



Identification of Bacterial Species from Injured Cuticle of Mud Crab, *Scylla serrata*

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Abstract

Crustacean aquaculture production is one of the major economic activities worldwide with prime importance in the developing countries. Infectious diseases from viral, bacterial, and in some cases, fungal origin affecting the crustacean aquaculture production due to intensification of farming activities. The microbial population of 11×10^5 to 215×10^3 CFU were observed in the scraps of naturally injured/wounded cuticle of mud crab, *Scylla serrata*. Three predominant bacterial species were identified including *Bacillus sp.*, *Escherichia coli* and *Pseudomonas aeruginosa* in the scraps based on the series of biochemical analysis as well 16s rDNA sequence analysis. These findings may provide the baseline to reveal the pathogenicity of host specific and invading pathogenic bacteria in the cultivable crustaceans.

Keywords: Crustaceans; Mud Crab; *Scylla serrate*; Bacterial Diseases

Abbreviations: MR&VP: Methyl Red and Voges Proskauer; PCR: Polymerase Chain Reaction; EMB: Eosin Methylene Blue; TCBS: Thiosulphate Citrate Bile Salt Agar.

Introduction

Crustaceans are the second largest group, next to insects, in the phylum arthropoda comprising approximately more than 30,000 known species including lobsters, crabs, shrimps, prawns, krill, copepods etc., and have a predominant role in aquatic food chain, especially as primary and secondary consumers as well as food for human [1,2]. The semi and intensive culture of edible crustaceans was started in 1970s as an industrial activity, developed rapidly with a huge increase in the number of hatcheries as well as aqua farms [3]. Even though, crustacean aquaculture sector is still highly dependent upon marine capture fisheries for sourcing key dietary nutrient inputs, including fish meal and fish oil. This dependency is particularly strong within compound aqua feeds and farmed marine shrimps [4].

Diseases in Cultivable Crustaceans

The aquaculture industries witnessed major growth till 1980s, followed by a sudden decline in its production due to environmental degradation and infectious as well as non-infectious diseases caused by many microbes including bacteria, viruses and fungi [3].

A much larger proportion of bacterial diseases are actually external infections, where a variety of microorganisms attack the exoskeleton and affect crustacean aquaculture production [5]. These chitinolytic microorganisms do not penetrate into the soft tissue underlying the exoskeleton [6] but may provide a portal entry to epidermal tissue for secondary invaders. The shell disease, which is most often reported among brachyuran crabs, appears to be associated with the microbes such as *Vibrio sp.* and *Pseudomonas sp.* [7]. Vibriosis, caused by bacteria belongs to family *Vibrionaceae*, is one of the predominant diseases in shellfish and finfish aquaculture, often responsible for mass mortality in aqua

farms with high economic loss worldwide [8-12].

Materials and Methods

The scraps obtained from naturally injured/wounded cuticle site on the dorsal side of crabs were diluted in sterile sea water, plated on nutrient agar medium. The colonies grown were counted after 24-48 hours by colony counter wherein pigmentation, relative size and shape of the colonies were analyzed. The bacterial isolates detected were subjected to staining procedures, biochemical and other tests to study the characteristic features by culturing in various media for their identification.

Staining Methods

Gram's stain was used to study the morphology and staining properties of the bacterial isolates obtained from the mud crab. The endospores of bacterial isolates were stained with malachite green and then counter stained with safranin [13].

Biochemical Analysis of Bacterial Isolates

Motility test was carried out to determine the differences, if any, among the bacterial isolates, in their motility in a liquid medium [13]. The basic biochemical test for the identification of bacterial isolates from crab were analyzed by following tests including, Oxidase test, Catalase test, Citrate utilization test, Methyl red and Voges Proskauer (MR & VP) test, Indole production test, Urease test, Starch hydrolysis test, Nitrate and nitrite reduction test Hugh-Leifson oxidative fermentative test [14]. Bacterial isolated colonies were grown in nutrient agar and some of the selective media (EMB agar, Cetrimide agar base medium and TCBS agar) for further identification based on colony morphology, size and pigmentation variability.

