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Enumeration, Isolation and Identification of Crustacean Surface Attachment Bacteria

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ABSTRACT

The presence of microbial epibionts on marine and freshwater zooplankton (crustaceans) and other invertebrates have been documented frequently. The ecological context and impact of these intricate relationships are not well understood so far. Recent studies have examined the interrelationship of bacterial epibionts on freshwater crustaceans.Presently,eighty-three bacteria were isolated from three different aquatic environmental study areas and seven of them were primarily screened on account of their colony characters, morphological and biochemical character. Among the seven isolates, SA-5 and SA-7 strains showed maximum different morphology with respect to others isolates.Molecular characterization of two isolates indicates that SA-5 and SA-7 are *Aeromonas sp.*.The present study provided the baseline information regarding the pathogenic and non-pathogenic bacteria in association with crustacean zooplankton. Present study also tries to find out the differences and commonalities across epibionts in the realm of epibiont-host nutrients cycling which in turn to generate relevant hypothesis in the context of host microbial interaction.

INTRODUCTION

Several human pathogens and fecal-pollution indicators may persist as viable organisms in natural environments, owing to their ability to activate different types of survival strategies. These strategies include adhesion on both abiotic and biotic surfaces and the entrance to the so-called viable but non-culturable (VBNC) state (Coveney et al., 1977; Hickman et al., 1977). In an 18-month survey for the detection of enterococci in both lake water and seawater(Signoretto et al., 2004) have shown that *Enterococcus faecalis* was detected mostly bound to plankton and in the VBNC state.Bacterial function in aquatic systems encompasses two major processes: (1) the degradation of organic matter; and (2) the regeneration of soluble nutrients to the biological system (McCoy et al., 1969). Organic matter emanating from the zooplankton and phytoplankton of lakes constitutes a sizable fraction of the organic materials pool which bacteria utilize (Ormerod et al., 1978). Detailed examinations of the relationships between plankton and bacteria are necessary to determine the various pathways of organic matter flux. Bacteria-phytoplankton interactions have been the focus of many studies (Henrici

et al., 1938;Niewolak *et al.*, 1971; Nalewajko *et al.*, 1972; Rieper *et al.*, 1976). Phytoplankton provides organic substances for the heterotrophic activities of bacteria by excreting carbonaceous materials during growth and by releasing them through lytic processes when the population undergoes senescence. Studies concerning zooplankton and bacteria have been concerned primarily with the utilization of bacteria as food for (Coveney et al., 1977). Investigators interested in the distribution of bacteria in river pond and cannels have demonstrated the importance of surfaces in the ecology of lake bacteria; in fact, some of the apparently high concentrationsof bacteria encountered may be surface-induced (McCoy et al., 1969&Hillbricht-Ilkowska *et al.*, 1966). Microorganisms, both sessile and free-swimming, provide surfaces that can support microbial films (Sieburth *et al.*, 1976). The objective of the present experimental research effort was to study the interrelationship ofbacterial community of zooplankton (copepods and cladocera), including the distribution and relative abundance of these bacteria from investigation site of Midnapore, West Bengal, India.

MATERIALS AND METHODS

Study site:

The present investigation was carried out from aquatic ecosystems of freshwater lotic zone, and around certain wetlands of Midnapore, PaschimMedinipur, West Bengal, India. The samples were collected from six study sites, two river sites located outside of the town which are used for domestic and agricultural purpose, two ponds located at heart of the town which are heavily used by colonies people of the town and two cannels(present within town), used mainly municipality sewage drainage system.

Collection of the environmental samples:

Water and plankton sample were collected from the six-study site in the month, of june, 2018. Zooplankton samples were collected from subsurface zone of the five sub- sampling sites in the water body of sampling site, using Nylobolt plankton net (25 µm mesh size). A total hundred liters of water were filter from each sampling site (Midya *et al.*, 2018).

Isolation and screening:

The zooplankton were identified to the lowest taxonomic level following standard references, Emir (1994), Ruttner-Kolisko (1974), Segers (1995), Nogradyand Pourriot (1995), and DeSmet (1998) for Rotifera and Kiefer (1978), Reddy Ranga (1994), and Dussart and Defaye (2001). Quantitative study of zooplankton was done under a phase contrast microscope with the help of Sedgewick rafter counting cell and the values was expressed in number per litter.

