Available online at: www.mbai.org.in doi:10.6024/jmbai.2021.63.2.2278-18

Occurrence of fluorescent bacterium *Pseudomonas aeruginosa* a putative biocontrol agent in Indian pompano *Trachinotus mookalee* Cuvier, 1832

Imelda Joseph*, Anu Mathew and V. Susmitha
ICAR-Central Marine Fisheries Research Institute, Kochi–682 018, Kerala, India.

*Correspondence e-mail: imeldajoseph@gmail.com

Received: 24 Mar 2021 Accepted: 25 Nov 2021 Published: 10 Dec 2021

Short communication

Abstract

Bacterial diversity studies in healthy marine finfish *Trachinotus mookalee* Cuvier, 1832 have lead to the isolation of six strains of *Pseudomonas* spp. The bacteria were isolated from the skin, gills and intestine of live and healthy fish collected from Karwar, Karnataka, India (N- 13°, 05.722'; E- 079°, 48.658'). Among the strains three were non-fluorescent and others were fluorescent *Pseudomonas* strains. Fluorescent strains are known to secrete pyoverdine, a yellow-green siderophore which has wide application in agriculture and allied sectors. Fluorescent strains were further characterized by 16S rDNA sequencing and were identified as *Pseudomonas aeruginosa* strain TRG1 (Genbank Accession No. KC109784).

Keywords*: Fluorescent bacterium, biocontrol, Pseudomonas aeruginosa, Indian pompano*

Introduction

Pseudomonas is part of a large, heterogeneous and ubiquitous group of microorganisms generally referred to as pseudomonads. They are characterized as being highly metabolically versatile, bioactive and prolific colonizers of surfaces. Pseudomonads are gram-negative, straight or slightly curved rods with polar flagella; they are chemo-organotrophs with a respiratory, non-fermentative type of metabolism and are usually catalase and oxidase positive. Pseudomonads represent a diverse group of medically, environmentally and biotechnologically important bacteria. They are characterized by enormous metabolic capacity, which is reflected by their capability to adapt to diverse and challenging environments, degrade recalcitrant compounds, and synthesize a variety of low-molecular weight compounds as well as biopolymer. Pseudomonads constitute a large part of the microflora of the skin, gills and intestinal tracts of live fish (Cahill, 1990; Strom and Ringo, 1993) and are only rarely reported as pathogens of fish (Hatai and Willoughby, 1988). Pseudomonads have been identified to be of importance in bioremediation as a result of their tremendous capacity for biodegradation. They also offer considerable promise in agronomic applications, since many strains are bioactive, fast-growing, prolific colonizers of plant surfaces and are able to suppress or out-compete pathogenic and other deleterious

microorganisms (Nakai *et al*., 1985). The genus *Pseudomonas* is capable of using different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils to produce rhamnolipid-type bio-surfactants (Desai and Banat, 1997; Koch *et al.,* 1991). Rhamnolipids produced by strains of *Pseudomonas aeruginosa* were shown to be highly effective against plant pathogens, including *Pythium aphanidermatum, Plasmoparalactucae-radicis* and *Phytophthora capsci*.

Fluorescent *Pseudomonas* spp. secrete pyoverdine, a fluorescent yellow-green siderophore under iron-limiting conditions. Such species 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, hydrogen cyanide, and iron-chelating siderophores. The biocontrol properties of *Pseudomonas fluorescens* strains (CHA0 or Pf-5 for example) were applied in protecting roots of some plant species against parasitic fungi such as *Fusarium* or *Pythium,* as well as some phytophagous nematodes (Chigusa *et al*., 1996; Huan *et al.,* 1999). *P. aeruginosa* has been studied in more detail than any other pseudomonad using genetic techniques. The objective of the present study was to map the bacterial diversity of selected economically important marine fishes of Indian waters and to characterize the strains by phenotypic and genotypic characteristics. Indian pompano *Trachinotus mookalee* Cuvier, 1832 is a demersal carangid fish having good local market and it forms a moderate fishery resource in Karnataka. It is also having good mariculture potential and the seed production has been commercialized in India.

Material and methods

Sample collection and processing

Live and healthy Indian pompano *T. mookalee* Cuvier, 1832 were collected from Karwar, Karnataka, India (N- 13°, 05.722'; E- 079°, 48.658') during 2010. For screening of the bacteria they were anaesthetized using clove oil and were transported live to the laboratory. For gut isolates, the fish were dissected within laminar air flow cabinet on ice and the alimentary canals were removed as quickly as possible. The parts of the alimentary canal were cleaned, cut into pieces and by a longitudinal incision the contents were transferred to sterile petridishes and flushed with chilled sterile saline (pH 7.4; 0.89 % (w/v) sodium chloride). Further, the parts of the alimentary canal were also homogenized in sterile saline (10:1; volume: weight). The homogenate thus obtained was used as inoculum for bacterial culture.

