# A survey on functionally diverse bacterial strains from marine

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Prokaryotic microorganisms compromise a large portion of the organic biomass of the world's ocean and play an important role in the biogeochemical cycles and food webs of this ecosystem. Surface colonization by microorganisms is ubiquitous in marine systems with a large proportion of microbes occurring as complex communities. Despite their importance, comparatively little is known about the phylogenetic composition of this complex microbial population and the functional roles of their members. Living surfaces are ideal to explore colonization by microorganisms because eukaryotes are subject to a constant bombardment from the millions of microbial cells typically found in a millilitre of seawater. Alternatively, disease-causing microbes might already be present on fishes and their surroundings. So a survey and analysis of bacteria associated with marine fish can give an indication of the environment like water quality, feed availability, productivity and the presence of pathogens which cause havoc to the system or to the consumers. Also, many associated bacterial strains which find wide application in agriculture and allied sectors based on their biochemical/ physiological characteristics can be documented.

**Sampling protocol**

The survey was conducted for a period of eight years (2007-14) to assess the diversity of functionally important bacteria from selected marine fishes of Indian coast.The screening of bacteria was restricted to those from economically important finfish and shellfish species. The sampling sites selected were from the maritime states of Tamil Nadu, Puducherry (UT), Andhra Pradesh (East coast); and Kerala, Karnataka, Goa and Maharashtra (West coast). A total of 15 sites were covered for fish sampling. For stability of the data and its reliability for comparison, samplings were carried out only during early morning hours on sunny days (not in winter or rainy season). In majority of cases, only one time sampling was carried out during September to May months.During 2007-2014, 73 species of marine fishes, 7 species of shrimps, 2 species of crabs and one lobster species of commercial importance were collected. Finfishes collected live from shore seines were anaesthetized using clove oil (3-5 ppm) and were transported under sterile conditions to the laboratory for screening of the associated bacteria. The anaesthetized fishes were dissected and the alimentary canal was removed, cleaned, cut into pieces and slit open by longitudinal

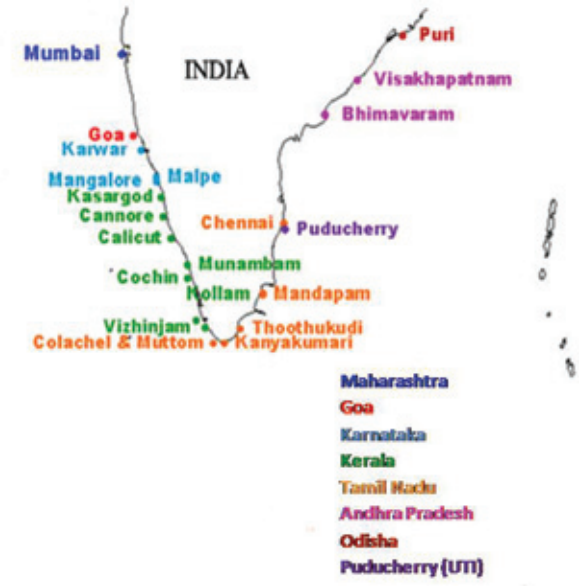


Fig. 1. Sampling sites along the Indian Coast

incision. The pieces of the alimentary canal were homogenized with sterile saline and the homogenate thus prepared was used as inoculum for plating on culture media. Mucus extracts was swabbed aseptically for screening of skin isolates. For gill isolates, gills were macerated in normal saline and centrifuged and the supernatant was used as inoculum for microbial culture. Characterization of bacterial strains based on phenotypic data was done for all culturable strains. Characterization of bacterial strains using 16S rDNA sequence analysis was followed for selected strains that were of distinct functional properties.

**Phenotypic Characterization :**

Morphological tests like Gram staining and Motility were determined in 24-hours (h) cultures in nutrient broth. The morphology and size of colonies were examined in nutrient agar after 3 days incubation period. Fluorescence was examined in King's B medium after 24 - 48 h. Physiological tests for observation of growth at different salinity were determined by inoculating in nutrient broth with NaCl concentrations of 0, 5, 9, 12% (w/v). The growth at different pH was determined in nutrient broth with the pH adjusted to 5 and 8 or 9 and 10 using either 1N Hydrochloric acid (HCl) or sodium hydroxide (NaOH). Biochemical tests conducted for bacterial 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**

Selected strains with specific functional properties like salt tolerance, thermal tolerance, pigmented strains, starch and cellulose hydrolyzing strains were subjected to genotypic characterization. Pure bacterial cultures were cultivated in tryptone soya broth (TSB, Oxoid) for 2 days. Bacterial DNA was extracted using Genomic DNA Purification Kit (Genie, Bangalore, India). Identification of fluorescent strains was performed

