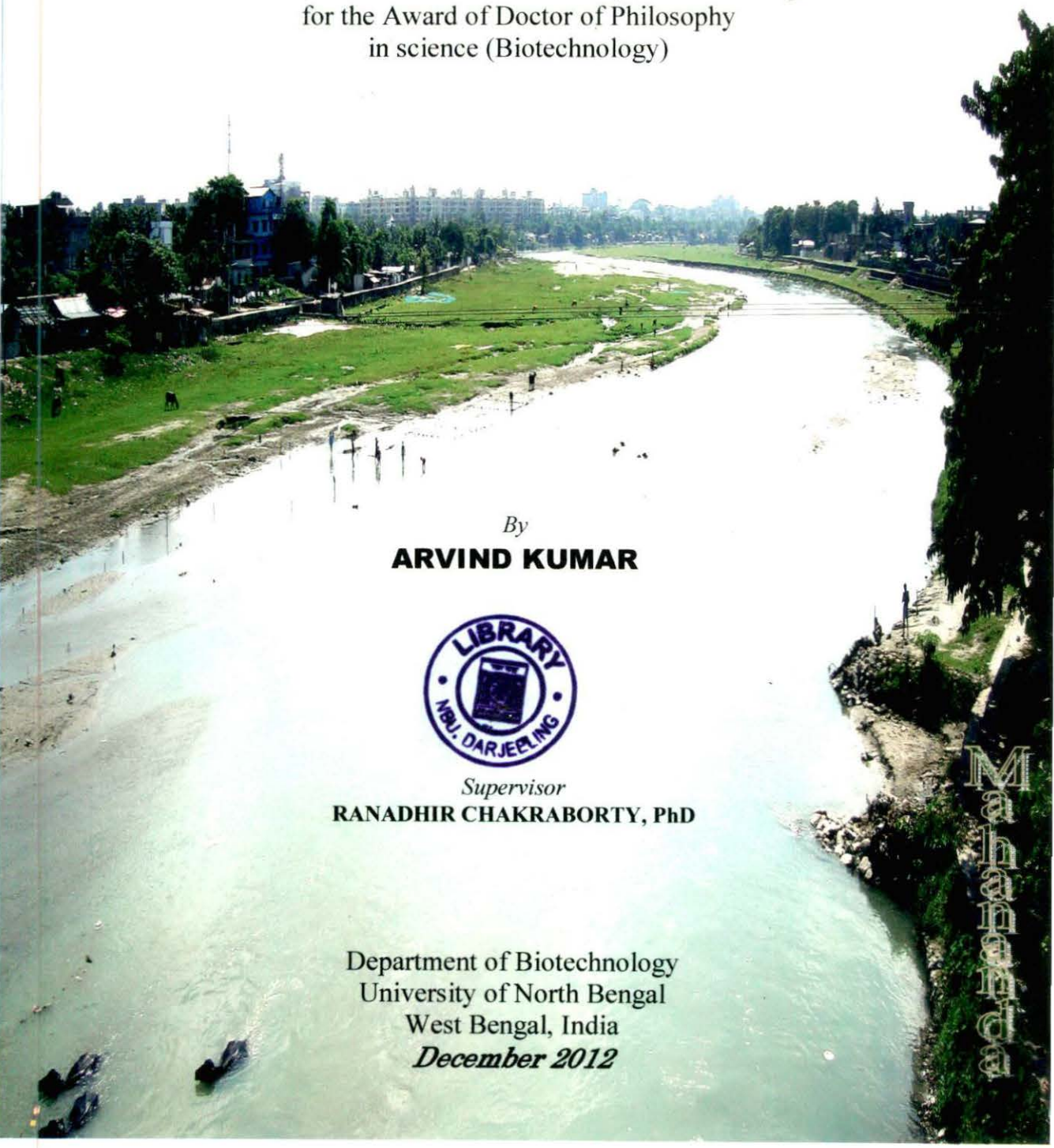
STUDIES ON OLIGOTROPHIC BACTERIA OF RIVER MAHANANDA OF NORTHERN WEST BENGAL WITH SPECIAL EMPHASIS ON GENOMICS OF INTEGRONS

A Thesis submitted to the University of North Bengal
for the Award of Doctor of Philosophy
in science (Biotechnology)



By

ARVIND KUMAR

LIBRARY
NBU, DARJEELING

Supervisor
RANADHIR CHAKRABORTY, PhD

Department of Biotechnology
University of North Bengal
West Bengal, India
December 2012

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"Roshni ki rah, to chaltein hain sabhi.....,
Rah roshan kar chalo, to koi baat hai....."