Molecular Characterization of Isolated Bacterial Species

Total DNA was extracted from bacterial cells using phenol chloroform method [15]. DNA concentration was measured spectrophotometrically at 260 nm in a spectrophotometer. Aliquots of the DNA (100 ng) were then used for Polymerase Chain Reaction (PCR). The 16S rDNA was amplified from the total genomic DNA by PCR using a universal primer. The forward and reverse primer sequences used were 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492r 5'-TACGGTTACCTTGTTACGACTT-3' [15]. The PCR products were gel purified and sequenced in one direction only with forward primer using Ocimum Bio solutions DNA Sequencing Services, Hyderabad, India. The gene sequences were aligned in FASTA format and then analyzed with ClustalW using MEGA 4 software. The program was then used to calculate

the best match for selected sequences, similarities and differences were identified, through viewing the phylograms based on N-J phylogeny. Finally, based on the morphological, biochemical and other characteristic features, the colonies were identified by using Bergey's Manual of Determinative Bacteriology [16].

Results

Detection and Identification of Bacterial Species

In order to detect and isolate the microbes from the scraps of injured/wounded cuticle of the mud crab, *S. serrata*, and total microbial count was analysed for 24 to 48 hours, based on the extent of bacterial colonies grown in nutrient agar plates. The data showed the presence of a strong microbial population ranging from 11×10^5 to 215×10^3 CFU (10^5 to 10^3 dilutions) in the scraps obtained from naturally injured/wounded cuticle. In addition, a dilution of 10^5 appears conducive to get a maximum 23 types of colonies which could be detected under hand held lens or colony counter (Table 1).

Dilution factor	Number of colonies (CFU/ml)
3-Oct	215×10^3
4-Oct	48×10^4
5-Oct	11×10^5

CFU: Colony Forming Units.

Table 1: Total aerobic microbial count (bacterial load as CFU) grown in nutrient agar from scraps of naturally injured/wounded cuticle of the mud crab, *Scylla serrate*.

Of the twenty three bacterial colonies detected from the injured/wounded cuticle of the mud crab, *S. serrata*, at least four rich and morphologically (colour, growing pattern and size of the colony) distinct colonies were isolated.

Morphological Phenotypic Characteristics of Bacteria

Isolates of these four distinct colonies were sub cultured in nutrient agar medium showed the distinct pigmentation, size and a pattern of growth. As shown in Figure 1, colony 1, 2 and 3 (A, B and C) appeared large with white colouration ranging from cloudy to bright white. Among these three, colony 1 and 2, although appeared similar in overall colouration, dry surface and the size of colonies varied distinctly (Table 2). Further, while both these colonies (colony 1 & 2) grew very well and appeared large, raised with irregular margin, colony 3, which was also white but with wet surface did not grow as raised but with distinct surface appeared with a smooth margin. On the other hand, colony 4, appeared green in colour but were relatively small sized compared to colony 1,

2 and 3, although all the four colonies appeared to be bacilli under light microscope.

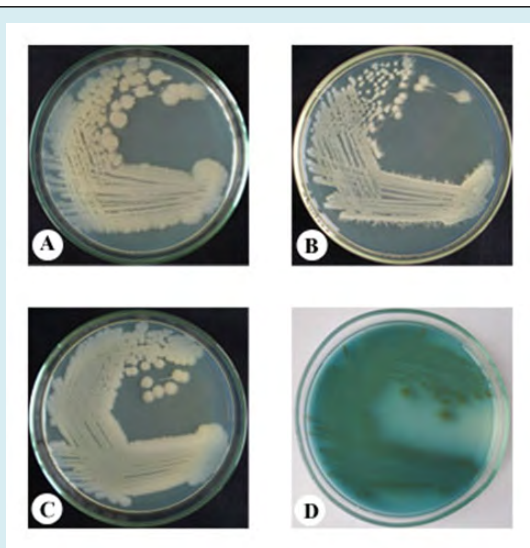


Figure 1: Bacterial species grown in nutrient agar medium isolated from the naturally injured/wounded cuticle of mud crab, *Scylla serrata*.