Table 1: list of fishes sampled in various maritime states

| Fish Species | States / UT | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MAH | GOA | KAR | KER | TN | AP | OD | PUD |
| **Finfishes** | | | | | | | | |
| *Abalistes* sp. | | | | ✓ | | | | |
| *Acanthurus* sp. | | | | ✓ | ✓ | | | |
| *Anodontostoma* sp. | ✓ | | | | | | | |
| *Arius maculatus* | | | | | | ✓ | | |
| *Arius* sp. | ✓ | | | | | | | |
| *Atule mate* | | | | | ✓ | | | |
| *Auxis rochei* | | | | ✓ | ✓ | | | |
| *Auxis thazard* | | | | ✓ | | | | |
| *Carangoides ferdau* | | | | | ✓ | | | |
| *Carangoides* sp. | | | | | ✓ | | | |
| *Caranx* sp. | | | | | ✓ | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Caranx tille* | | | | | ✓ | | | | |
| *Cephalopholis* sp. | | | | | ✓ | | | | |
| *Chanos chanos* | | | | | ✓ | | | | |
| *Coilia* sp. | ✓ | | | | | | | | |
| *Coryphaena equiselis* | | | | | ✓ | | | | |
| *Cynoglossus bilineatus* | | | | | | | | | ✓ |
| *Cynoglossus* sp. | | | | ✓ | ✓ | | | | |
| *Daysciaena albida* | | | | | ✓ | | | | ✓ |
| *Decapterus russelli* | | | ✓ | ✓ | | ✓ | | | |
| *Dussumieria acuta* | | | | ✓ | | | | | |
| *Epinephelus faveatus* | | | | ✓ | | | | | |
| *Gerres filamentosus* | | | ✓ | | | | | | |
| *Hemiramphus lutkei* | | | ✓ | | | | | | |
| *Johnius amblycephalus* | | | | | | ✓ | | | |
| *Johnius* sp. | ✓ | | ✓ | ✓ | | | | | |
| *Lactarius lactarius* | | ✓ | ✓ | | | | | | |
| *Lagocephalus gloveri* | | | | ✓ | | | | | |
| *Leiognathus* sp. | | | | | ✓ | | | | |
| *Lepturacanthus savala* | | | ✓ | | | | | | |
| *Liza macrolepis* | | | | ✓ | | | | | |
| *Lutjanus johnii* | | ✓ | | | | | | | |
| *Lutjanus lutjanus* | | | | ✓ | | | | | |
| *Lutjanus* sp. | | | | ✓ | | | | | |
| *Megalaspis cordyla* | | | | ✓ | ✓ | | | | |
| *Mugil cephalus* | | ✓ | ✓ | | | | | | ✓ |
| *Nematalosa nasus* | | | ✓ | | ✓ | | | | |
| *Nemipterus japonicus* | | | | ✓ | ✓ | ✓ | | | |
| *Nemipterus randalli* | | | ✓ | | | | | | |
| *Nibea* sp. | | | | ✓ | | | | | |
| *Opisthopterus tardoore* | | | | | | | ✓ | | |
| *Oreochromis* sp. | | | | | | ✓ | | | |
| *Parascolopsis aspinosa* | | | ✓ | | | | ✓ | | |
| *Pempheris mangula* | | | | ✓ | | | | | |
| *Polynemus* sp. | | | | ✓ | | | | | |
| *Priacanthus hamrur* | | | ✓ | | ✓ | | | | |
| *Pseudorhombus arsius* | | | | | ✓ | | | | |
| *Raconda russeliana* | | | | | ✓ | | | | |
| *Rastrelliger brachysoma* | | | | | | | ✓ | | |
| *Rastrelliger kanagurta* | | | | ✓ | ✓ | ✓ | | | ✓ |
| *Rastrelliger* sp. | | | | | ✓ | | | | |
| *Sardinella fimbriata* | | | | | | ✓ | | | |
| *Sardinella gibbosa* | | | | | | ✓ | | | |
| *Sardinella* sp. | | | | | ✓ | | | | |
| *Saurida undosquamis* | | | | ✓ | | ✓ | | | |
| *Scomberoides tol* | | | | | ✓ | | | | |
| *Secutor insidiator* | | | ✓ | | | | | | |
| *Siganus* sp. | | | | ✓ | ✓ | | | | |
| *Sillago sihama* | | | | | | | | | ✓ |
| *Sillago* sp. | | | | | ✓ | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Sphyraena obtusata* | | | | ✓ | | |
| *Sphyraena putnamae* | | | | | | ✓ |
| *Sphyraena* sp. | | | ✓ | | | |
| *Symphurus* sp. | | | ✓ | | | |
| *Tetraodon* sp. | | ✓ | | | | |
| *Therapon* sp. | | ✓ | ✓ | | | |
| *Thryssa* sp. | | ✓ | | | | |
| *Trachinocephalus myops* | | ✓ | | | | |
| *Trachinotus mookalee* | ✓ | | | | | |
| *Tylosurus crocodilus* | | | ✓ | | ✓ | |
| *Upeneus moluccensis* | | | | ✓ | | |
| **Crustaceans** | | | | | | |
| *Fenneropenaeus indicus* | | ✓ | | | | |
| *Litopenaeus vannamei* | | | | ✓ | | |
| *Metapenaeus dobsoni* | ✓ | ✓ | | | | |
| *Metapenaeus monoceros* | ✓ | | | ✓ | | |
| *Panulirus homarus* | | ✓ | | | | |
| *Parapenaeopsis stylifera* | | ✓ | | | | |
| *Penaeus monodon* | | ✓ | | | | |
| *Portunus pelagicus* | | ✓ | | | | |
| *Portunus sanguinolentus* | | ✓ | | | | |
| *Solenocera crassicornis* | | | | ✓ | | |

