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Intestinal bacterial diversity of *Charybdis* (*Charybdis*) *feriata* (Linnaeus, 1758) from Kerala coast

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Original Article

Abstract

Considering the increasing demand and scope for aquaculture of crucifix crab *Charybdis (Charybdis) feriata* (Linnaeus, 1758) an attempt was made to study its intestinal bacterial diversity. The intestinal bacterial diversity of *C. feriata* from three different sites along the Kerala coast was examined using conventional biochemical techniques as well as by 16S rRNA gene sequencing. The total bacterial count in the intestine of *C. feriata* ranged from 1.5×10^6 to 3.2×10^6cfu.g-1 (colony forming unit per g tissue). The predominant isolates were genera *Pseudomonas, Bacillus, Microbacterium* and *Dermacoccus*. Among these two isolates one having protease and other with amylase and cellulase activity were selected for molecular characterization by 16S rRNA gene sequencing. They were identified as *Microbacterium oxydans* KU937308 and *Dermacoccus abyssi* KU937309 respectively. Since protease, amylase and cellulase are important in the areas of food processing, detergent, textile, pharmaceutical products, dairy, beverages and feed industries the isolates can be further screened for such applications. *Dermacoccus abyssi* has been found to be useful in biocleaning of industrial dye baths and *Microbacterium oxydans* in reutilization of brown-seaweed waste.

Keywords: *Charybdis feriata, intestinal bacterial diversity, Microbacterium oxydans, Dermacoccus abyssi*

Introduction

Crustacean fishery makes a noteworthy impact to the worldwide nutritional security and livelihoods of people residing in coastal areas (Bondad Reantaso *et al.*, 2012). Among marine crustaceans, crabs occupy vast position in terms of export capacity and dietary value (Varadharajan and Soundarapandian, 2014). Exploration of intestinal bacterial range in crab forms a topic of interest due to its importance in isolating and identifying potential bacteria having high specificity for recalcitrant compounds. Due to its benthic habitat, crabs receive bacteria in the intestine from the aquatic environment through water and the food it consumes.

There are reports on intestinal bacterial diversity of marine crabs like *Scylla serrata*, *S. tranquebarica, Portunus pelagicus*, *P. sanguinolentus*, *Charybdis helleri* (Ravichandran and Kannupandi, 2005; Rameshkumar *et al.,* 2009) and *Eriocheir sinensis* (Li *et al.,* 2007). Kannathasan and Rajendran (2010) have reported *Acinetobacter baumanii*, *Bacillus subtilus*, *Escherichia coli, Klebsiella pneumonia, Micrococcus luteus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Salmonella typhi*, and *Vibro cholera* from carapace, gills, hepatopancreas, gonad, muscle and alimentary canal of *Charybdis natator.* The intestinal bacterial diversity represents an atmosphere of the

highest complexity and our understanding of this biochemical system and its interactions are constrained (Reiji *et al.,* 2004; Gatesoupe, 2007). An understanding of the host intestinal bacterial floral interactions is significant for the improvement of farming conditions and to optimize the species growth in aquaculture (Oxley *et al.,* 2002). Intestinal microorganism also has specific role in nutrients digestion and offer the host with physiologically active substances, like enzymes, amino acids and vitamins (Sugita *et al*., 1997). Symbiotic bacteria in an animal's digestive tract often produce enzymes that could supplement for digestion of foods as well as synthesize compounds that are assimilated by the host (Saha *et al*., 2006). Screening microbes from commercially important marine crab species in opposition to aquatic pathogens has been reported as useful in developing autochthonous probiotics for the farming of those animals (Verschuere *et al*., 2000).

Charybdis feriata is an ecologically important portunid crab species, widely distributed in Pacific and Indian oceans. Rajendran *et al.* (2008) identified five bacterial species from different tissues of ice stored *C. feriata*. However, it remains incomplete without studying the bacterial flora in the fresh intestine of *C. feriata*. Such information will be useful to understand the immunity aspects of the animal, its nutritional requirement and also to ensure the safety for human consumption. In view of its application in aquaculture, a study was conducted to estimate the bacterial diversity in *C. feriata* and to screen out some bacteria having biotechnological applications.

