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Characterization of functionally diverse intestinal bacterial flora of *Panulirus homarus* (Linnaeus, 1758) along the southwest coast of India

Anu Mathew* and Imelda Joseph
ICAR - Central Marine Fisheries Research Institute, Post Box No.1603, Ernakulam North P.O., Kochi - 682 018, Kerala, India.

*Correspondence e-mail: anuchalilm@gmail.com

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Original Article

Abstract

Rock lobster *Panulirus homarus* is a commercially important species exploited in India and abroad, and has proven mariculture potential. The present study was focused on isolation, screening, biochemical and molecular level characterization of putative probiotic bacteria from the intestine of *P. homarus* sampled along the south west coat of India. The total plate count in the intestine ranged from 8×10^4 to 3.8×10^6 cfu g^{-1} (colony forming unit per g tissue). The intestinal bacterial flora as identified by the physiological and biochemical characters, consisted of strains belonging to *Enterobactericeae*, *Bacillaceae*, *Vibrionaceae, Pseudomonadaceae*, *Micrococcaceae* and *Moraxellaceae* families. Among these, four strains which were functionally diverse *viz.* pigmented, proteolytic and denitrifying strains were characterized by molecular methods following 16S rDNA sequencing and were identified as *Bacillus aerophilus* KU296018, *Micrococcus aloeverae* KU296019, *Psychrobacter sanguinins* KU296021 and *Pseudomonas caeni* KU296020. In aquaculture beneficial bacteria could be introduced into artificial diets. The enzyme producing microorganisms isolated from the crustacean digestive tracts in the present study may be beneficially used as a probiotic supplement while formulating the diet, especially in the larval stages.

Keywords: *Panulirus homarus, intestinal bacterial flora, Psychrobacter sanguinis, Bacillus aerophilus, Micrococcus aloeverae, Pseudomonas caeni*

Introduction

An understanding of community structure and pattern of distribution of microbial diversity in the sea will be helpful in studying the intestinal bacterial flora of marine organisms. Analysis of microbial biodiversity also helps in isolating and identifying new and potential microorganisms having high specificity for different biogeochemical functions. Like in any other organisms, in crustaceans, gut microorganisms are considered important for the nutrition (Harris, 1993). Gut microorganisms grow in a stable environment inside the animal, while the host is benefited either from microbially mediated digestion of ingested food or from essential nutrients.

Though not big in volume, lobster is an important crustacean resource from the Indian seas. It is one of the most valuable

and highly priced crustaceans in India, having high export value. The major fisheries are located along the north-west, south-west, and south-east coasts of India. Among the 12 species recorded, *P. polyphagus*, *P. homarus, P. ornatus* and *Puerulus sewelli*, are the most significant commercial species. Among these, *P. ornatus* and *P. homarus* are more affected stock owing to its high vulnerability to fishing activities. The aim of this study was to investigate the intestinal bacterial flora in *P. homarus* dwelling along the Kerala coast and characterisation of selected strains with different functional diversity. The studies on intestinal bacterial communities provide useful insights into the feeding and nutritional behaviour of *P. homarus*, especially in commercial rearing efforts that are suggested as alternative to the overexploitation and stock management. In lobsters, especially *P. homarus*, data on the gut microflora and their role on the health status of the host are perhaps little or nil. The present work aimed at a detailed study on isolation, screening, biochemical and molecular level characterization of bacteria from the intestine of *P. homarus* which have potential application in aquaculture or any other related sectors.

Material and methods

Sample collection and processing

Five specimens of rock lobster, *P. homarus* were collected from each collection site at four locations along the Kerala coast. Sampling was done during June 2012-May 2013 from Kannur- Azhikkal (N 11° 56′ 32.028′′ E 75° 18′ 23.58′′) Calicut-Beypore (N 11° 11′ 25.08′′ E 76° 47′ 12′′) Fort Kochi (N 9° 58′ 39.636′′ E 76° 14′ 31.848′′) and Alappuzha Thottapally (N 11° 29′ 5.28′′ E 75° 19′ 20.28′′). Live samples were collected from local fishermen and washed thoroughly in fresh seawater and then by freshwater. The specimens were wiped clean with tissue paper and transported live to the laboratory of Central Marine Fisheries Research Institute (CMFRI) under aseptic conditions.