Fresh plankton sample were further concentrated and homogenized. These concentrated samples were incubated in alkaline peptone water (APW) and incubated at 37°C for 18-24 hrs. Pellicle growth from the surface of APW was subsurface on to selective chitin agar media and incubated at 37°C for 48-72hrs (Midya *et. al.*, 2019)

Effect of pH, temperature and salt concentration on native isolates:

The effects of pH on newly isolated bacterial growth were determined by adjusting the pH of the nutrient agar media at different level of pH (4.0 -12.0). The effect of temperature on bacterial growth was determined by incubating inoculated medium at different temperatures (15, 18, 22, 28, 35, 37, 40, 50 and 55°C) in nutrient broth through separate incubation. In order to determine the effect of salt concentration on bacterial growth the selected bacterial strain was grown in presence of varied level of NaCl (1-10%) with nutrient agar media (Kuddus M.S., *et al*., 2013)

Tests for arginine, lysine, and ornithine decarboxylases:

Inoculate tube of each of 3 decarboxylase broth media (HiMedia, India: having composition (g/l):Peptic digest of animal tissue- 5.0, Beef extract- 5.0, Dextrose- 0.5, Bromocresol purple- 0.01, Cresol red- 0.005, Pyridoxal- 0.005, Final pH- 6.0 ±0.2, at 25°C] with loopful of TSA culture. After inoculation, add 10 mm thick layer of sterile mineral oil to each tube; include basal medium control. Replace caps loosely and incubate 24 hr at 35°C. Examine every 24 hr for 4 days.

Effect of substrate supplementation on bacterial growth:

The isolated bacteria were grown in phenol red broth(HiMedia), India: having composition (g/l):Proteose peptone- 10.0, Beef extract- 1.0, Sodium chloride- 5.0, Phenol red- 0.018, Final pH- 7.4±0.2, at 25°C], supplemented with four different substrates sugar disc such as fructose, maltose and lactose (disaccharide) respectively at 37°C for 96 hrs (Gerhardt, 1994 & Kuddus M.S., *et al.*, 2013).

Isolation of genomic DNA for 16S rRNA and PCR amplificationwith specific primer for molecular identification of the isolates:

Genomic DNA was extracted from the bacterial cells by using the standard phenol–chloroform method (Ruzzante, D.E. *et al.*, 1996).Extracted DNA was checked for its quality by agarose gel electrophoresis on UV transilluminator and concentration by nanodrop. The extracted DNA was amplified with 16S rDNA specific primer pair, Forward primer (27F) 5¹-AGAGTTTGATCMTGGCTCAG-3¹, and Reverse primer (1492R) 5¹-TACGGYTACCTTGTTACG-ACT-3¹ (Lane et al. 1991).Each 25 µ1 reaction mixture contained: 1.5 µ1 of 10 X Taq polymerase buffer contains (100 mMTris (pH 9), 500 mMKCl , 15 mM MgCl2 , 0.1% Gelatin)(Genei) , 1.5 µ1 of dNTP mixture ((dATP, dCTP, dGTP, dTTP10 mMconectration) (Genei),Enzyme: Taq polymerase-0.5 µ1 (3 U/ìl) (Genei), 1 ìl each primers (5pM/ ìl) (Eurofin), Template DNA-2µ1 (100 ng) and 17.5 µ1sterile molecular biology Grademilli-Q water (Hi-media). The reaction conditions were 94°C for 5 min, 94°C for 45 s, 56°C for 1 min, 72°C for 1 min (for 35 cycles), and then 72°C for 10 min and final holding at 4°C. The PCR-amplified product

was analyzed on 1.5% agarose gel containing ethidium bromide (0.5 mg/mL) and 1 kb DNA molecular weight marker and documented using a gel documentation system. The PCR amplicon for the partial 16S rDNA gene was further processed for sequencing. Sequencing was carried out using the same set of primers in both the directions to check the validity of the sequence. Sequencing of the 16S rDNA gene amplicons were done by outsourcing (Scigenom, Kochi). After that the homology of the partial 16S rRNA gene sequence of these two isolates was analyzed using the BLAST algorithm in Gen-Bank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Only the highest-scored BLAST result was consideredfor identification.

