ABSTRACT

Natural environments generally contain trace amounts of nutrients as opposed to high nutrient concentrations in culture media used in bacteriological laboratories. Bacteria, adapted for growth under low-nutrient conditions are termed as oligotrophs. These organisms generally fail to grow on rich nutrient media. Several methods have been innovated to isolate these bacteria from environmental and non-environmental settings. One of the suitable habitats for oligotrophic bacteria is river, which often undergoes nutrient fluctuations due to its dynamicity. In the past few decades the water quality of rivers have extensively deteriorated due to addition of various kinds of pollutants and nutrients through the agencies like sewage, industrial effluents, agricultural runoff etc which brings a series of changes in the physicochemical and other characteristics of water. The present study was basically focused to understand the population dynamics of oligotrophic bacteria over a continued stretch of three years at regular intervals. Also it was imperative to look into the pool of antibiotic-resistance genes present in these bacteria because few studies done earlier have reported that these bacteria were often resistant to different antibiotic(s), and quite often the isolates were found to resist two or more antibiotics called multiple-antibiotic-resistant. With an overall aim to reveal phenotypic and genotypic data of the oligotrophic bacterial population of a city-waste polluted river, Mahananda, of northern West Bengal, India, in the light of genomics of gene cassettes borne by class 1 integrons, five major objectives were set in this study: (I) to provide detailed descriptive information about the nature of antibiotic resistance in culturable oligotrophic bacteria from the water samples of river Mahananda; (II) to study the diversity of the oligotrophic bacteria of Mahananda river; (III) to apply molecular systematics in ascertaining taxonomic status of the isolates; (IV) to explore the incidence of resistance integrons in oligotrophic bacterial population; and (V) to explore the molecular diversity of the antibiotic resistance gene cassettes.

Culturable oligotrophic bacteria and fraction of their population resistant to antibiotics used were enumerated on nutrient-poor-agar (NPA) medium and NPA amended with defined concentration of each antibiotic tested. A new protocol for determination of antibiotic resistance cut-off points in case of oligotrophic bacteria by selecting proper reference strain was developed. For assessing antibiotic sensitivity, five sensitive reference strains, from the facultatively oligotrophic isolates, were chosen for calculating LD_{50} (the dose where half of the bacteria were killed with respect to control devoid of any antibiotic). The break points for antibiotic resistance were set as five times greater of the calculated LD_{50} for resistance determination. A total of ninety composite water samples (10 samples/year/site) were analyzed. The oligotrophic bacterial load of river Mahananda at three sites of sampling (SS I, upstream; SS II, midstream; SS III, downstream; at Siliguri) ranged from 1×10^3 to 5.9×10^4 CFU/mL. From the pool of total oligotrophic (obligate and facultative) bacteria, facultative ones were selected by replica plate method for further study. 76.2% of the total facultatively oligotrophic bacteria isolated from river Mahananda were resistant to one or more than one antibiotics and 23.8% were sensitive to all the antibiotics tested. Within antibiotic-resistant facultative oligotrophic bacterial population, 47% were SAR (single-antibiotic-resistant) and 53% were MAR (Multiple-antibiotic-resistant).

In order to understand the gene cassette diversity associated with class 1 integrons irrespective of their origin, in hosts, be it sensitive, single or multiple antibiotic resistant, a total of 2188 randomly selected facultatively oligotrophic bacterial isolates were examined for the presence of class 1 integrons, using a highly reproducible PCR strategy. Ninety (4.1%) isolates were found to carry class 1 integron, and amongst them 18 (22%) were sensitive to all the twelve antibiotics, 07 (7.8%) were SAR (single-antibiotic-resistant), and 65 (72.2%) were MAR (resistant to two or more antibiotics). The amplified amplicon lengths of variable region varied from 0.15 to 3.45 kb. Amplicon of size ~1.0 kb was predominating and was detected in 24.4% of the total integron positive isolates; however very short sequence of 153 bp were also detected from two isolates, MB62 and MB63 which did not carry any gene cassette within variable region. It was observed that the occurrence of

isolates bearing class 1 integron(s) was highest at sampling site II. The results showed that maximum incidence of class 1 integrons were in the isolates that corresponded to the resistance index (RI) between 0.5-0.9.

Sequence analyses and cassette characterization showed that ~29% isolate carried gene cassettes, bearing ORFs not related to any of the reported antibiotic resistance and ~71% isolates were having the gene cassettes encoding antibiotic resistance. The most common carriages in gene cassettes bearing antibiotic resistance genes were observed for aminoglycoside adenyltransferase gene cassettes such as *aadA, aadA1, aadA2, aadA4,* and *aadA5* conferring resistance to streptomycin/ spectinomycin antibiotics followed by dihydrofolate reductases (type-A: *dfrA1, dfrA5, dfrA7, dfrA12, dfrA16, dfrA17,* and type-B: *dfr-IIe,* a single gene cassette) conferring resistance to trimethoprim. The study revealed two novel dihydrofolate reductase genes, *dfrA28* and *dfrA30.* The *dfrA30* gene was expressed in *E. coli* JM109 showing trimethoprim resistance up to the level 1000 mg/L. Besides antibiotic resistance gene cassettes, a number of gene cassettes (~29% of the total) bearing ORFs coding for unrelated function (not for any antibiotic resistance) were also observed. Bioinformatic analyses were done to characterize these gene cassettes yielding novel information.

Bacterial taxonomy revealed that the Integron-positive isolates largely belonged to two main classes, *Betaproteobacteria* and *Gammaproteobacteria.* Class *Betaproteobacteria* was represented by two isolates belonging to the genus *Comamonas* and *Acidovorax* of the family, *Comamonadaceae.* The representative genera of class *Gammaproteobacteria* were constituted by families, *Moraxellaceae, Pseudomonadaceae, Aeromonadaceae* and *Enterobacteriaceae.* Majority of the identified integron positive oligotrophic bacteria of super class *Gammaproteobacteria* were detected from the family *Enterobacteriaceae.* Following genera comprised by family *Enterobacteriaceae*: *Shigella, Kluyvera, Klebsiella, Salmonella, Citrobacter, Serratia, Enterobacter, Proteus, Providencia* and *Escherichia.* Despite of several known bacterial genera, nine isolates, MB25, MB28, MB41, MB44, MB48, MB54, MB81, MB83 and MB12 could not be placed into any of the known genera. One novel Gram positive bacterium belonging to the genus *Brevibacterium* was finally assigned a status of a novel species, *B. siliguriense* sp. nov. employing polyphasic approach

A preliminary attempt was made to seek alternative strategy for the control of sprawling MAR bacteria. Evaluation of antibacterial property of zinc oxide Quantum dots with surface adsorbed acetate ion (ZnO-Ac) revealed that growth of facultatively oligotrophic, multiple-antibiotic and human serum-resistant *Klebsiella penumoniae* strain MB45 was completely arrested at concentration of 500 mg/L ZnO-Ac QDs.