A: Colony 1; B: Colony 2; C: Colony 3; D: Colony 4

Bacterial colony [#]	Morphological characteristics		
	Pigmentation	Relative size	Shape of the cell
Colony 1	cloudy white	large raised, irregular margin	short bacilli
Colony 2	white	large raised, irregular margin	short bacilli
Colony 3	milky white	large	bacilli
Colony 4	green	small	short bacilli

Colonies grown in nutrient agar medium.

Table 2: Morphological characteristics of bacterial colonies isolated from the scraps of naturally injured/wounded cuticle of the mud crab, *Scylla serrate*.

Primary Staining and Biochemical Characteristics of Isolated Bacterial Colonies

In primary test, all the 4 colonies appeared motile and aerobic. Among them, while colony 1, 2 and 3 grew well at

anaerobic condition, colony 4 did not show any significant growth. Gram's and endospore staining showed that colony 1 and 2 were Gram-positive and endospore forming, while colony 3 and 4 were negative for Gram's and endospore staining (Table 3).

Primary tests	Bacterial isolates			
	Colony 1	Colony 2	Colony 3	Colony 4
Gram's reaction (young culture)	+	+	-	-
Aerobic growth	+	+	+	+
Anaerobic growth	+	+	+	-
Endospore	+	+	-	-
Motility	+	+	+	+

+ Positive

- Negative

Table 3: Primary tests for identification of bacterial colonies isolated from the scraps of naturally injured/wounded cuticle of the mud crab, *Scylla serrate*.

Biochemical characteristics, as presented in Table 4, showed that, while all the four colonies are positive for catalase, colony 1, 2 and 4 are positive to oxidase. Tests with

methyl red, indole production or nitrate reduction indicated positivity for colonies 3 and 4 only.

Biochemical tests	Bacterial isolates			
	Colony 1	Colony 2	Colony 3	Colony 4
Oxidase reaction	+	+	-	+
Catalase test	+	+	+	+
Citrate utilization	+	-	-	+
Methyl red	-	-	+	+
Voges-Proskauer	-	-	-	+
Indole production	-	-	+	+
Urease	-	+	-	+
Starch hydrolysis	-	+	+	-
TSI	ND	ND	-	+
Nitrate reduction	ND	ND	+	+

ND: not determined; + Positive; - Negative; TSI: triple sugar iron test.

Table 4: Biochemical characteristics of bacterial colonies isolated from the scraps of naturally injured/wounded cuticle of the mud crab, *Scylla serrata*.

As shown in Table 5, the carbohydrate fermentation tests showed that all the four bacterial isolates invariably reduced or fermented all the 15 sugars tested. The colonies 1, 2, 3 and 4 reduced six sugars namely D-xylose, sucrose, D-glucose, D-sorbitol, L-arabinose and L-rhamnose while, melibiose was reduced by colony 1, 2 and 3. Further, colony

1, 2 and 4 did reduce the carbohydrates such as D-fructose, inositol, trehalose, D-mannose. D-galactose was reduced by colonies 1 and 4, while colony 3 and 4 reduced the sugar D-mannitol. Furthermore the colonies 1 and 3 reduced the sugars maltose and lactose respectively.

Biochemical test	Bacterial isolates			
	Colony 1	Colony 2	Colony 3	Colony 4
Carbohydrate fermentation				
D-Xylose	+	+	+	+
Sucrose	±	±	+	+
Lactose	-	-	+	-
D-Glucose	+	±	+	+
D-Fructose	±	±	-	+
Maltose	+	-	-	-
D-Galactose	+	-	-	+
D-Sorbitol	±	±	+	+
Inositol	±	±	-	+
L-Arabinose	+	+	+	+
D-Mannitol	-	-	+	+
Melibiose	±	±	+	-
Trehalose	±	±	-	+
L-Rhamnose	±	±	+	+
D-Mannose	±	±	-	+

+ Positive.

- Negative.