-unknown

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Arvind Kumar

Arvind Kumar

List of abbreviations

dNTP	2′-deoxynucleoside 5′-triphosphate
X-gal/ BCIG	5-bromo-4-chloro-indolyl-β-D-galactopyranoside
α	Alpha/proportional
ATCC	Americantype culture collection
aac	Aminoglycoside acetyltransferase gene
ARP	Antibiotic resistance profile
~	Approximate
be	base element
bp	base pair
BLAST	Basic Local Alignment Search Tool
β	Beta
β-ME	Beta mercaptoehtanol
B	Boron
Ca	Calcium
C	Carbon
attC	cassette associated attachment site
Cl	Chloride
CLSI	Clinical and Laboratory standards Institute
CFU/ or cfu	Colony forming unit
P_c	common promoter
CDD	Conserved domain database
CS	conserved segment
CS-PCR	Conserved segment-polymerase chain reaction
Cu	Copper
°C	degree celsius
DNA	deoxyribonucleic acid
dfr/DFR	Dihydrofolate reductase
DTT	Dithiothreitol
E. coli/E.C	*Escherichia coli*
EDTA	ethylene-diamine-tetra-acetic acid
≤	equal/and or less
≥	equal/and or greater
EUCAST	European committee on antimicrobial susceptibility testing
EMBL	European molecular biology laboratory
FAME	Fatty acid methyl ester
γ	gamma
GC	Gene cassette
g	gram
h	hour
H	Hydorgen
IMViC	Indole, Methyl red, Voges-Proskauer, Citrate
IS	insertion sequence
IntI	Integrase
attI	Integron associated attachment site
ICNB	International Code for the Nomenclature of Bacteria
Fe	Iron
IPTG	isopropylthio-β-galactoside
KAN	Kanamycin
LD	Lethal dose
L	Liter
LB	Luria Bertani broth/Luria broth
Mg	Magnesium
Mn	Manganese
MP	maximum parsimony
MK	menaquinone (MK)
*m*DAP	*meso*-diaminopimelic acid
µg	microgram
mg	milligram

List of abbreviations: continue...

mL	milliliter
mM	milimolar
MIC	Minimum inhibitory concentration
Mo	Molybdenum
MAR	Multiple-antibiotic-resistant
NP	nano-particle
NCBI	National Center for Biotechnology Information
NJ	neighbor-joining
Ni	Nickel
N	Nitrogen
NB	Nutrient broth
NPB	Nutrient poor broth
NPA	Nutrient-poor-agar
NRA	Nutrient-rich-agar
orf/ORF	open reading frame
O	Oxygen
PG/DPG	phosphatidylglycerol /diphosphatidylglycerol
P	Phosphorus
PCR	Polymerase Chain Reaction
K	Potassium
KCl	Potassium chloride
PAGE	Polyacrylamide gel electrophoresis
PDB	Protein data Bank
QD	Quantum dot
RI	Resistance index
R	Resistant
RNA	Ribonucleic acid
rRNA	ribosomal RNA
SS	Sampling site
Se	Selenium
S	Sensitive
Si	Silicon
SAR	Single-antibiotic-resistant
Na	Sodium
NaCl	Sodium Chloride
SDS	Sodium dodecylsulphate
NaOH	Sodium Hydroxide
SPEC	Spectinomycin
STR	Streptomycin
SI	Super Integron
TOBA	Taxonomic Outline of the Bacteria and Archaea
TLC	Thin-layer chromatography (TLC)
Tm	Transmembrane (Tm)
Tn	transposons
TMP	Trimethoprim
TAE	Tris-acetate-EDTA
TSB	Tryptone soy broth
W	Tungsten
UV	Ultra violet
UPGMA	unweighted pair group method with arithmetic means
V	Vanadium
v/v	Volume/volume
w/v	Weight/volume
Zn	Zinc
ZnO	Zinc oxide

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.

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Oligotrophic Bacteria: A General Introduction

Moderate terrestrial environment (environments with near neutral pH, temperature between 20-40 °C, air pressure 1 atm ; and sufficient water with solutes comprising of nutrients and inorganic salts) is considered most favorable to sustain life except the extreme habitats. However, a variety of microbes survive and grow well in other unusual habitats too, such as deserts and the ocean beds, acidic or hot springs, saline and/or alkaline lakes, glaciers, and very low nutrient milieu (Satyanarayana *et al.*, 2005). Tolerance of these microbes to extreme environments is an intrinsic property that helps to survive; for example, acidophiles show optimum growth at or below pH 3, alkaliphiles demonstrates an optimum growth at pH 9 or above, endolith can live inside rocks, thermophiles can thrive at 60 °C or above, halophiles requires at least 2M NaCl for their growth, psychrophiles grow optimally at 4 °C or below, piezophiles grow in high hydrostatic pressure (very common in deep sea surfaces), xerophiles prefers to grow in dry condition like deserts, oligophiles/ or oligotrophs grow in very low nutrient condition etc. Except in rare instances, nature does not offer bacteria such a verdant life like that when grown under laboratory condition. In the natural environment, there exists sharp competition among the competing bacteria for retrieval of nutrient from available sources. Therefore, the growth of pure cultures under laboratory conditions does not accurately mimic the situation found in nature. Bacteria in less nutrient environment struggles to adapt the fluctuating nutrient availability and thus compromises to modify its growth and tends to remain viable for a longer period of time rather than to perish (Poindexter, 1981; Mortia, 1988). Bacteria termed as 'oligotroph' [term "oligotroph" was introduced in by Weber (1907)] employ novel strategies to cope with circumstances of poor nutrient availability in their milieu and modify their growth according to nutrient conditions. Oligotrophic bacteria can therefore be defined as the "bacteria that grow and multiply in poor nutrient environments using low concentration of organic substrates". The studies of oligotrophic bacteria dates back to1920s; where survival and persistence of bacteria in absence of carbon, energy or other essential growth nutrients was reported by Winslow and Falk (1923). Later on many authors have studied the physiology of oligotrophic bacteria (Postgate and Hunter, 1962; Akagi *et al.*, 1977; Poindexter, 1981; Roszak and Clewell, 1987; Button, 1991; Button, 1993; Mortia, 1997). Earlier studies have described that an oligotrophic bacterium dominating under low nutrient condition possess mechanisms to assimilate substances present in the low-nutrient environment. Such bacteria are ubiquitous in nature and have been reported from diverse sources including clinical samples (Akagi *et al.*, 1977; Mallory *et al.*, 1977; Kuznetsov *et al.*, 1979; Tada *et al.*, 1995; Watve *et al.*, 2000; Nagarkar *et al.*, 2001; Miyake *et al.*, 2003; Pramanik *et al.*, 2003; Katsunori and Masafumi, 2006; Hu *et al.*, 2007; Ishii *et al.*, 2011). Experimentally, the ability of aquatic bacteria to develop at minimal concentration of organic matter was shown by Zobell and Grant (1943), and Jannasch (1967). Nonetheless, it was also found that many bacteria isolated on nutrient-poor media from environmental samples possess the ability either spontaneously or by adaptation, to grow on rich media. The possible explanation of the failure of obligate oligotrophic bacteria to grow on nutrient-rich media is still obscure. One possible reason could be because of their inability to cope up with the toxic products of metabolism e.g hydrogen peroxide. The growth of *Leptothrix pseudoochraceae*, *Siderocapsa eusphaera* and *Metallogenium personatum* was found inhibited due to accumulation of hydrogen peroxide when cultured on nutrient-rich media; when catalase was supplemented to the rich medium it enabled growth of those bacteria (Kuznetsov *et al.*, 1979). In *Pseudomonas oxalaticus* for example, it was found that enzymes involved in active transport of substrate into the cell or respiration) are inhibited in presence of high nutrient concentrations (Dijkhuzen and Harder, 1975; Kuznetsov *et al.*, 1979). Studies have also