Material and methods

Collection of crab and preparation of inoculum

Five numbers of *C. feriata* from each centre were collected from three locations along the Kerala coast. Sampling was done from Kollam–Neendakara harbour, Alappuzha-Thottapally harbour and Calicut–Beypore harbour. Samples were collected and transported live to the laboratory and the digestive tracts were removed, cleaned and cut into pieces. The pieces were transferred to sterile petri-plates and thoroughly flushed with chilled sterile saline (pH 7.4; 0.89% NaCl), and homogenized using normal saline (10:1; volume: weight). The homogenate thus obtained was used as inoculum for culture.

Enumeration and characterization of bacterial isolates

The numbers of cultivable bacterial cells present in crab intestine were estimated after isolation and growth on nutrient agar medium supplemented with 2% (w/v) sodium chloride and incubated at 35±2°C for 48 h. Morphological, biochemical and physiological tests were performed for characterization of the bacterial isolates up 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. All bacterial isolates characterized up to genus level were analysed for enzymes like amylase, (Sanchez-Porro *et al.,* 2003), protease, gelatinase and cellulase (Kasana *et al.,* 2008). The presence of the enzymes was recorded as growth, or a clear zone around the colony with/without the addition of respective reagents after incubation at 30 ± 2ºC for 24 h. 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 sodium chloride (Nacl, 0, 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 to 60ºC. The pH tolerance was examined by growing the isolates in nutrient agar medium at different pH ranging from 4 to12.

Molecular characterization of selected bacterial strains

For sequencing and phylogenetic analysis, DNA was extracted from the selected culture using phenol chloroform extraction method (Sambrook and Russell, 2001). PCR amplification of the 16S rRNA genes were carried out with the universal primers 27F (5'- AGAGTTTGATCCTGGCTCAG–3') and 1492R (5'- GGTTAC CTTGTTACGACTT–3') (Lane *et al*., 1985). The purified amplicons were custom sequenced by using the sequencing facility of M/S Sigenome, Kochi. The nearest taxa of the 16S rRNA gene sequence (1418-1542 bases) were identified by BLAST sequence similarity (https:// blast.ncbi.nlm.nih.gov/Blast.cgi). The CLUSTAL W software was used to align 16S rRNA gene sequences and Maximum Likelihood (ML) and neighbour – Joining methods with MEGA version 5 (Tamura *et al*., 2011) were used to construct the phylogenetic tree.

Results

Total bacterial count

The total bacterial load from the *C. feriata* intestine ranged between 1.5×10^6cfu.g^{-1} and 3.2×10^6cfu.g^{-1}. The maximum count of 3.2×10^6cfu.g^{-1} was obtained in the sample from Kollam and minimum 1.5×10^6cfu.g^{-1} from Calicut sample.

Bacterial isolates from intestine

A total of 6 bacterial strains were isolated from the intestine of

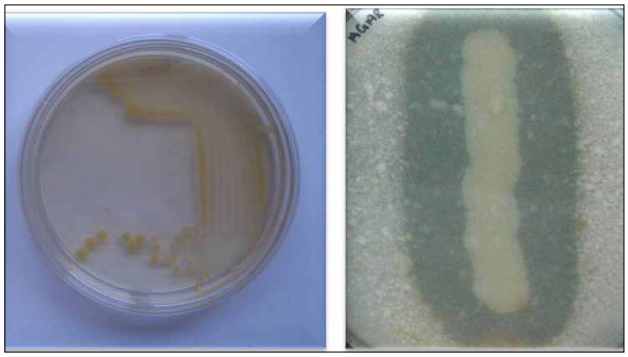

Fig.1. Yellow pigmented and Protease activity of *Microbacterium* sp.