RESULTS & DISCUSSION

Planktons biodiversity and microbial load in study area:

In this present study the zooplanktons number in different study area were reveals that the number of copepoda was higher than the cladosera. In case of cannel water both average number and standard deviation of copepoda and cladocera were 69.40 & 1.13; 50.30 & 0.93, respective Biodiversity of copepoda and cladocera were to much higher in cannel water with respect to pond and river water aquatic systems. But in case of river water average numbers of copepoda decrease, where the average number of cladocera is lowest in pond water aquatic systems (Fig-1). Length of copepod and cladocera were 906.96 µm 1848.84,respectively (Fig-2). Dynamics of culturable microbiological parameter was interestingly varied in different auatic system. Microbial abundance i.e.the culturable total bacterial Colony Forming Unitper ml (CFU/ml) was higher in cannels aquatic(5.61 X 10^5) sysytem than pond (5.34 X 10^5) and river (5.52 X 10^5) aquatic systems (Fig-1). So, the present study reveals that the microbial and planktons abundance both are higher in cannel aquatic system. A number of eighty-three bacteria were isolated from three different aquatic environmental study area and seven of them were primarily screened on account of their colony characters (Table-1). Among the seven isolates, SA-5 and SA-7 strains showed maximum different morphology with respect to others isolates. All the isolates were pure cultured and kept in refrigerator for further use.

Bacterial growth depends on pH, temperature and salt concentration*:*

Comparison of pH of these two strains showed that pH-7 was optimum for strain SA5 and SA7, but it’s favor for growth between pH 6–8 and pH 5–8, respectively. At higher the pH values, we may presuppose that the pooled enzymes and proteins were not in same structural configuration that’s why further enhance the growing environment, for that one which has diminished the bacterial growth.Generally, an increase in temperature will increase enzyme activity.But if temperatures get too much high then enzyme activity will diminish and the protein (also enzyme) will denature. On the other hand, lowering temperature will decrease enzyme

activity.Every bacterial species has a specific growth temperature requirement which is largely determined by the temperature requirements of its enzymes. In this study, both isolates were grown in mesophiles (i.e. 15 to 45ÚC) condition.Water passes out of a bacterium so as to balance salt concentrations on each side of its cell membrane. Without water, bacterial proteins such as enzymes cannot function and eventually the cell collapses in on itself. Some bacteria can tolerate salt; they are called as halotolerant bacteria. Certain strains of *Staphylococcus*, *Vibrio* and *Aeromonas* were responsible for infections, blood poisoning, and even death, these are also halotolerant. These pathogens have a salt alert system that uses sponge-like molecules to prevent water loss. Here both strains were growing simultaneously up to 6% NaCl, after that the growth rate was diminish with increasing the salt concentration, these strains are also called as salt tolerant bacteria (Fig-3).

Biochemical efficacy of native isolates:

Both the native isolates strains (mentioned above) were examined for evaluating their various types of biochemical efficacy. It was found that both the strains were Gram negative, non-spore forming, motile, catalasepositive, indole and methyl red test positive, also capable to hydrolyze starch agar medium.But both the isolates were Voges-Proskaure test negative and can't utilize Urease, also do not produce H_2S gas. On the other contrary varied results were observed incase of citrate utilization. Strain SA-5 can utilize citrate but strain SA-7 can't utilize citrate.(Table-2).Utilization of different sugar and oxidation of mannitol results showed that both isolates are utilizing all sugar and also oxidize mannitol.