shown that the cells grown in low-nutrient medium take up leucine more efficiently at low concentrations than the cells grown under high-nutrient conditions (Yoshinaga, 1990; Yoshinaga and Ishida, 1992). To investigate this physiological adaptation to low-organic nutrient conditions, Maeda *et al.* (2000) examined the protein composition of the bacterial cells by growing them into different organic nutrient concentrations. The induction of certain specific proteins designated as OlgA, -B and –C, were observed under low- organic nutrient conditions. The most significant ecological factor responsible for the development of oligotrophic bacteria is the concentration of dissolved organic substances which is indicator for the heterotrophic bacteria to assess the amount of energy substrates available for its sustenance (Al-Talhi, 2000). The effective substrate uptake system which renders the ability to acquire nutrients from very low nutrient environment is an important characteristic of the oligotrophic bacteria. Thus, oligotrophic bacteria are expected to be equipped with large surface area to volume ratio, high-affinity uptake systems with broad substrate specificities, and an inherent resistance to environmental stresses that provide them to grow, sustain and maintain their structure in low-nutrient environments. Due to potential biotechnological, medical and environmental importance, oligotrophic bacteria merit attention.

I. Nutrient availability for bacterial growth in oligotrophic environment

Growth is integrated phenomena of every live cell to increase in number and biomass; for which energy has to be derived from substrates present in its niche (Morita, 1988; Tranvik, 1988). Besides energy substrates, various elements like C, H, O, P, K, N, S, Ca, Na, Mg and Fe (macro-elements) and the trace elements; Mn, Zn, Mo, Cu, Co, Ni, V, B, Cl, Se, Si, W as well as others growth factors for example vitamins are essential for growth. Minimally these elements are acquired from the environment where bacteria sustain. Lack of one or more of these essential nutrients may wipe out a bacterial species from that environment. Hence, growth of an organism in an environment is self-explanatory that all nutrients were initially present, even if analytical techniques fails to detect the presence of any such component (because of rapid utilization of the component in extremely diluted conditions) (Al-Talhi, 2000). Life of bacteria is often difficult due to scarcity of substrates (which are vital for cellular function) to allow copious growth without any interruptions. Oligotrophic (low nutrient) environments are often created in (i) soil; (ii) marine water; and (iii) fresh water.

Soil

Nature of soil present elsewhere in the earth is not at all uniform and immensely diverse where the organic and inorganic components, that sustains bacterial life in it, are often present in extremely low concentrations (Ohta and Hattori, 1983). Much of the organic matter enters into the soil are utilized by higher living systems. Even in locations around roots of higher plants where there is regular supply of energy yielding substrate, input is often found inadequate to maintain an enormously active microbial community (Barber and Lynch, 1977). Water extract from soils usually contain < 4.2 µg/L of carbo-hydrate (glucose equivalents) and 1.9 µg/L amino acids (Ko and Lockwood, 1967). Hence, soil indisputably is a poor medium in comparison to conventional media, like nutrient agar which contains approximate 4000 mg/L carbon, used for routine bacterial culture. So we may conclude that bacteria growing in nature are more prone to oligotrophic condition than copiotrophic laboratory culture conditions.

Marine Water:

Natural seawater is characterized by extremely low nutrient concentrations, generally around 0.5 mg of carbon per liter that is, far less than conventional bacterial culture media, which range from 1,000-20,000 mg of carbon per liter (Maeda *et al.*, 2000). Oceanic volume, under 200-300 m in depth, the dissolved organic carbon concentration ranges from 0.35 to 70 mg/L and the particulate organic

carbon from 0.3-1.0 mg/L (Martin and Macleod, 1984). Bacteria that thrive in low-nutrient conditions also have mechanism to survive in starved condition (Morita, 1982; Kjelleberg *et al.*, 1987). During starved conditions, physiological and morphological changes occur that induce several characteristic proteins that helps bacteria to adapt in the low nutrient environment (Amy and Morita, 1983; Jouper-Jaan*et al.*, 1986; Morton and Oliver, 1994). It was found that even in the presence of viable bacteria there was no significant variation in the concentration of dissolved organic carbon in marine water over an incubation period of two months (Barber, 1968).