The lobsters were cut open and the digestive tracts were removed and homogenized in a sterile mortar with pestle under laminar airflow system. The required amount of resultant homogenate was suspended in 10 ml of normal saline (0.85% NaCl solution) and diluted serially up to 10^{-6}.

Isolation of bacterial strains

One millilitre of the homogenized sample from each dilution (10^{-1}-10^{-6}) was pour plated in nutrient agar medium (Himedia, Mumbai) supplemented with 2% (w/v) sodium chloride and incubated at 35±2°C for 48 h. In each sampling, the colony forming units (cfu) were counted and the prominent colonies were sub-cultured and identified. In order to determine the percentage composition, after incubation the bacterial colonies were divided into different types according to colony morphology *viz.* shape, size, elevation, surface, edge, colour etc. and counted. From such distinct colonies, 3-5 representatives of each type were streaked onto nutrient agar plate repeatedly until pure culture is obtained. Purified strains were then inoculated onto nutrient agar slants and maintained as stock cultures at 4°C for further analysis. Stock cultures were transferred to a new nutrient agar slant in every 6 weeks.

Characterization of bacterial isolates

Morphological, biochemical and physiological tests were performed for characterization of the selected bacterial isolates to genus level by following Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994). The regular tests followed were gram stain, motility, oxidase activity, catalase activity (3% hydrogen peroxide solution), oxidation/fermentation nitrate reduction, sugar tests and pigment production.

Enzyme analysis

All bacterial isolates characterized up to genus level were analysed for enzymes like amylase, (Sanchez-Porro *et al.,* 2003), protease, lipase (Sirisha *et al.,* 2010), gelatinase, cellulase (Kasana *et al.,* 2008), phenolphthalein phosphate agar (Himedia) for phosphatase and urea agar (Himedia) for urease. The presence of there enzymes were recorded as growth, or a zone around the colony with/without the addition of respective reagents after incubation at 30 ± 2°C for 24 h.

Physiological analysis

The isolates were also screened to study their tolerance to varying degrees of temperature salinity and pH. The tolerance to salinity was tested in a nutrient broth medium with different concentrations of NaCl (0, 2, 5, 10, 15, 20, 25 and 30% w/v) (Yeon *et al*., 2005). The temperature resistance of the isolates was studied on nutrient agar plates by growing them in a wide range of temperatures ranging from 4°C to 60°C. The pH tolerance was examined by growing the isolates in nutrient agar medium in different pH gradients from 4 to12.

Molecular characterization of selected bacterial isolates

Strains characterized up to genus level by following different tests having different functional properties were selectively characterized by following 16S rRNA sequencing.

DNA extraction

DNA was extracted from selected cultures using phenol chloroform extraction method (Sambrook and Russell, 2001).

The extracted DNA was checked through 1.2 % agarose gel (10 x 4 cm) electrophoresis with ethidium bromide incorporated in 1X TAE buffer for 30 minutes at constant voltage (90V). After electrophoresis, the gel was observed in ultraviolet light and documented using the BioDoc-It™ Imaging system (UVP).

Amplification of 16S rRNA gene using PCR

The polymerase chain reaction (PCR) permits the synthetic amplification of a minute amount of DNA in millions of copies in a few hours. PCR reactions were carried out in MyGene™ Thermal Cycler (Long Gene) employing specific universal primers for amplifications of partial sequences of 16S rRNA gene. Universal bacterial 16S rRNA primers (Lane *et al.*, 1985) were used to amplify a fragment of 16S rRNA.