Molecular identification of bacteria:

The isolates SA-5 and SA-7 were identified as *Aeromonas sp.* through biochemical test. The strains were rod shaped, Gram negative, motile and non-spore forming (Table 2). The identity of the strains was further confirmed by 16S rDNA analysis. The sequences of the 16S rDNA region of twelve isolates have been submitted to the NCBI database. The DNA sequences of 16S rDNA region were searchedfor homology with Basic Local Alignment Search Tool (BLAST) against the nucleotidedata base maintained by National Centre for Biotechnological Information (NCBI), NIH,USA. Isolates are used in the study exhibited 97 to 100% sequence similarity to the *Aeromonas sp.* vailable in NCBI database with lowest E-value and maximum query coverage andmaximum identity (Fig-4).

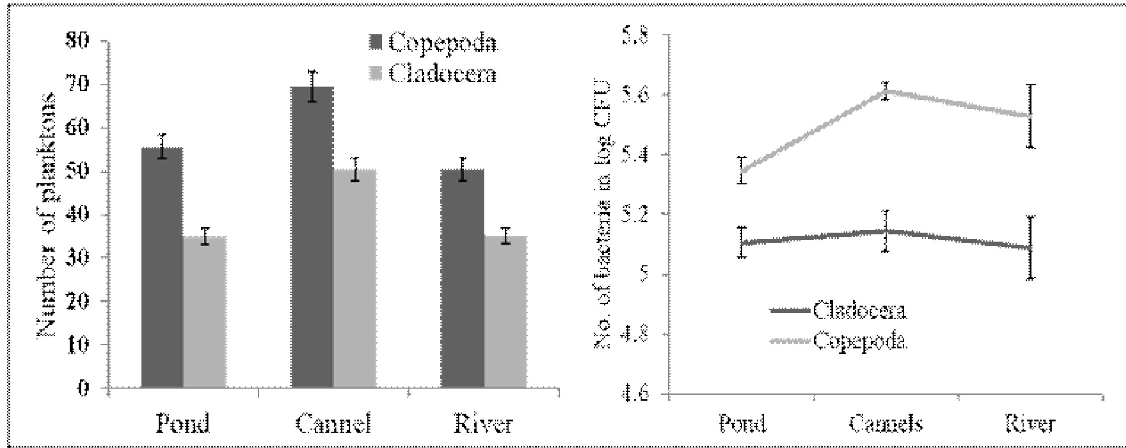

Fig-1: Temporal variation in zooplankton composition in different wetlands of study sites.