For skin isolates, mucus extracts were isolated by swabbing, aseptically within laminar air flow cabinet. The extracts were homogenized in a solution of 50 ml normal saline, and the supernatant was used for culture. Similarly, for gill isolates, gills were macerated in 50 ml normal saline and centrifuged aseptically and the supernatant was used as inoculum for microbial culture as in the above cases.

Isolation of bacterial strains

The samples were subjected to 10-fold serial dilution and aliquots (0.1 ml) were plated onto nutrient agar, supplemented with 2% (w/v) NaCl, in duplicates. The plates were incubated at 35°C for 48 h. After incubation, discrete and well isolated bacterial colonies with varying morphological characteristics were selected and streaked onto nutrient agar plates to obtain pure culture. The pure cultures of bacterial strains were re-streaked onto nutrient agar slants (supplemented with 2% NaCl) and stored at 4°C for further studies. Nutrient broth with 2% (w/v) NaCl and nutrient agar medium with 2% (w/v) NaCl was used to maintain the bacterial strains, to prepare the inocula and as the basal medium for phenotypic tests.

Culture medium

King's B medium (proteose peptone: 20 g L^{-1}, K_2HPO_4: 1.5 g L^{-1}, $MgSO_4.7H_2O$: 1.5 g L^{-1}, agar: 20 g L^{-1}, glycerol: 10 g L^{-1}; pH 7.2 $\pm$1 and incubation temperature: 35°C) specific for fluorescent and pigmented bacteria was used for culture and maintenance of fluorescent *Pseudomonas* spp. The pH of the medium was adjusted to 7.2 $\pm$ 0.2 with 1N NaOH or 1N HCl. The bacteria were preserved in 20 % glycerol at–80°C as stock culture until further use.

Characterization of bacterial strains

Phenotypic Characterization: The bacteria were identified by following Holt *et al.* (1994). For phenotypic characterisation 18-24 h old cultures in nutrient broth and nutrient agar (HiMedia, Mumbai) were used. Fluorescence was examined under UV using 48 h culture grown on King's B medium. Growth at different concentrations of salt were determined by inoculating the bacteria in nutrient broth containing 0, 5, 9, 12% (w/v) sodium chloride (NaCl). The growth at pH 5, 9 and 10 was determined in nutrient broth with the pH adjusted using 1N hydrochloric acid (HCl) or sodium hydroxide (NaOH). Biochemical tests carried out for characterization include catalase, cytochrome oxidase, penicillin sensitivity, H & L glucose O/F, sugar fermentation tests, cellulose hydrolysis, gelatin liquefaction, starch utilization and phosphate solubilization test.

Genotypic Characterization: DNA extraction-The strains which produced fluorescence in King's B medium were subjected to genotypic characterization. Pure cultures in tryptone soya

broth (TSB, Oxoid) for 2 days were used for this. The pure cultures were centrifuged for 10 min at 5031 × *g*. A total of 10–20 mg of each bacterial culture was placed in a 1.5-mL microcentrifuge tube and re-suspended at 200 μL of TE buffer. Bacterial DNA was extracted using Genomic DNA Purification Kit (Genie, Bangalore, India).

Amplification of partial 16S rRNA gene sequences and phylogenetic analysis

Identification of fluorescent strains was performed by sequence analysis of DNA coding for the 16S rRNA. Universal bacterial 16S rDNAs primers were used to amplify a fragment of 16S rDNA 760 bp in length. The PCR reaction mixtures contained 10× PCR buffer with $(NH4)_2SO_4$, 2 mM dNTP Mix, 1.5 mM $MgCl_2$, 20 pmol of each primer, 1 ng of DNA in 10 μL, and 2 Units of *Taq* DNA polymerase in a total volume of 50 μL. The PCR reactions were performed using initial denaturation during 3 min at 95°C followed by 30 cycles of denaturation for 1 min at 95°C, primer annealing for 1 min at 60°C, and primer extension for 1 min at 72°C. This procedure was followed by a final extension reaction at 72°C for 10 min. For negative controls for PCR reactions, sterile distilled H_2O instead of DNA was used. The PCR product was bi-directionally sequenced using the forward, reverse and internal primers. The multiple sequence alignment program Clustal W (Chenna *et al*., 2003) was used to align the 16S rRNA sequence of the strains. Sequences of rRNA genes, for comparison, were obtained from the NCBI Gene Bank and RPD data base. Evolutionary distance matrices were calculated by using the algorithm of the Kimura two-parameter model (Kimura, 1980). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou and Nei, 1987) with bootstrap re-sampling (data re-sampled 100 times) to assess the degree of support for the phylogenetic branching indicated by the optimal tree.