Table 5: Biochemical characteristics of bacterial colonies isolated from the naturally injured/wounded cuticle of the mud crab, *Scylla serrata*.

Growth in Selective Media

The isolated colonies were grown in certain specific selective media, including Eosin Methylene Blue (EMB), Cetrimide agar and Thiosulphate citrate bile salt agar (TCBS) showed that colony 1 and 2 did not grow at any of the three

selective media tested, while colony 3 and 4 grew well in EMB agar with metallic green colour and in cetrimide agar with green colour respectively. Interestingly, none of these 4 colonies grew in TCBS agar, showing that the isolated colonies did not belong to *Vibrio* species (Table 6).

Media	Growth of bacterial isolates			
	Colony 1	Colony 2	Colony 3	Colony 4
EMB agar	-	-	+ (metallic green)	-
Cetrimide agar base	-	-	-	+ (green)
TCBS agar	-	-	-	-

EMB agar: Eosin Methylene Blue agar

TCBS agar: Thiosulphate Citrate Bile Salt agar

+ Growth; - No growth

Table 6: Growth of bacterial colonies isolated from scraps of the naturally injured/wounded cuticle of the mud crab, *Scylla serrata* in selective media.

Based on these results, it may be safely concluded that colony 3 and 4, bacterial isolates from the scraps of injured/wounded cuticle of the mud crab *S. serrata* belongs to enterobacteriaceae and colony 3 could be identified as *Escherichia coli* as it grew well in EMB agar media with metallic green coloured colonies. The possible growth of colony 4 in cetrimide agar with green colour colonies along with its ability to reduce 12 of the 15 sugars tested (Table 5) indicates that the colony 4 could be identified as *Pseudomonas aeruginosa*. The ability to produce endospores and positivity for Gram's staining, biochemical, phenotypic characteristics and inability to grow in any of the three selective media tested indicate that colony 1 and 2 may belong to genus *Bacillus*.

Molecular Characterization of Bacterial Isolates

In order to identify the species of bacterial colonies 1 and 2 (*Bacillus* spp.), an attempt was made to characterize the bacterial isolates by amplifying 16S rDNA of the colonies using specific primers. The DNA was isolated from colony 1 and 2 by phenol chloroform method and subjected to electrophoresis on 0.8% agarose gel. The quantity of genomic DNA obtained was approximately 200 ng/μl (Figure 2A). The 16S rDNA amplified from the bacterial genomic DNA by PCR using specific primers produced a single band at approximately 1500 bp in agarose gel (Figure 2B). The purified PCR product was then sequenced and the nucleotide sequences were analysed using BLAST search. The results revealed the sequence similarities with *Bacillus* sp. and *Bacillus flexus* in the NCBI database. The sequence data obtained in the present study was deposited in the GenBank database and accession numbers were obtained, *Bacillus* sp. N1 (HM997150) and *Bacillus flexus* N3 (HM997151).