Fresh Water

Fresh water samples from diverse sources and locations like fast flowing rivers or comparatively static lacustrine system or pristine water bodies (both static and dynamic) or distilled water storage systems display a wide spectrum of low nutrient conditions. It was found that the concentration of organic matter from numerous American lakes ranged from 1 to 26 mg of carbon per liter, and averages of 1.36 mg of carbon for suspended organic substance and 15.24 mg of carbon for dissolved organic substance per liter (Birge and Juday, 1927; Birge and Juday, 1934); while Canadian lakes, the lake Opinikon contained approximately 5-10 µg of sucrose and 2-5 µg of glucose per liter (Vallentyne, 1954). Similar ranges of sugars and glucose were also found in oligotrophic lakes, Rudnlake and hypolimnion (Kuznetsov *et al.*, 1979).

II. Oligotroph: Definition

The Bacteria that have an ability to grow in very low nutrient concentration are labeled as oligotrophs, but there is no generally accepted definition of oligotrophic bacteria. Yanagita *et al.* (1978) define oligotrophs, organisms that able to grow at nutrient concentration of 5 mg C/L but not at concentration of 7.5 g C/L. The bacteria from the environmental sample which are able to form colonies when directly plated on media containing organic matter approximate 1-15 mg carbon per liter are termed as oligotroph; in spite of their ability to grow on rich-media at subsequent re-cultivations (Kuznetsov *et al.*, 1979). Other authors have recommended the use of 10 mg or 1 mg C/L in the media for the cultivation criterion specific for oligotrophic bacteria (Akagi and Taga, 1980a; Ishida and Kadota, 1981). However, Poindexter (1981) is of view that oligotrophy is a phenomenon demonstrated by bacteria to survive in ecological niche having a nutrient flux from near zero to a fraction of milligram of carbon per liter per day. Some of the definitions proposed by different authors are given in Table I.I.

Table I.I: Definitions of oligotrophic bacteria by different authors (Schut *et al.*, 1997)

- Oligotrophic micro-organisms are prokaryotic and eukaryotic organisms that are evolutionarily adapted to exploit ecological niches characterized by low substrate concentrations and low energy fluxes. Oligotrophs may develop in rich as well as in poor environments.
- Oligotrophic bacteria are heterotrophic bacteria capable of growth in the presence of organic nutrients equivalent to 16.8 mg/L.
- Bacteria capable of growth on un-amended BWA (agar-solidified Chesapeake Bay water).
- Bacteria which can grow at substrate concentration of less than1 mg C/L.
- Oligocarbophilic bacteria are capable of to grow on media containing only minerals, and they meet their carbon and energy requirements from trace amounts of organic substance found in the air.
- A trophic group of bacteria that can grow only in the presence of minor amount of nutrilites and not in the presence of a large amount.
- Those bacteria that develop at the first cultivation on the media with the minimal content of organic matter of about 1-15 mg C/L and that grow on such media on subsequent re-cultivation through they can grow on richer media.

Table I.I: continue....

- Organism that grow in media containing organic matter at a concentration of 1 mg C/L. Obligate oligotrophs may decrease in number or disappear with the onset of man-made eutrophication, facultative oligotrophs can tolerate or rapidly adapt to the higher concentration of organic substances.
- Oligotrophic bacteria can be conceived of as those whose survival in nature depends on their ability to multiply in habitats of low nutrient fluxes (approaching zero to a fraction of mg C per liter per day.
- Bacteria that can be isolated on a low nutrient medium (unsupplemented Bushnell Haas agar) and that are restricted to growth at low nutrient concentrations.
- Oligotrophs are defined as those organisms known to be able not only to survive but particularly to multiply under conditions of extremely low and often discontinuous supply of nutrients. In other words, organism adapted to low and irregular fluxes of substrates.
- Obligately oligotrophic bacteria are capable of growth in $SF10^{-1}$ (0.2 mg C/L) but not in $SF10^{-1}$(200 mg C/L).
- Obligate oligotroph as an organism which does not grow in rich (200 mg C/L) media, and the facultative oligotroph as an organism grow in not only in poor (0.2 mg C/L) but also in rich media.
- Oligotrophic isolates are defined as bacteria capable of growth on OEMS agar (0.4 mg C/L).
- Oligotrophic bacteria can broadly be defined as organisms that grow on low concentrations of organic substrates. Obligate oligotrophs cannot grow at substrate concentrations above 6 g C/L.

