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Cyanobacteria from Indian Sundarbans as a source of Antimicrobial Compounds

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***Abstract*—Cyanobacteria are an incredibly old group of prokaryotic organisms that produce a variety of industrially important secondary metabolites such as antibiotic, algicide, cytotoxic, immunosuppressive and enzyme inhibiting agents. Tropical seas are well known to be areas of high biological diversity and therefore, logical sites of high chemical diversity but till now no systematic explorations have been conducted to assess the biotechnological applications of the cyanobacterial biodiversity of tropical *Sundarbans* of India. Cyanobacteria were isolated from the intertidal soil of fourteen different locations of Sagar Island (21°44'7"N 88°7'2"E), *Sundarbans*. Methanolic extracts of 40 cyanobacteria were screened for their antimicrobial activity against two Gram-positive bacteria, *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96) and two Gram-negative bacteria, *Escherichia coli* (MTCC 739), Pseudomonas *aeruginosa* (MTCC 424). Of total cyanobacteria, 27.5% (11 cyanobacteria) exhibited antimicrobial activity. Isolated cyanobacteria with positive antimicrobial activity were identified morphologically as *Lyngbya-Phormidium-Plectonema* Group B, *Oscillaroria* sp., *Leptolyngbya* sp., *Phormidium* sp.**

Keywords- cyanobacteria; Sundarbans; antimicrobial; Mangrove

I. INTRODUCTION

Marine bacteria are emerging as an exciting resource for the discovery of new classes of therapeutics. If properly developed, marine bacteria could provide the drugs needed to sustain us for the next 100 years in our battle against drug-resistant infectious diseases. The current increase in scientific and commercial interest in the use of marine genetic resources is also of significance to international marine policy makers. Of the Gram-negative marine bacterial phyla, Proteobacteria and filamentous cyanobacteria are the most prolific producers of potentially novel molecules. Extremely high levels of structural modifications are typical in cyanobacterial compounds, particularly the inclusion of polyketide derived units and each strain produces an array of bioactive compounds, making drug discovery from these sources a fruitful endeavor [1,2].

Intertidal estuarine systems are a vital interface of the ocean, atmosphere, and terrestrial environments. They are characterized by frequent fluctuations in temperature, ion concentration, dessication, UV-irradiation, and wave action [3]. A previous study related to cyanobacteria of the intertidal regions focused on the effect of extreme environmental conditions on community distribution along various locations of oil polluted transect across the intertidal zone of Abu Ali Island located in the Arabian Gulf [4]. Similarly, the effects of salinity fluctuation on bacterial diversity, rates of gross photosynthesis and oxygen consumption in the light and in the dark were investigated by Abed et al. [5] in three submerged cyanobacterial mats from a transect on intertidal flats of Abu Dhabi (United Arab Emirates). The frequency of the fluctuating environmental conditions creates physical and biochemical challenges to microorganisms which inhabit this ecosystem and one way to respond to such stress could be the production of antimicrobial substances. To the best of our knowledge antimicrobial activity of marine cyanobacteria adapted to changes of salinity and temperature isolated from the estuarine intertidal locations has not been intensively investigated.

Our study area is the *Sundarbans*, the world's largest tidal mangrove forest, which lies on the delta of the Ganges, Brahmaputra and Meghna rivers off the Bay of Bengal. The forest covers 10,000 sq km of which about 6,000 sq km is in Bangladesh and the rest in India. In this communication we report the isolation of marine cyanobacteria and their antimicrobial activity, as well as identification of the active strains.

II. MATERIALS AND METHODS

A. Sampling

Fourteen microbial mat samples were collected during November 2006 from the different location of Sagar Island (21°44'7"N 88°7'2"E). Sampling was done in the post monsoon periods to avoid the effects of rain water. All samples were collected aseptically in plastic sterile bags and carried to the laboratory under refrigeration within 24 hours.

B. Isolation and culture conditions

Soil samples were cultured by usual methods [6]. Cyanobacteria were grown in 250 ml conical flasks containing 100 ml of ASN-III medium [7] adjusted to pH 7.4. The cultures were grown at 25±2°C and illuminated (50 µmol photons m^{-2} s^{-1}) under cool fluorescent lights of 12:12 L:D cycle. The culture media were bubbled with 0.3% CO2-enriched air. Standard plating and streaking techniques were

used for isolation and purification of cyanobacteria [8]. Identification of cyanobacteria with antimicrobial activity was done according to Desikachary [9], Prescott [10], Anagnostidis & Komarek [11], and John et al. [12].

C. Preparation of cyanobacterial extract

Cyanobacterial biomass were harvested in the stationary growth phase by centrifugation at 5000×g for 15 min. 1 gm of dried biomass of the isolates was extracted with methanol in a mortar pestle and kept overnight at 4°C for complete extraction. The supernatant was collected after the centrifugation at 10000×g at 10 min. The solvent extracts were concentrated under reduced pressure at 40°C. Dry residue was re dissolved in dimethylsulfoxide (DMSO) and kept at 4°C until use for bioassay.