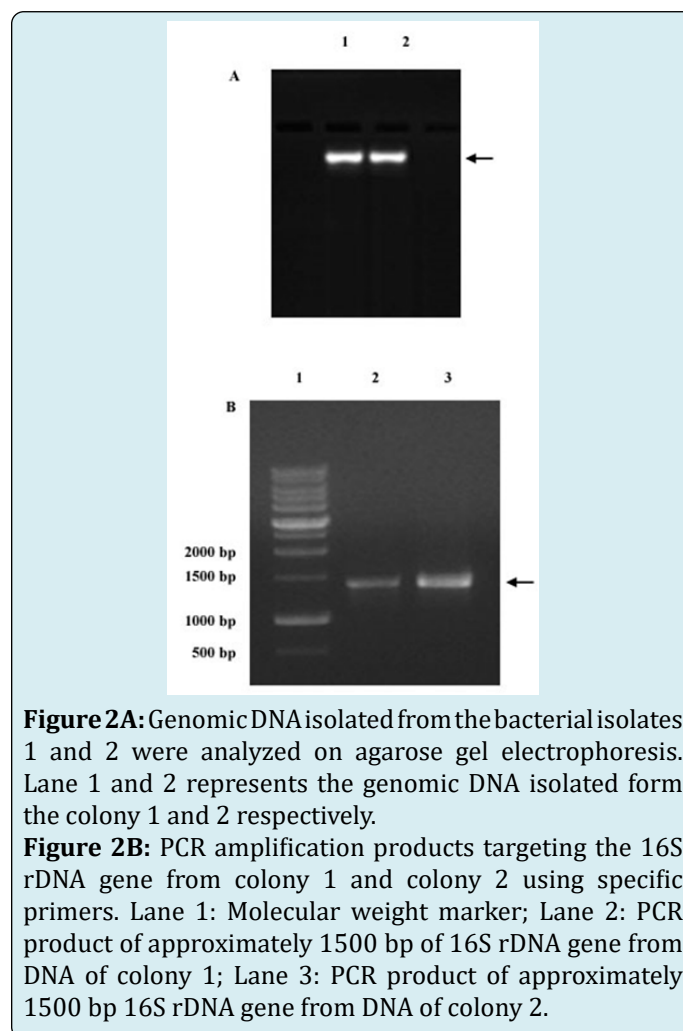


Figure 2A: Genomic DNA isolated from the bacterial isolates 1 and 2 were analyzed on agarose gel electrophoresis. Lane 1 and 2 represents the genomic DNA isolated from the colony 1 and 2 respectively.

Figure 2B: PCR amplification products targeting the 16S rDNA gene from colony 1 and colony 2 using specific primers. Lane 1: Molecular weight marker; Lane 2: PCR product of approximately 1500 bp of 16S rDNA gene from DNA of colony 1; Lane 3: PCR product of approximately 1500 bp 16S rDNA gene from DNA of colony 2.

The sequence data of *Bacillus* sp. N1 and *B. flexus* N3 obtained for our sample were aligned against similar such *Bacillus* nucleotide sequences available in the sequence data

from NCBI Gen Bank and evolutionary relationships were analysed using MEGA software version 4 (Figures 3&4).

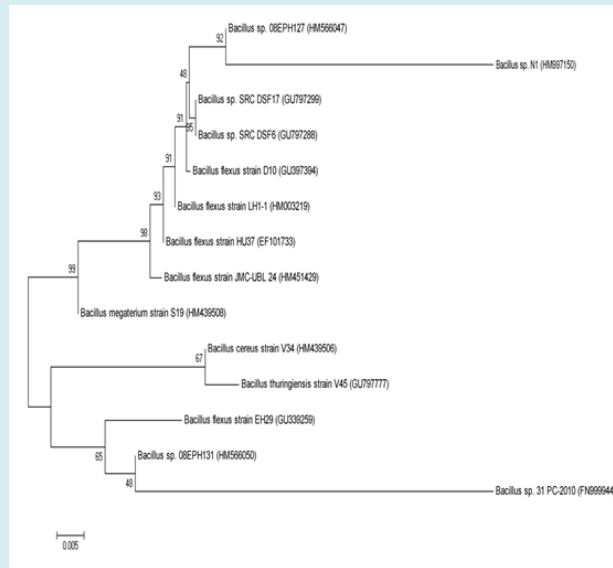


Figure 3: Phylogenetic relationships of colony 1 (*Bacillus* sp. N1) derived from 16S rDNA gene sequence homology. The sequences have been retrieved from NCBI databases and the tree was drawn using neighbour joining algorithm with kimura 2 parameter distances in MEGA 4 software. The bar represents distance values calculated in MEGA and values at nod represent percentage of 1000 bootstrap replicates. Numbers in bracket represent GenBank accession numbers.