III. Classification of oligotrophic bacteria

Oligotrophic bacteria can be classified broadly in two classes- obligate and facultative. Ishida *et al.* (1986) differentiated facultative oligotrophs from obligate oligotrophs that fails to grow in substrate concentration above 0.3 g C/L. Obligate oligotrophs grow only at low nutrient condition and fail to grow on a richer media while facultative oligotrophs have the flexibility to grow in low as well as on the nutrient-rich media and can enjoy life in both environments. Horowitz *et al.* (1983) used the term 'euryheterotroph' for facultative oligotroph, later on Baxter and Sieburth (1984) replaced the term 'facultative oligotroph' by eurytroph. Kuznetsov *et al.* (1979) divided oligotrophs in four categories (Table I.II). The 16S rDNA probe-based hybridizations with environmental DNA revealed multitude of uncultured organism (Pace, 1997; Hugenholtz *et al.*, 1998). These unculturable bacteria constitute fourth class of non-cultivable organisms (Kuznetsov *et al.*, 1979). The fact that these bacteria could not be cultured may be because of the following reasons: (i) the deficiency of knowledge-base for designing suitable media; (ii) non-availability of knowledge of existing consortia of different kinds of bacterial communities that aid symbiotic association to each other; (iii) existence of bacteria in the form of inert cell or spore with an ability to revert to vegetative form by switching infrequently for short periods to the growing state in a particular ecological niche (Koch, 2001). These uncultivable bacteria may also include the "obligate oligotrophs". The prototypic organisms generally used for studying oligotrophs are *Caulobacter crescentus* and *Arthrobacter* spp. (Poindexter, 1981); but the fact remains that they are 'facultative oligotroph' and not as "obligate oligotroph" as they could grow both in nutrient-poor and nutrient-rich medium. Hattori's (1976) and other workers observed that frequency of obtaining colonies from soil and environmental samples is more in diluted organic nutrient agar medium than the usual rich nutrient medium.

IV. Predicted properties of oligotrophs

Proposition of a model oligotroph with possible attributes on the basis of nutrient uptake and utilization was one of the outcomes of the Dajhelm conference (Hirsch *et al.*, 1979). The predicted properties that a bacterium should possess to be branded as an oligotroph are as follows: (a) having a shape pertaining to high surface per volume ratio (also expected to be small or bear prostheca); (b) possessing an intrinsic preference to utilize metabolic energy for uptake of nutrients during periods of stagnancy of growth; (c) having ability for nutrient uptake which are expressed constitutively; (d)

existence of high affinity, low-specific transport system to facilitate simultaneous uptake of mixed substrate; and (e) having mechanisms of storing nutrients after the uptake.

Table I.II: Categories of oligotrophic Bacteria

Category	Characteristic	Species	References
1.	Demonstrates formation of colonies on sterile-water agar medium but fails to grow when sub-cultured.	Bacteria of unusual morphology.	(Kuznetsov *et al.*, 1979)
2.	Initially could be grown on nutrient-poor media and do not readily grow in rich media, but can be adapted slowly to grow in nutrient-rich media.	Some species from *Pseudomonas, Agrobacterium, Photobacterium, Vibrio, Aeromonas, Flavobacterium, Micrococcus roseus, M. luteus, M. rajahs, Staphylococcus sapro. phyticus, Corynebacterium, Arthrobacter.*	-do-
3.	Isolated and subsequently re-cultivated in special nutrient-poor media.	Species *of Hyphomicrobium, Caulobacter, Microcyclus, Leptothtix, Ochrobium, Metallogenium, Pasteuria* etc.	-do-
4.	Detected in natural water reservoirs, only under electron microscope; could not be cultivated under laboratory conditions.	Prosthecate bacteria; a number of bacteria with gaseous vacuoles.	-do-

The small size of cells was predicted to provide a distinct advantage in terms of grazer dodging (Ostrowski, 2006) and increased efficiency of nutrient uptake. It is also expected that broader specificity in nutrient uptake would be present in oligotrophic bacteria. Button (1998) observed that the ability of organisms to compete for substrates at low concentrations is influenced by the quantities and types of transport proteins and enzymes like different permeases as opposed to conditions when bacteria are exposed to high nutrient growth environments where metabolic cost will not be borne by the cells in expressing enzymes that are not required when nutrients are in abundance. The regulation of biosynthetic routes operating in an oligotroph would be in line with nutrient uptake rates (Poindexter, 1979). Oligotrophs were also expected to have certain savings and therefore predicted to have the ability to store diverse nutrients in reserves (Hirsch *et al.*, 1979). Physiological studies on oligotrophs are still meager to support and validate the above-mentioned predictions.

V. Oligotroph versus Copiotroph

Oligotrophic bacteria have the ability to grow and multiply in extremely nutrient poor environments; often defined to have the ability to grow when carbon flux is 1 to 15 mg soluble carbon per liter while copiotrophic bacteria grow in carbon-rich environments that provide about 1000 mg soluble carbon per liter (Paul and Clark, 1996). In concept, the oligotrophic bacteria survive in a perennially meager environment. Copiotrophs, or eutrophs, are coupled with richer environments and are usually adapted to utilize resources quickly when available. Habitats with continually low levels of nutrients (oligotrophic), of course, are a foremost and important extreme environment. Button (1991) demonstrated that copiotrophic bacteria have high V_{max} and K_m values and adapted to high nutrient environments, while oligotrophic bacteria have high substrate (carbon) affinity as evidenced by the low K_m values of their transport systems, therefore, oligotrophic bacteria have competitive advantages over copiotrophs when substrate is low. To sustain life in a nutritionally robed environment must involve the expression of genes that have evolved over long periods of good and bad environmental conditions. The way of life of oligotrophs, especially facultative oligotrophs are very important to understand the microbial evolution. Although both oligotrophs