D. Screening of cyanobacterial extracts for antibacterial activity

The antibacterial activities of cyanobacterial extracts were evaluated by agar plate diffusion test. The following bacteria were used as test organisms: Gram-positive bacteria, *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96) and Gram-negative bacteria, *Escherichia coli* (MTCC 739), *Pseudomonas aeruginosa* (MTCC 424). Filter-paper disks (5 mm) were saturated with 20 µl of 1mg. ml^{-1} test solution, dried, and placed on nutrient agar plates with a lawn of the test microorganisms. Plates were incubated at 37°C and inhibition zones were measured.

III. Results And Discussion

A. Field data

The average pH of water of the 14 stations sampled was 7.5±0.1; the average salinity was 17.5±0.5 ppt while the average soil temperature was recorded as 23±2 ºC. Aerial view of the sampling site was presented in Figure 1.

The *Sundarbans* is characterized by frequent variations in surface temperature over a 24 hour period as well as salinity during tides or after rainfall. High evaporation rates during summer (April to July) too results in high salinity in localized regions. These spectacular shifts in soil temperature and osmotic/ionic potential create a highly variable or "poikilotrophic" environment [13]. Such an environment is considered to be truly extreme because resident organisms must acclimatize not only with the high salt and desiccating conditions of a terrestrial hypersaline habitat, but also the rapid changes in temperature, salinity and water availability when rain and/or flooding occurs especially during the monsoon (June to September) in this geographical region.

Figure 1. Aerial view of investigation site. (Source: Google earth)

B. Morphological characteristics of the isolated cyanobacteria

Based on morphological characters (Table I) four of the eleven filamentous cyanobacteria were assigned to the *Lyngbya-Phormidium-Plectonema* (LPP) Group B. *Oscillatoria sp.* and *Phormidium sp.* also present in the isolated cyanobacteria. One *Lyptolyngbya sp.* was found. The photomicrographs of isolated cyanobacteria are presented in figure 2.

From the description of the environment of the Great Salt Plains (GSP) in north-central Oklahoma, USA, [13] it appeared that in the *Sundarbans* and in the GSP, the highly variable nature of the environmental factors was common. Thus, it would be interesting to compare the properties of the cyanobacteria isolated from this (GSP) region and our isolates. Most of the strains collected from the GSP were non-halophilic, tolerating salinity up to 5% while the halophilic ones (*Cyanodictyon* sp., *Geitlerinema* sp., *Halomicronema* sp.) could tolerate up to 15% salinity. The *Leptolyngbya* sp. was found to be halointolerant and a large number of *Phormidium* species was found of which *Phormidium keutzingianum* and another *Phormidium* species could tolerate up to 15% salinity. They, however, had no obligate requirement of salinity for growth.

In a hypersaline environment, the evaporation ponds of the saltern in Guerrero Negro, Baja California, Mexico *Phormidium hypolimneticum* was isolated from by Nubel et al. [14] which was related to *Oscillatoria limnetica*. Interestingly, heterocyst formers (data not shown) were absent in our collection which is a characteristics of hypersaline environments. Our isolates can also be termed euryhaline, because they were able to grow with close to optimal rates over wide ranges of salinities. A remarkable ecophysiological uniformity in the salinity tolerance has been observed. Growth of our isolates as biofilms can also offer protection against changing environmental conditions (e.g., temperature, salinity and desiccation). Our isolates differed from the previous authors' strains in being obligately halophilic and tolerant to elevated temperatures

and pH conditions. In addition, our isolates possessed broad-spectrum antimicrobial activity.

TABLE I. MORPHOLOGICAL FEATURES OF ISOLATED CYANOBACTERIA BASED ON LIGHT MICROSCOPY.

Isolates	*Locations*	*Morphotype*	*Cell width (μm)*	*Motility*	*Taxonomic affiliation*
AP-3	Kachuberia2	Straight, cells deeply constricted	1.4-1.6	Yes	*LPP group B*
AP-8	Harinbari	Straight, cells constrictions are missing	1.2-1.4	No	*Phormidium* sp.
AP-9	Rudranagar1	Straight, not separated by constrictions	0.8-1	Yes	*Oscillatoria* sp.
AP-13	Light house1	Straight, not separated by constrictions	0.8-1	No	*Oscillatoria* sp.
AP-16	Mangrove forest near Gangasagar1	Straight, not separated by constrictions	0.8-1	Yes	*Oscillatoria* sp.
AP-17	Mangrove forest near Gangasagar2	Straight, not separated by constrictions	4.8-5	Yes	*Oscillatoria* sp.
AP-20	Kachuberia1	Straight, not separated by deep constrictions	1.4-1.5	No	*LPP group B*
AP-22	Light house2	Straight, shallow constriction	1.4-1.6	No	*Lyptolyngbya* sp.
AP-24	Light house3	Straight, not separated by deep constrictions	0.8-1	No	*LPP group B*
AP-25	Light house4	Straight, not separated by deep constrictions	0.8-1	No	*LPP group B*
AP-40	Rudranagar2	Straight, cells constrictions are missing	1.2-1.4	No	*Phormidium* sp.