27F, 5′ - AGA GTT TGA TCC TGG CTC AG-3′
1492R, 5′ - GGT TAC CTT GTT ACG ACT T-3′

The amplifications were performed in 25 μl reactions containing 1X assay buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin) 200μM of each dNTP (Genei, Bangalore, India), 5μM of each primer, 0.5mM $MgCl_2$ (Genei, Bangalore, India), 1.5U *Taq* DNA polymerase (Sigma) and 1μl (~20ng) of template DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. The following thermo-cycler conditions were used: initial preheat at 95 ºC for 3 min, denaturation at 95 ºC for 45 s, annealing at 58 ºC for 40 s, extension at 72 ºC for 1 min 30 s, repeated for 30 cycles, followed by a final extension for 10 mins at 72 ºC and final hold at 4 ºC for 10 mins.

16S rRNA sequence analysis

Aliquots (25 μl) of the amplified 16S rRNA products were electrophoresed in 1.2% agarose gel (10x4 cm) using 1X TAE buffer with ethidium bromide 0.05μg/ml. Samples mixed with sample dye were loaded and the electrophoresis was carried out in a sub marine electrophoresis unit (Hoefer Inc.) with a constant voltage of 80V. DNA molecular markers (100 bp and 1kb ladder) were run parallel to the samples. The gels were documented in gel documentation system. The purified PCR products were used as the template for sequencing PCR. The isolated PCR products were sequenced at the sequencing facility of M/S Sigenome, Kochi.

Phylogenetic analysis

The 16S rRNA gene sequences were analysed and the relative phylogenetic positions were determined by searching GenBank database using BLASTn algorithm (Altschul *et al.*, 1997). The alignment of multiple sequences were performed with CLUSTALW (Thompson *et al.*, 1994). A phylogenetic tree was constructed from evolutionary distances using neighbour-joining DNA distance algorithm (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis of 1,000 data sets with MEGA5 (Tamura *et al.*, 2011) to validate the reproducibility of the branching pattern. This algorithm calculates distance matrix using a Kimura 2-parameter. The sequences obtained were submitted in GenBank for accession.

Results

Isolation and enumeration of bacteria

A total of 19 bacterial strains were isolated from the intestine of *P. homarus* collected from four locations along Kerala coast. The average number of culturable bacteria was 2.7×10^6 cfu g^{-1}. Among sampling sites, samples from Calicut recorded the highest bacterial count of 3.8×10^6 cfu g^{-1} and the lowest count observed was 8×10^4 cfu/g from Kannur.

Characterization of bacterial strains

The 19 bacterial strains were identified up to genus level by following morphological, biochemical and physiological tests. The bacterial isolates belonged to 7 genera *viz. Enterobacte*r (21.05%), *Acinetobacte*r (21.05%), *Bacillu*s (21.05%), *Vibrio* (15.78%), *Pseudomonas* (10.52%), *Micrococcus* (5.26%) and *Moraxella* (5.26%). Among the isolates two strains were yellow pigmented, three were producing cellulase, two were producing protease, two expressed amylase activity, gelatin liquefaction by two, urease activity by one and nitrate reduction by four strains (Table 1).

Molecular characterization

Of the 19 isolates, 4 putative bacterial isolates namely AN-01, AN-03, AN-04 and AN-05 were further characterized by molecular methods by following 16S rRNA sequence analysis.

Isolation of DNA

The DNA of 4 selected strains were extracted and used as template for further studies. DNA extraction resulted in bright bands in the very high base pair range of a gel electrophoresis. An OD260:280 ratio between 1.8 to 2.0 was obtained for the extracted DNA samples, thereby indicating that DNA preparation of the isolates was proper, and the samples were pure and free from protein or phenol contamination.