and copiotrophs can survive in a low nutrient environment, but only oligotrophs can persist in chronic starvation conditions and, conversely, may not be able to persist for long periods in richer environments. Study of Hu *et al.* (1999) illustrated an inverse relation between soil copiotrophs and oligotrophs in response to carbon availability. It was shown that high carbon availability inhibited oligotrophs in natural soils. Earlier workers (Akagi *et al.*, 1980b; Fry, 1990) have reported that high carbon concentrations were detrimental to the oligotrophs on agar or liquid media. Akagi *et al.* (1980b) showed that the colony number of marine bacteria was maximal at 400 mg peptone-carbon per litre of medium and then decreased significantly as carbon concentration increased. There are no environments that are really exhibit constant nutrient concentration except for a perfect chemostat or turbidostat culture. In nature there are reasonably upswings and downswings in the nutritional state in the immediate environment hence microorganism must have to develop mechanisms to resist, capitalize on, and exploit such bonuses and survive deficits. The term 'oligotroph' is approximately equivalent to the term 'autochthonous' which was introduced by Winogradsky in 1924, it's because of many bacteria inhabiting soil environment grow in sparse and difficultly metabolizable substrates (Koch, 2001). Thus according to the terminology these autochthonous bacteria can be placed in oligotrophic group, and can be differentiated from "copiotrophs", a term introduced by Poindexter (Poindexter, 1978; Poindexter, 1981).

Table I.III: Some possible reasons that, why oligotrophs failed to grow on rich nutrient media and copiotrophs on poor nutrient media.

Possible reasons for oligotrophs to succumb during challenges by too high nutrition*	**Possible reasons for copiotrophs to succumb during low nutrition or starvation***
➢ **OSMOTIC CHALLENGES** **Transport Raises the Internal Osmotic Pressure** • Cell dies by osmotic swelling because solute pumping is too rapid for consumption • Cell, inappropriately, pumps a substrate inward that the cell cannot use. • Collective import of many substances makes the osmotic pressure too high. • Cell pumps a specific substance that harms the cell because growth is blocked by absence of other growth factors • Wall growth is blocked, but not import of nutrients • (Example-Cell forms bulges in presence of low levels of penicillin and ruptures at division site) ➢ **GROWTH IMBALANCE** **Energy depletion of ATP and/or proton motive force** • Too many transportable non-metabolic substances suddenly available **Rigid and slowly-enlarging wall may rupture due to cytoplasm accretion** • Wall enlargement rate may not be adequate to prevent wall rupture (Counter example- *S. mutans* in presence of penicillin type inhibitors) **Cell insertion of inappropriate membrane proteins** • The cytoplasmic membrane has too high protein to lipid ratio not permitting growth • Too many integral membrane protein may weaken membrane and block growth ➢ **TOXIC SUBSTANCE** **Lactose Death** (Example-Galactoside excess is caused in an unknown way) **Killing due to free radicals** • Higher rate of free radicals formation generated from nutrients in the presence of oxidants, such as oxygen, generation **Inappropriate SOS-like response** • DNA synthesis blocked, but cell never recovers	➢ **LACK OF ADEQUATE REGULATORY SYSTEMS** • Cells have lost, or inactivated, their starvation mechanisms • Cells never did evolve starvation protection mechanisms and have no other strategies • Stripped-down cells that have jettisoned protection Mechanisms ➢ **LACK OF ADEQUATE TIME TO ADAPT TO POOR NUTRITION** • Regulatory mechanisms may need adequate time to make protective proteins ➢ **HOLDUP DUE TO LACK OF REPAIR OF DNA DAMAGE DUE TO CELL'S INADEQUATE SUPPLIES OF NUCLEOTIDES** ➢ **SOS SYSTEM FAILS TO FUNCTION AND FAILS TO BLOCK CHROMOSOME REPLICATION AND CELLS DIVISION AFTER DNA DAMAGE HAS TAKEN PLACE** ➢ **UNDER ENERGY STARVATION, SYNTHETIC REACTIONS ACT IN THE REVERSE DIRECTION** ➢ Enzymes (and Protein Synthesis Machinery) Have to Catalyze Reversible Processes and Unless Specifically Blocked, Hydrolysis or Leakage May Occur ➢ **METABOLIC PRODUCTION OR NON-UTILIZATION OF A SUBSTANCE POISONS CELL** • (Example-Galactose-1-phosphate killing in both humans and bacteria is due to metablic imbalance) • Cell depletes a necessary consumable cofactor and cannot grow

Table I.III: Continue.....

Unbalanced syntheses • Blockade of DNA synthesis because failure of chromosome initiation Blockade of cell division because failure of constriction or septum formation **Cell depletes a necessary cofactor for protein synthesis and cannot grow or recover** • (Example-This is one possible explanation of how streptomycin kills, suggested by Robert Harvey, unpublished) **Generation of inactive cells** • (Example-Extension of quiescent cells formation as in low-dilution rate chemostats) **Possible role of a VBNC** of a syndrome or a shut-down cell phenomenon***** • Cell regulatory systems turn down metabolism, but • inappropriately and not easily reversed *That is, either no growth, slow growth, or dying. **Variable but not cultivable. ***Cell limited to motility in its metabolic role.	➢ **COMPETITION WITH OLIGOTROPHS** • In nature, the concentration of certain nutrients may be maintained at quite small levels because the oligotrophs deplete the resource and compete with any copiotroph entering the habitat ➢ **MAINTENANCE COSTS ARE TOO BIG** • Energy for maintenance is needed to repair and possible resynthesize of 'worn out' cellular components. Oligotrophs may have adapted to need little energy for such purposes, while copiotrophs have not. *No growth, slow growth, or dying

[adapted without any modification from Koch A.L. 2001. Oligotrophs versus copiotrophs. *BioEssays* 23: 657-661]

Oligotrophs are characterized both by the ability to grow in low concentrations of substrates, and inability to grow and prosper in environments with high levels of nutrients. Some doable rationales for the oligotrophic and copiotrophic life strategies pointed by Koch (2001) are presented in the table I.III.