Results and discussion

Seven bacterial strains were isolated from the skin, gills and gut of *T. mookalee* Cuvier 1832, collected from Karwar, Karnataka, India. Out of seven strains, six were identified as *Pseudomonas* spp. Of these, three were non-fluorescent and another three fluorescent strains, which secreted pyoverdine, a yellow-green siderophore on King's B medium (Fig. 1 and Fig. 2). The biochemical characters are given in Table 1. All were Gram-negative, motile short rods. Colonies on nutrient agar were light brown, translucent, oval or circular, flat or convex with smooth and shiny texture. They were catalase-positive, oxidase-positive, citrate-positive, penicillin-resistant, non-sporulating and non-lactose fermenting. They gave negative tests for indole, methyl red, Voges-Proskauer, starch utilization and cellulose hydrolysis. They were able to reduce nitrate. The morphological, physiological and biochemical characteristics of the strains are

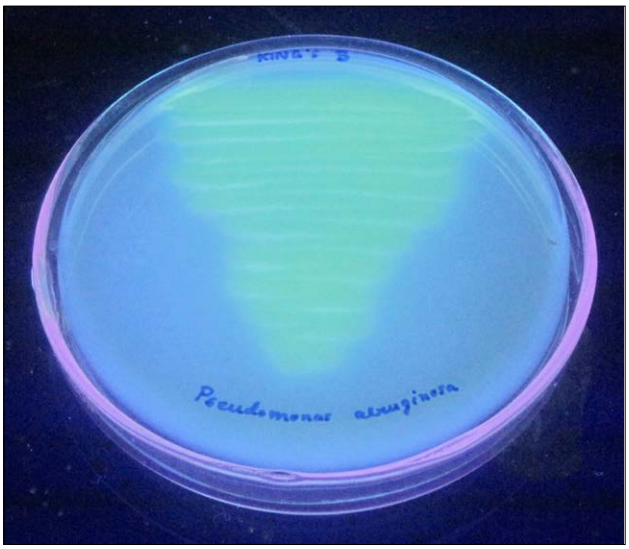

Fig.1. Fluorescent colonies of *P. aeruginosa* on King's B agar medium observed under UV light

Fig. 2.Colonies of non-fluorescent *Pseudomonas* spp. on King's B agar medium observed under UV light

almost similar, as reported in Table 1. However, a few among these characteristics differentiated the strains from each other. Fluorescent strains showed oxidative type of metabolism in H&L medium, whereas each of the non-fluorescent strain showed different reactions like oxidative, alkaline and no change.

Genotypic characterization

The 16S rRNA gene sequence of the fluorescent strains were aligned and compared with all sequences currently available for members of the Genus *Pseudomonas* and related taxa (Fig. 3) by using the NCBI BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST). Based on the nucleotide homology and phylogenetic

Table 1. Phenotypic characteristics of fluorescent and non-fluorescent *Pseudomonas* spp. isolated from *T. mookalee* Cuvier, 1832

Characteristics	Fluorescent strains	Non fluorescent strains
Morphological		
Light brown, translucent, oval or circular, flat or convex colonies on nutrient agar with smooth and shiny texture		
Biochemical		
Gram-negative, motile, short rods		
Catalase	+	+
Oxidase	+	+
Citrate utilization	+	+
Fluorescence	+	-
Spore forming	-	-
Lactose fermenting	-	-
Indole	-	-
Methyl Red	-	-
Voges-Proskauer	-	-
Starch Utilization	-	-
Cellulose hydrolysis	-	-
Gelatin liquefaction	+	+
Penicillin sensitivity	Resistant	Resistant
Nitrate reduction	+	+
Oxidation fermentation	Oxidative	Oxidative/alkaline
Physiological		
Growth in NaCl (%)	0-5%	0-9%
Growth in different pH	5.0-10.0	5.0-10.0

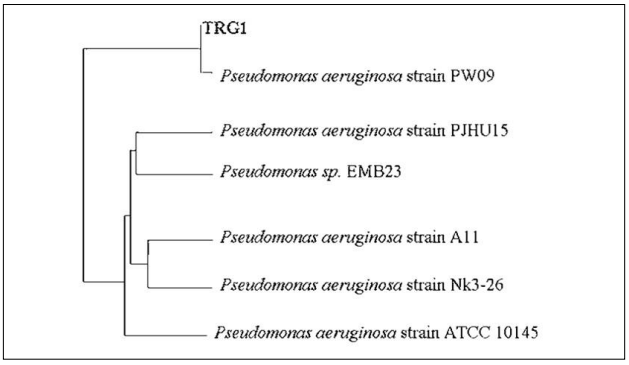

Fig. 3.Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain TRG1 and other related *Pseudomonas* species. The tree was constructed using the neighbour-joining algorithm based on 16S rRNA gene sequences.