Identification and phylogeny

Sequence analysis of PCR products revealed that, for all

Table 1. Biochemical characteristics of the bacterial isolates

Sampling locations	Bacterial Isolates	Pigments	Nitrate reduction	Enzymatic activity							Physiological activity		
				A	B	C	D	E	F	G	H	I	J
Kannur	*Enterobacter* sp.	White	-	-	-	+	-	-	-	-	5-10	0-12	10-40
	Acinetobacter sp.	Off white	-	-	-	-	-	-	-	-	9-10	0-9	30-37
	Bacillus sp.	White	-	-	-	-	-	-	+	-	5-10	0-11	8-37
	Vibrio sp.	Off white	-	-	-	-	-	-	-	-	5-10	0-9	5-43
Calicut	*Enterobacter* sp.	Off white	-	-	-	-	-	-	-	-	5-10	0-9	10-40
	Acinetobacter sp.	Off white	+	-	-	-	-	-	-	-	5-10	0-5	30-37
	Bacillus sp.	Off whit	-	+	-	-	-	-	-	-	9-10	0-9	25-35
	Vibrio sp.	Off white	-	-	-	-	-	-	-	-	5-10	0-5	8-43
	Micrococcus sp.	Yellow	-	-	+	+	-	-	+	-	5-12	0-10	15-41
	Moraxella sp.	Off white	+	-	+	-	-	-	-	+	9-10	0-9	4-37
Fort Kochi	*Enterobacter* sp.	Off white	+	-	-	-	-	-	-	-	5-10	0-9	10-40
	Acinetobacter sp.	Off white	-	-	-	-	-	-	-	-	5-10	0-12	30-37
	B*acillus* sp.	Off white	-	-	-	-	-	-	-	-	5-10	0-9	25-35
	Vibrio sp.	Off white	-	+	-	-	-	-	-	-	5-10	0-12	5-43
	Pseudomonas sp.	Pale yellow	-	-	-	-	-	-	-		6-10	0-3	4-37
Alappuzha	*Enterobacter* sp.	Off white	-	-	-	-	-	-	-	-	5-10	0-5	10-40
	Acinetobacter sp.	Off white	-	-	-	-	-	-	-	-	5-10	0-15	30-37
	Bacillus sp.	Off white	-	-	-	-	-	-	-	-	5-10	0-5	25-35
	Pseudomonas sp.	Off white	-	-	+	-	-	-	-	-	5-10	0-9	10-42

A— Amylase, B— Cellulase, C— Gelatinase, D— Lipase, E— Phosphatase, F— Protease, G—Urease, H—Hydrogen ion concentration (pH), I— Salinity (w/v), J— Temperature (^{0}C)

the isolates there were 1% or no differences with the most closely matched sequences in the databank. The isolates were identified as AN-01 as *Psychrobacter sanguinis,* AN-03 as *Bacillus aerophilus,* AN-04 as *Micrococcus aloeverae* and AN-05 as *Pseudomonas caeni* and the sequences were deposited at GenBank with the Accession numbers KU 296021, KU296018, KU296019, KU296020 respectively. The phylogenetic tree (Fig.1) was inferred from Kimura 2-parameter by the neighbour-joining method. The analysis of 16S rRNA gene sequence indicates the position of the native identified isolates in the same cluster with respect to their reference group.

AN-01 Psychrobacter sanguinis

They are non-motile, non-sporulating, gram-negative coccobacilli. Colonies are non-pigmented, circular and smooth with entire margins. They are positive for catalase, oxidase, cellulase, urease and are able to reduce nitrate to nitrite. No growth is observed on MacConkey agar and can grow at temperatures ranging from 4^{o}C to 37^{o}C. They are negative towards tests for utilization of Simmons' citrate, hydrolysis of gelatin, indole production, lipase and phosphatase activity. On the basis of 16S rRNA gene sequence, isolate AN-01 was closely related to *Psychrobacter sanguinis* 13983(T) as reported by Wirth *et al.* (2012) with 99.63% sequence similarity (Fig.1).

AN-03 Bacillus aerophilus

Colonies on nutrient agar were white, irregular, raised and 5-8 mm in diameter. Cells produced spores and fluorescent pigment on King's B medium. It was positive for utilization of Simmons' citrate and expressed protease activity. Growth was recorded at all temperatures ranging from 8^{0}C to 37 ^{0}C and between pH 5 and 10. It could tolerate salt (NaCl) up to 11%. 16S rRNA gene sequence of the isolate AN-03 was closely related to *Bacillus aerophilus* 28K (T) with 99.93% sequence similarity as reported by Shivaji *et al.* (2006) (Fig.1).