Fig. 2. Yellow pigmented, starch and cellulase activity of *Dermacoccus* sp.

C. feriata. The strains were belonging to the genera *Pseudomonas*, *Bacillus*, *Microbacterium* and *Dermacoccus*. Among these *Microbacterium* sp. was yellow pigmented and with protease activity and *Dermacoccus* sp. was yellow pigmented, starch utilizing and with cellulolytic activity (Fig. 1 & 2). These two strains were further characterized by following 16S rRNA gene sequence analysis.

Strains identified by 16SrRNA gene sequence analysis

Genomic extraction of *Microbacterium* sp. and *Dermacoccus* sp. resulted in bright bands in the very high base pair range of gel electrophoresis. An OD 260:280 ratios between 1.8 to 2.0 was obtained for the extracted genomic DNA samples, thereby indicating that DNA preparation of the bacterial isolates was proper and the DNA samples were pure, free from protein or phenol contamination. Sequence analysis of PCR products revealed that, for the isolates there was1% or no difference with the most closely matched sequences in the data bank. The isolates designated as AN-07 and AN-08 were identified as *Microbacterium oxydans* and *Dermacoccus abyssi* respectively. The sequences were deposited at Genbank with the Accession numbers KU937308 and KU937309. The phylogenetic tree (Fig. 3) was inferred from Kimura 2-parameter by the neighbour-joining method. The analysis of 16S rRNA gene sequence indicated the position of the native identified isolates in the same cluster with respect to their reference group.

AN-07 Microbacterium oxydans

Colonies on nutrient agar are yellow, round and with entire edge. They are gram-positive rods, motile with no endospores, measure1-2 μm in length. It is catalase-positive and oxidase-negative. It is gelatin, and protease hydrolyses positive with methyl red-negative, no nitrate reduction and no cellulose hydrolysis. Growth occurs at 20-30^0 C, with optimum growth at around 30^{0}C. The DNA G + C content of the type strain is 70 mol %. On the basis of 16S rRNA gene sequence, isolate AN-07 was closely related to *M. oxydans* KMDH15 with 99.78% sequence similarity (Fig. 3).

AN-08 Dermacoccus abyssi

Colonies on nutrient agar are yellow, circular, entire, convex and irregular clusters. Forms 0.8–1.5 mm in diameter. Cells produce fluorescent pigment on King's B media. It degraded cellulose and starch. Growth occurs at 10-35^{0}C, with optimum growth around 28^{0}C. It tolerates up to 10% NaCl. The DNA G+C