VI. Methods for studying oligotrophic bacteria

The effect of low nutrient habitats on bacteria is studied either by *in-situ* detection (direct observation of live bacteria in the environmental samples) or by cultivating environmental bacterial samples in media devoid of any energy source or by cultivating environmental bacterial samples in the sterile media containing the same constituents from where the samples were isolated [like sterile soil (for cultivating the soil bacteria); filtered sea water (for cultivating marine bacteria); and filtered river waters (for cultivating riverine bacteria)]. Due to troublesome operations, there are only few reports of the direct observation of bacteria in soils and rocks (Balkwill *et al.*, 1997). The difficulty in isolating oligotrophs from the environment has been pointed by several authors (Schut *et al.*, 1997a). The difficulties, faced by the experimenter in isolating oligotrophs and subsequently adapt them to the laboratory culture conditions for in-depth studies, are due to several factors like intolerance to high concentrations of nutrient levels in the conventional culture media; or inappropriate growth substrates used for enrichment; or the lack of appropriate growth factors/cofactors like specific microelements and or vitamins; or presence of undetected inhibitory growth substrates or other additives; or incompatibility to grow in close proximity to other cells (in colonies on agar plates); or inability to resist oxidative respiratory burst upon upshift and outgrowth in the sudden nutrient replenishment conditions when fresh nutrients are added during cultural practices; or because of the enrichment of the phage population that perishes the population. However, efforts to cultivate oligotrophs using different methodologies did not fade away and several trouble-shootings were innovated.

Isolation of oligotrophic bacteria by plating method

Oligotrophs mostly have been isolated on several fold diluted traditional media or on agar plates without any organic nutrients. Nutrient-poor media which contained 10 mg of polypeptone and either deionized water or aged sea water was used by Yanagita *et al.* (1978) to isolate oligotrophs. Brain heart infusion esturian salts agar (brain heart infusion 3.7g/L, mineral salts 15g/L) was used

by MacDonell and Hood (1982) to isolate oligotrophic aquatic bacteria. Bushell Haas agar which containing exclusively mineral salts was used by Horowitz *et al.* (1983). Some workers used agar amended with water from natural reservoir supplemented with minimal quantities of mineral nitrogen and phosphorus for cultivating oligotrophic bacteria (Kuznetsov *et al.*, 1979). Diluted (10^{-3} and 10^{-4}) Nutrient broth amended with agar was used by Tada *et al.* (1995) to isolate oligotrophic bacteria from clinical samples. Watve *et al.* (2000) and Nagarkar *et al.* (2001) used Ravan medium (glucose, 5 mg/L; peptone, 5 mg/L; sodium acetate, 5 mg/L; sodium citrate, 5 mg/L; yeast extract, 5 mg/L; sodium pyruvate, 2 mg/L; agarose, 10 g/L) . Reasoner and Gerldreich (1985) introduced R2A medium for cultivating environmental bacteria (oligotrophic) from potable water. Massa *et al.* (1998) compared results obtained from plate count agar (rich nutrient medium) and R2A medium for enumeration of heterotrophic bacteria; and found that bacterial counts on R2A agar were 34.3% greater than the bacterial counts on plate count agar. These results indicate that R2A medium was better than the plate counts agar for cultivating bacteria from natural mineral water. Some workers used 100- 10,000 fold diluted nutrient agar for isolating and cultivating oligotrophic bacteria from different sources (Tada *et al.*, 1995; Ishii *et al.*, 2011). Recently diluted Luria Bertani (LB) borth and diluted Luria broth was used to isolate oligotrophic bacteria (Oh *et al.*, 2009; Kumar *et al.*, 2010, 2011). Hu *et al.* (2007) in their study demonstrated that application of diluted LB produced more colonies representing diverse bacterial communities including the novel ones. Also, some workers used comparatively rich media to isolate facultative oligotroph. F5 agar (1 g/L polypeptone, 0.1 g/L yeast extract) was used for isolating facultative oligotrophic bacteria (Ishida and Kodota, 1981). However, growth on nutrient rich medium do not exclude the oligotrophic organism since facultative oligotrophs have the capability to grow on nutrient-rich as well as on nutrient-poor media. To differentiate colonies of oligotrophs from copiotrophs, replica plating on nutrient-rich and nutrient-poor plates is preferred. The facultative oligotrophic bacterial colonies will appear on the imprints of both nutrient-poor and nutrient-rich agar medium. The colonies of obligate oligotrophs on nutrient-poor master plates will show growth only on nutrient-poor replica plates. The bacterial colonies on nutrient-rich master plates which would show growth only on nutrient-rich replica plates will be differentiated as copiotroph.