AN-04 Micrococcus aloeverae

It was a non-motile, non-endospore-forming, gram-positive spherical bacterium. Colonies were yellow, circular and smooth with entire margins. Cells were catalase-positive but oxidase-negative. It was positive for protease, cellulase and gelatinase activity, but negative for nitrate reduction, urease and amylase production. Temperature range for growth was 15^{o}C-41^{o}C and pH between 5 and 12 and it could tolerate 10% NaCl in the medium. 16S rRNA gene sequence of AN-04 bacterial strain was closely related to *Micrococcus aloeverae* AE-6 (T) with 99.19% sequence similarity reported by Prakash *et al.* (2014) (Fig.1).

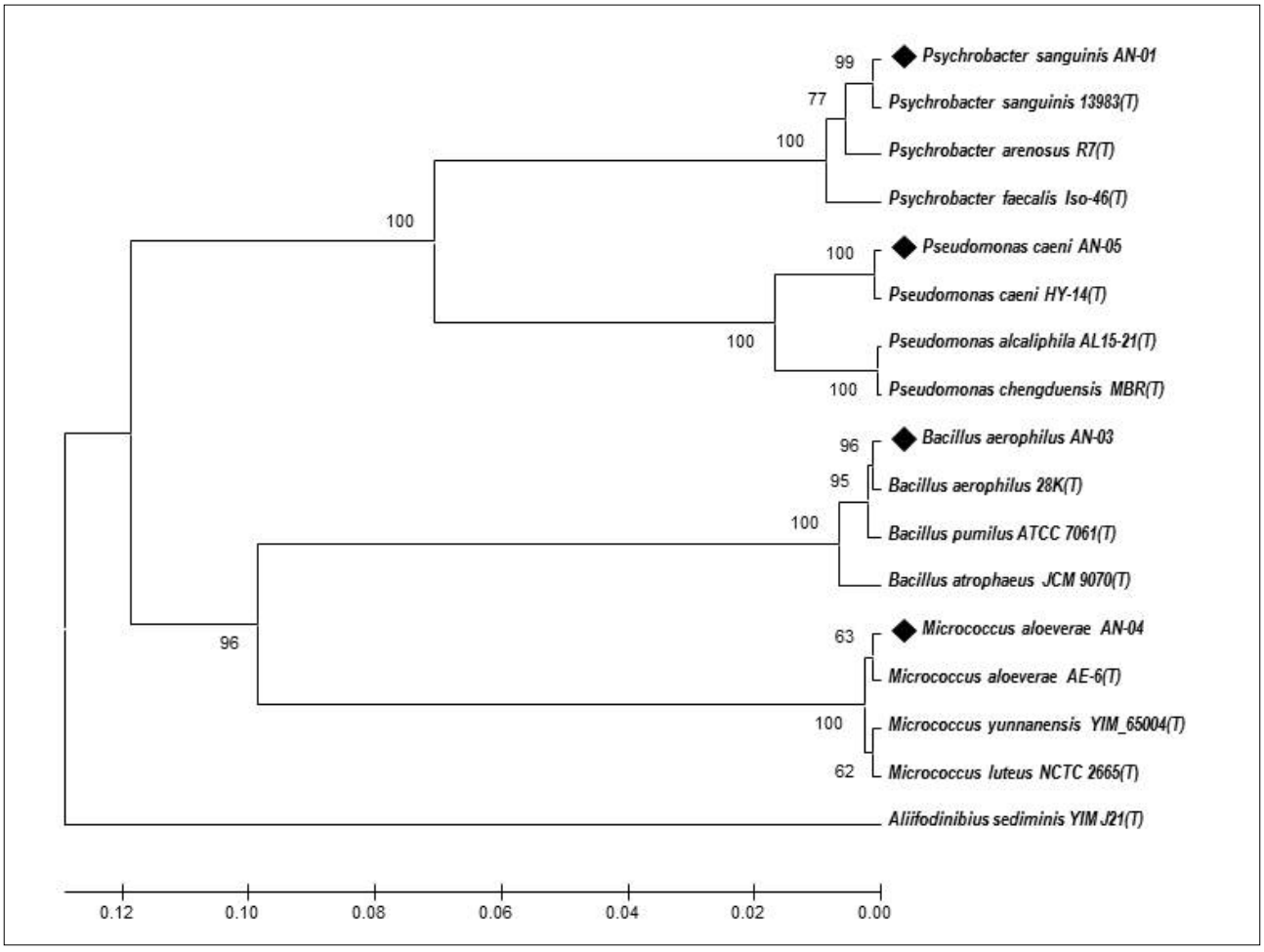

Fig. 1. Phylogenetic relationship of selected bacterial strains based on the neighbour-joining method