*MAH: Maharashtra; GOA: Goa; KAR: Karnataka; KER: Kerala; TN: Tamil Nadu; AP: Andhra Pradesh; OD: Odisha; PUD: Puducherry*

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 reactions were performed using standard procedures. For negative controls in 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 was used to align the 16S rRNA sequence of the strains. For comparison, sequences of rRNA genes, were obtained from the NCBI GenBank and RPD data base. Evolutionary distance matrices were calculated by using the algorithm of the Kimura two-parameter model. A phylogenetic tree was constructed by using the Neighbour-Joining method with bootstrap re-sampling (data re-sampled 100 times) to assess the degree of support for the phylogenetic branching indicated by the optimal tree.

A total of 732 bacterial strains were characterized phenotypically from marine fishes and crustaceans. About 26 bacterial strains comprising halophilic, pigmented, starch hydrolyzing (amylase producing), cellulase producing and heat tolerance were identified using 16S rDNA sequence analysis. These include *Halomonas marina* strain DSM 4741 (GenBank Accession No. AJ306890), *H. aquamarina* (GenBank Accession No. EU440965), *Planococcus maritimus* (GenBank Accession No.EU624446), *Sporosarcina saromensis* (GenBank Accession No.AB243864), *Arthrobacter* spp. (GenBank Accession No. EU797642), *Pseudomonas aeruginosa* (GenBank Accession No. FJ665510), *Stenotrophomonas* spp. (GenBank Accession No. EU816585), *Bacillus marisflavi* (GenBank Accession No. DQ105973), *Bacillus* spp. ZH4(GenBank Accession No. EU236750), *Microbacterium esteraromaticum* (GenBank Accession No. JQ581525), *Micrococcus luteus* ATCC = 4698 (GenBank Accession No. EU236750), *Bacillus circulans* (GenBank Accession No. JQ58152), *Bacillus cereus* (GenBank Accession No. JN793477), *Pseudomonas stutzeri* (GenBank Accession No. JQ581527), *Bacillus circulans* (GenBank Accession No. JQ581528), *Brachybacterium conglomeratum* (GenBank Accession No. JQ581529), *Staphylococcus warneri* (GenBank Accession No. JQ581530), *Bacillus nealsonii* (GenBank Accession No. JN710379), *Vibrio alginolyticus* (GenBank Accession No. JN710378), *Bacillus*

*atrophaeus* (GenBank Accession No. JN712298), *Pseudomonas* spp.(GenBank Accession No. JN710377), *Aeromonas hydrophila* (GenBank Accession No.JN712299), *Bacillus* spp. (GenBank Accession No. JN712300), *Bacillus subtilis* (GenBank Accession No. JN710380) and *Klebsiella oxytoca* (GenBank Accession No. JN712301).

Study on functionally diverse bacterial isolates from fishes/ crustaceans were a new concept and were carried out under the ICAR network project Application of Microorganisms in Agriculture and Allied Sectors (AMAAS). The exact role played by bacteria on fish skin, gills or viscera is unknown. The only known fact was that marine bacteria are potential source of unusual, novel bioactive compounds which can find wide application for industrial, agricultural, environmental, pharmaceutical and medical uses. Many bacterial strains of novel characteristics like extreme halophiles which can thrive and multiply in salt content ranging from 2% to 25%, fluorescent strains, pigmented strains, strains which can utilise cellulose and starch, that can produce carotenoids *etc* could be isolated and characterized during the study. By genotypic characterization, the variable portions of the 16S rDNA gene provided unique signatures that enabled the identification of selected bacteria up to species level. The strains that find application in agriculture and allied sectors were also deposited in the culture collection at National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, Uttar Pradesh for future reference by researchers.