et al., 2008) for the four selected strains with the five indicator strains was the same as autoaggregation assay.

**Biofilm formation by bacterial strains and screening for quorum sensing (QS) inhibition**

Scanning electron micrographs (SEM) of the biofilms formed on glass surfaces by the four selected strains were obtained (Lembke et al. 2006). The commonly used microtitre-plates method (Rode et al., 2007) was applied for the quantification of biofilm formations of the four selected strains and the five indicator strains separately. Chromobacterium violaceum MTCC 2656 was preliminarily used for screening the two selected Gram-negative strains (MBTU_PB1 and MBTU_PB4) for quorum sensing (QS) properties (thenmozhi et al., 2009).

**Result and discussions**

The use of probiotics is an important management tool, but its efficiency depends on understanding the nature of competition between species or strains. Antagonism to indicator strains was the first step for the screening of probiotics. Four isolated strains of guppy (MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4) showed moderate to strong antagonistic activity against the five indicator strains, and these isolates were further selected and identified using 16S rDNA gene sequence analysis (NCBI GenBank accession numbers JN247799, JN247800, JN247801 and JN247802 for MBTU_PB4, MBTU_PB3, MBTU_PB1 and MBTU_PB2, respectively).

In the present study, except WCP, the HKWCP, ECP and ICP of all the four isolated strains were equally effective, as revealed by the zone of inhibition, to all the tested indicator strains. The results obtained after the antibiotic sensitivity test on Muller-Hinton agar plates showed the wild nature of organisms. The isolates MBTU_PB2 and MBTU_PB3 were sensitive to all the tested antibiotics. However, MBTU_PB1 was sensitive to all the tested
antibiotics except *Tetracycline, Vancomycin, Methicillin, Erythromycin* and *Penicillin*, while MBTU_PB4 was sensitive to all the antibiotics except *Methicillin* and *Penicillin*.

Plasmids were produced only by the two Gram-negative isolates, MBTU_PB1 and MBTU_PB4. MBTU_PB1 harboured two plasmids with molecular weights of ~10 kb and ~5 kb, while MBTU_PB3 harboured plasmids with a molecular weight of ~13 kb. Application of ethidium bromide or acridine orange intercalating agents along with elevated temperature (40°C) was found to be effective in curing the plasmids. The percentage of plasmid curing was determined as 35.4% and 51.8% for MBTU_PB1 and MBTU_PB4, respectively. However, the plasmids have no role on the antagonistic properties of the isolated strains. This may limit the transfer of many antibiotic resistance genes between pathogenic and nonpathogenic Gram-negative bacteria (Moriarty, 1998).

A ranking index (RI) was developed using the parameters doubling time (t_d) and lag period (λ): RI = 100 / (λ t_d). The RI hypothesizes that a bacterium with a short t_d and short λ has a better chance of out competing other bacteria based on their growth characteristics. The RI values indicated that among the four selected strains, MBTU_PB2 had significantly higher out competing characters against the indicator strains.

Thermo sensitivity studies indicated that all the four selected strains produced heat tolerant antimicrobial substances. In our case, organic acid did not cause the inhibition observed, since the BLIS activity all four selected strains showed optimal inhibitory activity at pH 7±0.5. BLIS activity of all the four strains was found to be sensitive to different proteolytic enzymes (trypsin and proteinase K) which indicated the proteinaceous nature of bactericidal compound. The antimicrobial activities of all the four isolates, in general, were sensitive by treatments with organic solvents.

The UV-Vis absorption spectrums of the bacteriocins were examined which showed maximum absorbance for each of the four isolates in the range 220-240 nm, characteristic of peptide bonds. FTIR analysis of each of the four samples showed two characteristic absorption bands in the range 1660-1535 cm⁻¹ and 1200-1000 cm⁻¹, which correspond to peptide bonds and PO₂⁻ groups, respectively (Stewart, 1965). A wide band indicative of the presence of polar groups in each of the four selected strains appeared in the range 3700-3100 cm⁻¹ (Stewart, 1965). In addition, for each of the four selected strains, the C-H stretching in the range 2960-2920 cm⁻¹ and 1240-1530 cm⁻¹ indicated the presence of aliphatic chains, which may be related to the presence of a fatty acid in the structure. Therefore, these results
confirm the proteinaceous and phospholipid nature of the antimicrobial compounds produced by the selected four isolates.