AN-05 Pseudomonas caeni

The strain was motile, non-spore-forming, gram-negative rod shaped bacterium. Colonies were pale yellow, translucent and circular with entire margin. It was catalase and oxidase-positive, negative for urease, phosphatase, amylase, cellulase and protease. Growth occurred from 4^0C to 37 ^{0}C and pH 6.0-10.0. It could grow only within 0-3 % (w/v) NaCl. 16S rRNA gene sequence, isolate AN-05 was closely related to *Pseudomonas caeni* HY-14(T) as reported by Xiao *et al.* (2009) with 99.82% sequence similarity (Fig.1).

Discussion

In crustaceans, the intestinal microbial colonization has great importance in health and overall growth performance. The intestinal microflora which is highly complex in nature represents the surrounding ecosystem and it mediates many interactions with the chemical environment (Spanggaard *et al.,* 2000). The purpose of the present study was to understand the functional diversity of the selected intestinal bacteria of *P. homarus.*

The information regarding intestinal bacterial population in lobster is limited and therefore, adequate research in the field of its activity in lobster gastrointestinal tracts is necessary. Intestinal bacterial diversity in live *P. homarus* during transportation process has been reported by Immanuel *et al.* (2006). In this study, the data on total viable count (TVC) of intestinal samples was relatively more in the experimental lobsters, compared to that recorded from the unpacked control lobsters. The bacterial load associated with the intestine of freshly caught Japanese spiny lobster *P. japonicus* was reported to be ranging between 9.5×10^7 and 1.3×10^9 cfu.g^{-1} (Sujita *et al.,* 1987). In the present study, the total culturable bacteria in the gut sample of *P. homarus* ranged from 8×10^4 to 3.8×10^6 cfu/g and the maximum was recorded in samples from Calicut, and the minimum was observed in samples from Kannur. Factors such as bacterial host specificity, food type and water resource may be attributed to these differences (Verner-Jeffreys *et al.,* 2003). Bacterial population in the surrounding environment and feeding habit of the lobster also might have influenced the composition of the gastrointestinal bacteria.

The dominant bacterial species reported from the intestine of live *P. homarus* were *Pseudomonas aeruginosa*, *Vibrio haemolyticus, Bacillus circulans, Escherichia coli, P. damselae, Flavobacterium columnare* and *Micrococcus luteus* (Immanuel *et al.,* 2006). In the present observations species of *Enterobacter, Acinetobacter, Bacillus, Vibrio, Pseudomonas, Micrococcus* and *Moraxell*a genera were recorded. The species recorded were *Psychrobacter sanguinis, Bacillus aerophilus, Micrococcus aloeverae* and *Pseudomonas caeni.* Generally, bacteria are abundant in the environment in which lobster live and it is impossible to avoid them being a component of their diet. In the present investigation, the presence of a considerable population of bacterial flora has been found in the gastrointestinal tracts of the rock lobster *P. homarus* and certain strains were pigmented, some expressed nitrate reduction, some were amylolytic, cellulolytic, ureolytic, gelatinolytic or proteolytic in nature. The microbial enzyme activities may be useful in development of probiotics, drugs or in the industrial enzyme production (Gildberg *et al*., 1997). In commercial aquaculture beneficial bacteria could be introduced by incorporating them into artificial diets. The enzyme producing microorganisms isolated from the crustacean digestive tracts in the present study may be beneficially used as a probiotic supplement while formulating the diet, especially in the larval stages. However, much more research should be conducted to determine if the addition of such isolates to crustacean feeds do in fact provide some kind of benefit to the crustacean involved before advocating its use.

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