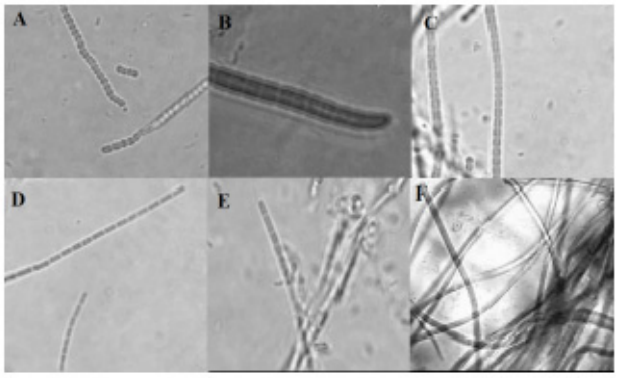

Figure 2. Photomicrographs of filament ornamentation of eight cyanobacteria (A) AP3 (B) AP17 (C) AP20 (D) AP24 (E) AP25 (F) AP40.

Despite marine cyanobacteria being projected as potential producers of novel biologically active molecules [15-18], till date most of the research has largely focused on few marine representatives of the genus *Lyngbya* [19]. In this study we report antimicrobial activity of genera such as *Leptolyngbya*, *Oscillatoria*, *Phormidium* and *LPP group B* which have not been intensively investigated for bioactivity. The few descriptions of bioactive compounds from these genera are briefly described.

We obtained forty cyanobacterial isolates from Sagar Island and this collection was screened for antimicrobial activity. Five from these eleven showed antimicrobial activity against all the test bacteria. The extracts were stored at 4°C for a maximum period of 24 hours before testing of antimicrobial activity. They were found to be active as reproducible results were obtained during the course of the investigation.

In preliminary biological activity screening, the 100% ethyl acetate eluting fraction of the marine filamentous cyanobacterium *Leptolyngbya* sp. collected from Panama was cytotoxic to human lung tumor cells and active against malaria, leishmaniasis and trypanosomal parasites as well. Further purification yielded optically active colorless oil, coibamide A [20]. Bioassay guided fractionation of the lipophilic extract of the marine cyanobacterium *Phormidium ectocarpi* yielded hierridin B and 2, 4-dimethoxy-6-heptadecyl-phenol. The isolate showed antiplasmodial activity towards *Plasmodium falciparum* [21]. An *in vitro* and *in vivo* analyses of a partially purified compound from a marine cyanobacterium *Oscillatoria willei* BDU 130511 on human peripheral blood mononuclear cells (PBMC) and Swiss albino male mice were performed [22]. While the *in vitro* study revealed enhanced count of PBMC up to 72 hours, effect of the extract on animal models by various mechanism based assays showed that the compound was recognized by the immune system of mouse and the immune response was augmented by enhancement of immunoreactive cells. Unicellular marine cyanobacteria *Synechocystis* and *Synechococcus* extracts induced apoptosis in eukaryotic cells and caused inhibition of Gram-positive bacteria. The different activity in different extracts suggested presence of different compounds with different polarities [23].

TABLE II. ANTIMICROBIAL ACTIVITY OF ELEVEN CYANOBACTERIA OBTAINED FROM THE SAGAR ISLAND AS SHOWN BY ZONE OF INHIBITION (IN MM). "-" INDICATES NO ACTIVITY.

	Zone of inhibition in millimetre			
Isolates	*Escherichia coli* (MTCC 739)	*Bacillus subtilis* (MTCC 441)	*Staphylococcus aureus* (MTCC 96)	*Pseudomonas aeruginosa* (MTCC 424)
AP-3	15	12	17	14
AP-8	12	-	13	14

AP-9	-	11	-	13
AP-13	11	-	12	10
AP-16	-	12	-	-
AP-17	13	11	15	12
AP-20	12	14	18	13
AP-22	11	-	-	12
AP-24	14	15	17	12
AP-25	12	15	17	13
AP-40	12	-	-	14

We believe the extreme conditions of the *Sundarbans* create a stress situation in the marine cyanobacteria which results in the production of the antimicrobial compounds. Although the role of bioactive molecules in the producer organism itself is poorly understood but, considering the wide spectrum of biological adaptations and tolerance to environmental stress revealed by cyanobacteria, some of these compounds can be produced in an attempt to confer advantages for their survival [23].

In conclusion, we have highlighted the potential of some lesser-known marine cyanobacterial genera as producers of antimicrobial compounds. We assume the intertidal location of these bacteria necessitates the development of certain adaptation strategies of which production of antimicrobial compounds is one. Therefore, intertidal locations and other poikilotrophic environments may be potential regions for bioprospecting. Some of the biochemical, physiological and taxonomical characteristics showed interesting divergences from the known cyanobacterial characteristics. Further taxonomical investigations and isolation of the active biomolecules is planned for the future.

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