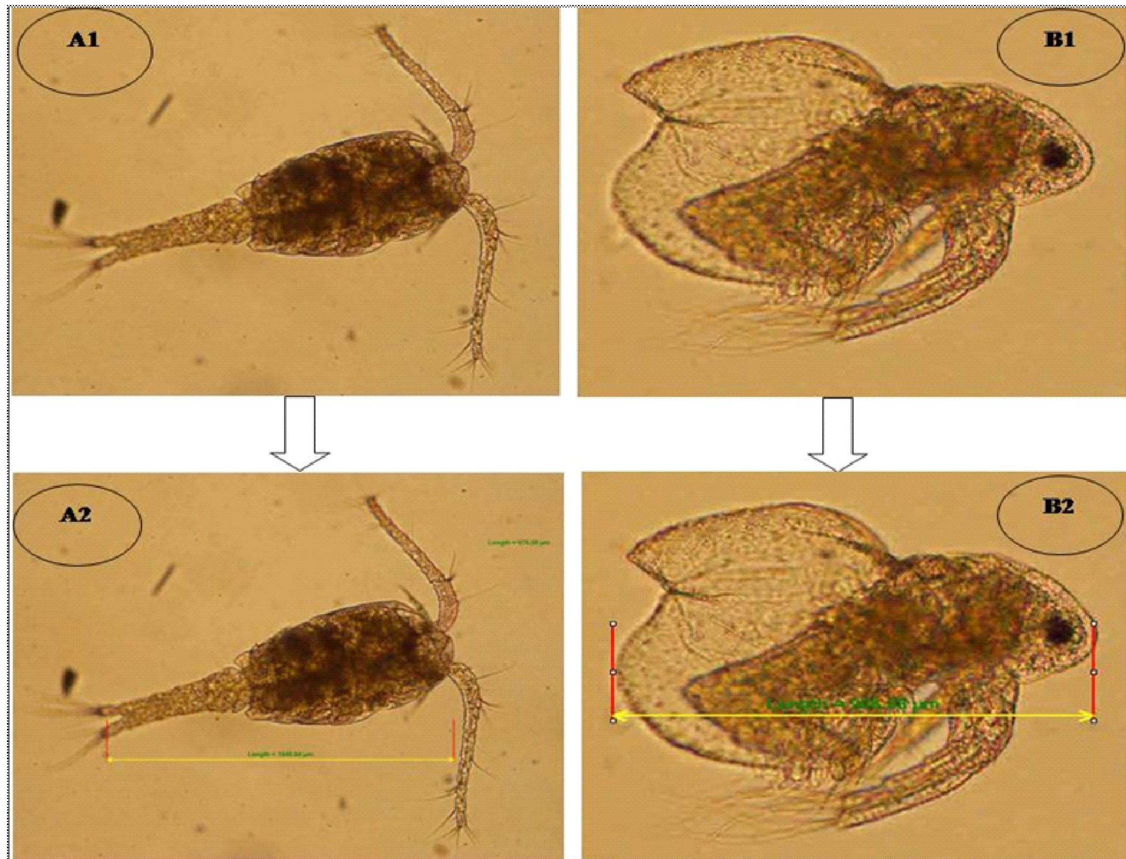

Fig-2: Microscopic view and body length of zooplankton (A) & (B)

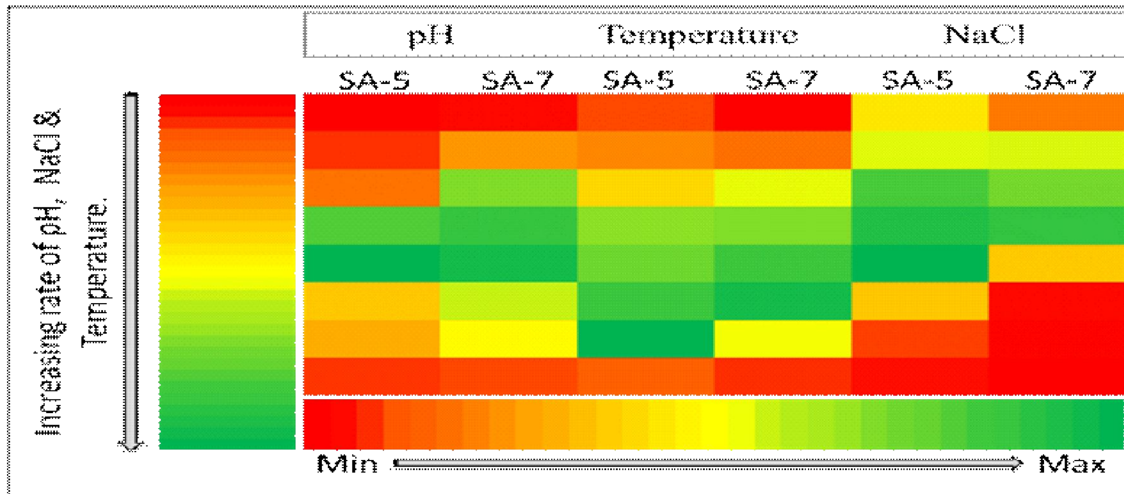

Fig-3: Favorable growth conditions of two isolates were obtained in different pH, temperature and NaCl concentration. Heat map generated data reflecting values in several conditions.