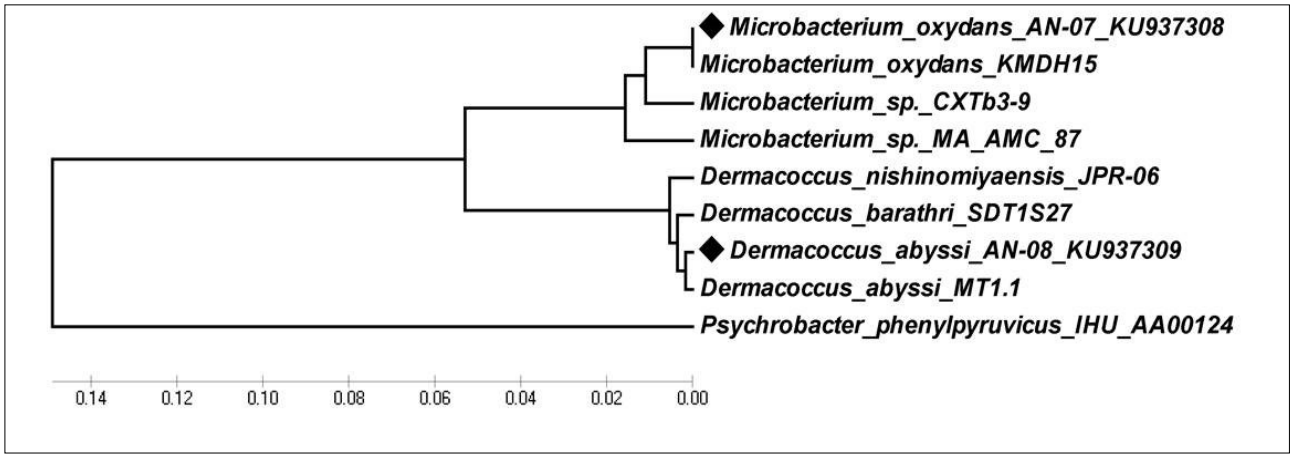

Fig. 3. Phylogenetic relationship of selected bacterial strains based on the neighbour joining method

content of the type strain is 65.2 mol %. On the basis of 16S rRNA gene sequence, isolate AN-08 was closely related to *D. abyssi* MT1.1 as reported by Wasu Pathom-aree *et al.* (2006) with 99.78% sequence similarity (Fig. 3).

Discussion

The purpose of this study was to isolate and enumerate the bacterial strains in the intestine of *C. feriata* sample from different places along the Kerala coast. Najiah *et al.* (2010) reported the total bacterial count in *S. tranquebarica*, *S. olivacea* and *S. serrata* as around 10^4 to 10^6. The total bacterial load between $0.7 \pm 0.49 \times 10^6$ and $8.9 \pm 0.13 \times 10^6$ Cfu.g^{-1} from intestinal sample of crabs have been reported by Sivasubramanian *et al.* (2017). In the present study the total culturable bacteria in the intestine of *C. feriata* ranged from 1.5×10^6 to 3.2×10^6cfu.g^{-1}. The maximum was recorded from Kollam, and the minimum from Calicut. Factors, such as bacterial host specificity, food type and water resource may be attributed to explain these differences (Verner-Jeffreys *et al.,* 2003). Bacterial diversity in the surrounding environment and feeding habit of the crab also influence the gastrointestinal bacterial count. *Pseudomonas* spp. was the predominant genus recorded in the present study. Other isolated genera were *Bacillus* sp., *Microbacterium* sp. and *Dermacoccus* sp. Venkateswaran *et al.* (1981) have reported that *Pseudomonas* sp. and *Micrococcus* sp. were dominant in the intestine of all crabs. In the present study genus *Microbacterium* sp. and *Dermacoccus* sp. were scanty, while *Pseudomonas* spp. was the dominant one. A recent study has found out that settlement by *Microbacterium* sp. in shrimp hatchery system has considerably improved the larval survival and has been taken into account as a unique probiotic microorganism (Xue *et al.,* 2015). Similarly, for crab the present *Microbacterium* sp. can be experimented for improved larval survival in hatchery system. The two bacterial strains selected for characterization up to species level were identified as *M. oxydans* and *D. abyssi*. Wasu Pathom-aree *et al.* (2006) have reported that *D. abyssi* was a piezo-tolerant species isolated from sediments of Mariana Trench in Philippines. *D. abyssi* MT1.1 had been reported to possess azo-reductase activity and potential for bio-cleaning of industrial dye baths (Weeranuch *et al.,* 2014). *M. oxydans* has been reported as an alginate and laminarin degrading bacterium for the reutilization of brown-seaweed waste (Eun *et al.,* 2013). They have also found *M. oxydans* capable of reutilization of brown-seaweed waste for the production of reducing sugars. In the present study *M. oxydans* and *D. abyssi* expressed amylolytic, cellulolytic and proteolytic activities. The 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 and probiotics could improve the digestibility of nutrients, increased tolerance to stress and encourage reproduction. The enzyme producing microorganisms isolated from the intestine of *C. feriata* in the present study may be beneficially used as a probiotic while formulating the diet, especially in the larval stages. However, much more research should be conducted to conclude that addition of such isolates to feeds do in fact, provide some kind of benefit to the animal involved before advocating their use.

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