analysis all three were found to be the same and was identified as *P. aeruginosa* strain TRG1 (Genbank Accession No. KC109784). The strain is alkali-tolerant, non-sporulating and gram-negative rod. Colonies on nutrient agar were light brown, translucent, oval or circular with smooth and shiny texture. The colonies on King's B agar medium emitted bluish green fluorescence due to the secretion of pyoverdine, a yellow-green siderophore (Fig. 1). Hussain *et al.* (2007) reported that *P. aeruginosa* are able to produce compounds of industrial interest like enzymes (alkaline protease, lipase, chitinase, thermostable keratinase *etc.*), biosurfactants, biocatalyst for biofuel cell application, chelating agents (siderophore), polymers (exopolysaccharides, polyhydroxyalkanoates) *etc.* They are also reported to play an important role in the bioremediation of endosulfan polluted soil and water environments. Rhamnolipids, produced by *P. aeruginosa*, represent an important group of biosurfactants having various industrial, environmental, and medical applications (Maier and Soberon-Chavez, 2000). The role of *P. aeruginosa* in marine fish has not yet been established and further studies are necessary in this aspect. However, this strain can be tested for application in agriculture as a biocontrol agent for crops. Also its occurrence in marine fishes needs future studies to establish its biochemical role in fishes.

Acknowledgements

The authors acknowledge Indian Council of Agricultural Research (ICAR), New Delhi, India for funding of the work through the National Network Project Application of Microorganism in Agriculture and Allied Sectors (AMAAS). The authors are grateful to the Director, Central Marine Fisheries Research Institute (CMFRI), Cochin, Kerala, India for providing the facilities to carry out this work.

References

Cahill, M. M. 1990. Bacterial flora of fishes: a review. *Microbial. Ecol.,* 19: 21–41.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins and J. D. Thompson. 2003. Multiple sequence alignment with the CLUSTAL series of programs. *Nucleic Acids. Res.,* 31: 3497–3500.

Chigusa, S., T. Hasegawa, N. Yamamoto and Y. Watanabe. 1996. Treatment of wastewater from oil manufacturing plant by yeasts. *Water Sci. Technol.,* 34: 51-58.

Desai, J. D. and I. M. Banat. 1997. Microbial production of surfactants and their commercial potential. *Microbiol. Molecul. Biol. Rev.,* 61: 47−64.

Hatai, K. and L. G. Willoughby. 1988. *Saprolegnia parasitica* from rainbow trout inhibited by the bacterium *Pseudomonas fluorescens*. *Bull. European Assoc. Fish Pathol.,* 8: 27–29.

Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams (ed.) 1994. Bergey's Manual of Determinative Bacteriology, 9th ed. Williams & Wilkins, Baltimore. p. 786-788.

Huan, D., S. Gao, S. Han and S. Cao. 1999. Purification and characterization of a *Pseudomonas* sp. lipase and its properties in non-aqueous media. *Biotechnol. Appl Biochem.,* 30: 251-256.

Hussain, S., M. Arshad, M. Saleem and A. Khalid. 2007. Biodegradation of α and αendosulfan by soil bacteria. *Biodegradation*, 18: 731–740.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Molecular Evol.,* 16: 111-120.

Koch, A. K., O. Kappeli, A. Fiechter and J. Reiser. 1991. Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *J. Bacteriol.,* 173: 4212-4219.

Maier, R. M. and G. Soberon-Chavez. 2000. *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. *Appl. Microbiol. Biotechnol.,* 54: 625-633.

Nakai, T., H. I-landa and K. Muroga. 1985. First records of *Pseudomonas anguilliseptica* infection in cultured ayu, *Plecoglossus altivelis. Fish Pathol.,* 20: 481-484.

O'Sullivan, D. J. and F. O'Gara. 1992. Traits of fluorescent *Psuedomonas* species involved in suppression of plant root pathogens. *Microbiol. Rev.,* 56: 662–676.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biol. Evol.,* 4 (4): 406-425.

Strom, E. and E. Ringo. 1993. Changes in the bacterial composition of early developing cod, *Gadus morhua* (L.) larvae following inoculation of *Lactobacillus plantarum* into the water. In: Wather B. and Fyhr H.J. (eds.) Physiological and Biochemical Aspects of Fish Larval Development. University of Bergen, Norway, p. 226–228.