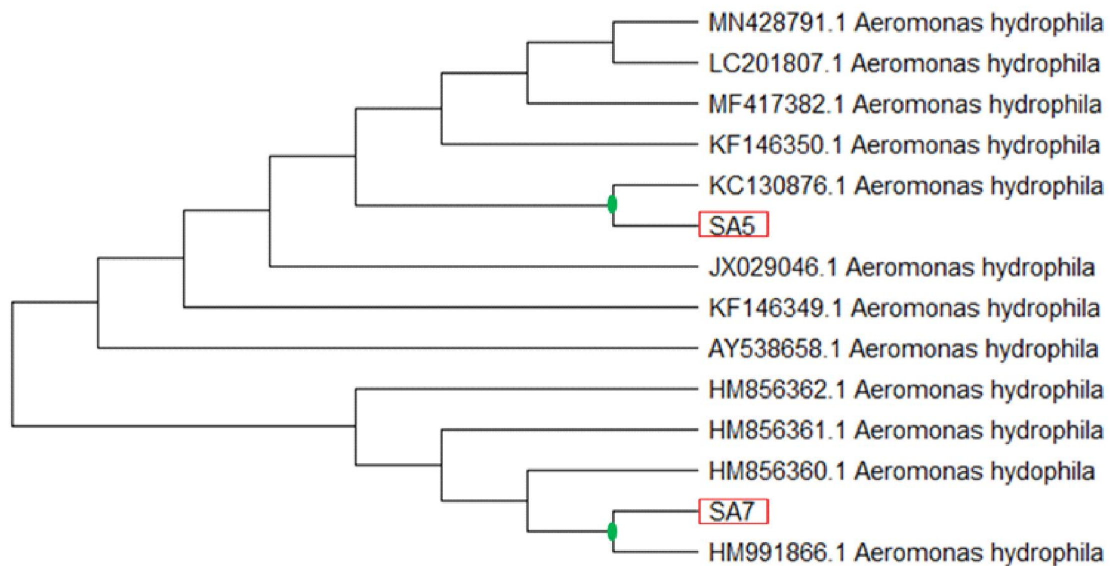

Fig-4: The evolutionary history was inferred using the Neighbor-Joining method.Phylogenetic tree of 14 taxa generated by comparing 16S rDNA homology in MEGA-X showing the location of isolates SA5 and SA7. The GenBank accession numbers are shown in parentheses.

Table-1: Colony characterization of native isolates.

Colony properties	SA-5	SA-7
Colony shape and size	Irregular, 2–3 mm	Circular, 2 mm
Elevation	Convex	Convex
Margin	Undulate,	Entire edge,
Gram reaction	Negative	Negative
Cellular morphology	Rod	Rod
Color	Creamish white	Creamish
Opacity	Opaque	Opaque
Texture	wrinkled surface	smooth surface

Table-2: Biochemical assay of native isolates, isolated from plankton surface area.

Biochemical assay	SA-5	SA-7
Utilization of lactose, Maltose and cellobiose	+ve	+ve
Oxidation of mannitol	+ve	+ve
Spore	-ve	-ve
Citrate utilization	+ve	-ve
Motility	+ve	+ve
Urease production	-ve	-ve
Catalase test	+ve	+ve
Starch hydrolysis	+ve	+ve
Indole	+ve	+ve
H_2S	-ve	-ve
Methyl red	+ve	+ve
Voges–Proskauer reaction	-ve	-ve
Decarboxylases of arginine, lysine, and ornithine	+ve	+ve
Growth at pH	6–8	5–8
Growth at temperature (ÚC)	15-40	18–37
Growth in NaCl	2–8%	2–6%

CONCLUSION

The result concluded that these two strains SA-5 and SA-7 are novel mesophilic andslightly salt tolerant bacterial strains that have the ability to producedifferent types of enzyme in short time. Both the isolated strains of *Aeromonassp.* have the ability to hydrolysisstarch in optimize temperature and pH.Infection of *Aeromonas* remains among those infectious diseases of potentially severe risk to public health. *Aeromonas* infectious disease outbreaks have to create a hurting awareness of the personal including economic, societal, and also public health expenses associated with the impact of contaminated drinking water in the aquatic environment. The evidences from many scientific reports suggest that the prevalence of diseases of *Aeromonas* infections may be noticeably underestimated in some developing nations and that schedule endemic exposure to waterborne and food-borne pathogens have been occurring more frequently than originally perceived. A variety of factors likely demographical, societal, environmental, and physiological emergence have to play significant roles in enhancing the occurrence of transmission of pathogens to hosts and the rising trend in antibiotics resistance property in some bacteria makes extensive studies on the particular group of bacteria.Previous study suggests that, the *Aeromonas* sp. have fungicidal efficacy. So, these strains may use in agriculture and different industrial purpose in different way.

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