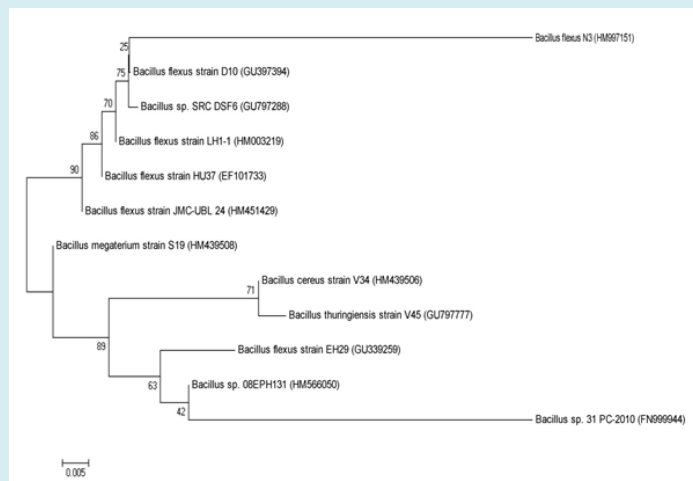


Figure 4: Phylogenetic relationships of colony 2 (*Bacillus flexus* N3) derived from 16S rDNA gene sequence homology. The sequences have been retrieved from NCBI databases and the tree was drawn using neighbour joining algorithm with kimura 2 parameter distances in MEGA 4 software. The bar represents distance values calculated in MEGA and values at nod represent percentage of 1000 bootstrap replicates. Numbers in bracket represent GenBank accession numbers.

Discussion

A variety of microorganisms, including bacteria and fungi, have been identified from shell lesions of the

crustaceans and it is probable that these microorganisms are involved in destroying the chitin [17]. Most often, shell diseases are caused by *Vibrio* spp. as well as by many other chitinolytic or chitinoclastic bacteria [6,18-21].

In the present study, bacterial colonies isolated from the scraps obtained from naturally injured/wounded cuticle of mud crab, *Scylla serrata*, were serially diluted, plated on nutrient agar medium and the growth was observed up to 5X dilutions (11×10^{-5} CFU), which indicated the presence of a strong microbial population in the injured/wounded site.

A detailed bacteriological study of these bacterial colonies upon isolation, demonstrated the presence of at least four rich and morphologically distinct colonies. Preliminary phenotypic and biochemical characteristics of these four bacterial isolates indicated that while the colony 3 and 4 appeared belonging to Enterobacteriaceae and Pseudomonadaceae, colony 1 and 2 appeared to be Bacillaceae type.

Based on the size, shape, staining for endospores and Gram's test and the biochemical characteristics, the colonies of 1 and 2 were identified as genus belonging to *Bacillus* and based on the capability to grow in selective media, including EMB agar and cetrimide agar, colony 3 and 4 were identified as *Escherichia coli* and *Pseudomonas aeruginosa* respectively.

Molecular characterization of the colony 1 and 2 using 16S rDNA sequence showed that colony 1 and 2 were identified as *Bacillus* sp. N1 and *Bacillus flexus* N3. It may be noted in this context that there are at least two earlier studies attempted to detect bacterial colonies in the shell diseases (shell disease lesion) of crustaceans including a crab, *Callinectes sapidus* [7] and moribund shrimps, *Penaeus stylirostris* [22]. While working on a marine blue crab, *C. sapidus*, [7] have reported the presence of at least six genera of bacteria with predominant presence of *Acromobacter* sp., *Aeromonas* sp., *E. coli*, *Plesimonas shigelloides*, *Pseudomonas* sp. and *Vibrio* sp.

The moribund shrimps, *Penaeus stylirostris* often infected with bacteria belonging to genus *Vibrio*, *Pseudomonas* spp. and *Bacillus* sp. in the hemolymph [22]. Thus, either in shell diseased marine crab or moribund shrimps, *Pseudomonas* sp., *E. coli*, *Bacillus* sp. or *Vibrio* sp. appears to be predominant in these animals. In the present study, the bacterial species isolated from the cuticle scraps of injured/wounded crab, *S. serrata* from Indian waters showed *Bacillus* sp., *E. coli* or *P. aeruginosa* as predominant bacterial species associated with the shell disease of these crabs, although, *Vibrio* sp. could be detected, it did not appeared as a predominant bacteria.

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