MALDI-TOF MS is effective for peptides and proteins with molecular masses ranging from 0.5 to 30 kDa. Molecular mass of each of the partially purified BLIS from the four selected strains was estimated to be approximately in the range between 3700-4900 kDa. Unlike in MALDI, peptides in LC-MS obtained multiple charges, which were ascertained in order to compute the peptide mass.

This work establishes the presence of a siderophore-based iron-sequestration mechanism in the isolates from guppy. All the four isolates produced siderophores in the presence of an iron-chelating compound, 2,2’-dipyridyl. The ability to synthesize siderophores would undoubtedly be an advantage for survival, growth and pathogenicity of the isolates. In this study, we observed that all the investigated indicator strains displayed growth along each of the four selected strains under both iron-sufficient and iron-limited conditions. However, the growth of the indicator strains was markedly reduced when grown in strains cultured without iron. The different color changes we observed in the CAS-blue agar (orange, purple, or purplish-red) suggested the production of siderophores of different natures by the microorganisms and the intensity could be related to siderophore concentration. Both FeCl₃ and tetrazolium tests produced positive results for all the four selected strains indicating the presence of hydroxamate siderophores. The spectral scans using siderophores and iron-perchlorate assay revealed that the siderophores produced by each of the isolates showed a peak between 425 and 520 nm, depending possibly on the iron-hydroxamate coordination structure. Of the four isolates under identical conditions, MBTU_PB2 produced maximum amount of siderophore (80%), while MBTU_PB1 produced the least amount (38%).

All the selected four strains had higher adhesion abilities than the indicator strains. However, no general correlation was observed between cell-surface hydrophobicity and the ability to adhere to the intestinal mucus. The four selected strains and *V. cholerae* were found strongly autoaggregating (autoaggregation percentage ≥ 80). Further, all the four selected strains showed strain-specific coaggregation abilities with the tested indicator strains.

At the optimum growth temperature (37°C), both MBTU_PB1 and MBTU_PB3 had the maximum abilities to form biofilms, while MBTU_PB2 and MBTU_PB4 displayed moderate biofilm productions. Both the Gram-negative selected strains (MBTU_PB1 and MBTU_PB4) were capable to degrade quorum sensing N-AHLs based on their ability to inhibit *violacein* production.
Conclusions
We have performed the isolation, identification and in vitro characterization of the four strains MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4 from the natural flora of guppy (Poecilia reticulata). The above four selected strains have a well defined role in the immune status of the host organism. Of the four strains, MBTU_PB1 and MBTU_PB3 can be considered as new species based on their BLAST search analysis. The selected four strains exhibited antagonistic activity against the five indicator strains. Thus, these selected isolates could be a promising source for biocontrol agents in aquaculture.

The RI assumption suggested the out competing nature of the selected strains against the indicator strains, which is desirable for probiotic strains. Each of the selected four strains produced siderophores and also showed BLIS activity against the indicator strains. Qualitative analysis of all the strains showed the hydroxamate nature of the siderophores, while quantitative analysis revealed the maximum amount of siderophore production by MBTU_PB2. The characterization of BLIS produced by all the four strains based on the action of proteolytic enzymes, and using UV-Vis and FTIR analysis revealed the protenaceous nature of the bactericidal compounds. Additionally, tricine-SDS, MALDI-TOF MS and LC-MS analysis suggested the peptide nature of these compounds.

The in vitro adhesion properties or the ability of colonization is often considered as a selection criteria for probiotics. All the selected four strains had higher adhesion abilities than the indicator strains. However, no general correlation was observed between cell-surface hydrophobicity and the ability to adhere to intestinal mucus. The four selected strains strongly autoaggregated and also showed strain-specific coaggregation abilities with the indicator strains tested. Further, these four strains had the ability to form biofilms on polystyrene surfaces. In our investigation, the Gram-negative strains MBTU_PB1 and MBTU_PB4 showed N-AHL degradation, which is needed to be confirmed.

Our studies on in vitro probiotic characterizations of these strains is equally promising to develop probiotic therapy in aquaculture as individual strain or in combination by formulating an aquaculture feed through desirable techniques such as bathing or solid state fermentation. In aquaculture, a probiotic mixture might be more effective than applying a single species, since not all desirable probiotic characteristics would be present in a single isolated strain. Today, aquaculture sector face problems in maintenance of proper culture conditions especially due to disease management, water quality and increasing nutritional value. Probiotic therapy could manage these problems very effectively.