Isolation of oligotrophic bacteria using glass fiber filter

A novel method using an indecisive utilizable organic material, devoid of agar but retained a solid surface in form of glass fibers was designed and used for isolation of oligotrophic bacteria (Akagi *et al.*, 1977). In this method annealed plates of pressed glass in sterile Petri dish (9 cm in diameter) filled with 15 mL of nutrient-poor media devoid of agar were placed at the bottom of dish. On the plates sterile membrane filters were placed through which volumes of sea water (0.5- 500 mL) was passed. The system which was devoid of agar (with an uncertain complement of utilizable organic material) retained the advantages of offering a solid surface. The dilute nutrient medium used contained 16.8 mg carbon per liter. The composition of the medium (in charcoal treated sea water) polypeptone,10 mg/L; protease; peptone, 5 mg/L; bacto soytone, 5 mg/L; yeast extract, 5 mg/L; sodium glycolate, 5 mg/L; sodium malate, 5 mg/L; D-mannitol; 5 mg/L; sucrose, 5 mg/L; and ferric citrate, 0.5 mg/L.

Isolation of oligotrophic bacteria using liquid media

Some of the workers used liquid media for the isolating oligotrophic bacteria by diluting bacterial suspension in low nutrient liquid medium followed by incubation at 20 °C for 15-30 days. But the original technique turned to be problematic for recording growth in dilute media. Ishida and Kadota (1981) resolved this problem by labeling substrate with ^{14}C in the medium; and growth was assessed by $^{14}CO_2$ evolution using a scintillation counter. Baxter and Sieburth (1984) detected growth by

epiflourescence microscopy after growing in a series of diluted inorganically supplemented sea water with 0.01 and 0.1 mg/L of glucose at 17 °C for 2 days. Sub culturing onto agar was done from higher dilutions to obtain isolated colonies. HuiXia *et al.* (2007) isolated an oligotrophic bacterium SGB-5 from biological soil crust using KH_2PO_4 0.2 g/L; NaCl 0.2 g/L; $MgSO_4$ 0.1 g/L; $CaCO_3$ 3 g/L; C 10 mg/L (quite glucose 0.025 g); neutral pH.

Isolation of oligotrophic bacteria by enrichment method

VenderKooj and Hijnen (1985) used batch culture enrichment technique to isolate facultative oligotrophs from drinking water. They incubated tap water with added substrates at 15 °C. *Flavobacterium* spp. was isolated from medium with the added starch, while strain 166 and strain S12 were isolated from medium containing 10-20 µg/L and 100 µg/L starch respectively. These isolates grew well on low concentrations of one or more of the substrates used in the enrichment medium. Hence this method may be a good alternative for isolating oligotrophs with specific nutrient requirements.

Isolation of oligotrophic bacteria by extinction dilution method

Extinction dilution method is the most successful isolation technique. The method was often used to obtain strains of *S. alaskensis* (Button *et al.*, 1993; Schut *et al.*, 1993) and *Cycloclasticus oligotrophus* (Button *et al.*, 1993, Wang *et al.*, 1996). In this method the samples (from where it originated, for example seawater) were diluted in the filtered sterile diluents (made from source) until only few bacteria remained in each dilution tube (Button *et al.*, 1993). The lack of additional substrate prevents the possibility of substrate toxicity and removed competition for substrates by less abundant indigenous copiotrophs allowing for long term incubation of potentially pure cultures in the highest dilutions. Long term incubation of these cultures (6-12 months) in the dark and at 5 °C initiated an unknown mechanism that enabled the cells to grow on a rich nutrient medium, i.e a transition from an obligate to a facultatively oligotrophic state (Schut *et al.*, 1993). This changing behavior from one state to another is still blurred, in an experiment conducted with an isolate, RB2256, which demonstrated that the time of exposure and dose of concentration of external nutrient is an imperative factor to determine whether an oligotrophic bacterium would survive on nutrient rich medium or not (Schut *et al.*, 1997b). The transition phase (from oligotrophic to copiotrophic) may involve gradual changes in cellular reaction or cellular composition that provide resistance to cell to protect from osmotic stress induced by the initial uptake of nutrients and/ or the initial oxidative respiratory burst (Ostrowski, 2006). Isolation of previously uncultivated members of the SAR11 clade, as well as novel Gammaproteobacteria representatives from coastal and ocean environments (Rappé *et al.*, 2002; Cho *et al.*, 2004) are some fruitful outcome of this technique.

Isolation of oligotrophic bacteria by micro-encapsulation method

This technique combines encapsulation of cells in gel micro-droplets for massively parallel microbial cultivation under low nutrient flux conditions, followed by flow-cytometry to detect micro droplets containing micro colonies (Zengler *et al.*, 2002). In this method filtered seawater for diluting seawater sample and PBS buffer (pH 7.2) were used for diluting soil samples. The diluted samples (10^7cells/mL) were mixed with preheated agarose (at 40°C) and added into Cell Mix emulsion matrix and emulsified at room temperature. On cooling, the oil-bacterial suspension resulted in the formation of 10^7 gel micro-droplets (GMDs). Microscopic monitoring ensures encapsulation of single cell. Incubation of GMDs (at least 3 h-5 weeks) into respective medium resulted into development of micro colonies that were either sorted individually or by flow cytometry.

Isolation of oligotrophic bacteria by filtration–acclimatization method (FAM)

Hahn *et al.* (2004) developed this technique for isolation and cultivation of bacteria which were unable to cultivate by standard methods. The original method involved two steps; first step was filtration which removed most of the readily cultivable bacteria that overgrow than the slow growers; and second step was acclimatization that provided a slow transition from the low to the high nutrient concentration. In this method sudden exposure to high nutrient concentration is avoided to prevent substrate shock there-by increasing the viability of the oligotrophs (bacteria adapted to low substrate concentration). Neither rich nutrient media nor solid media were used for culturing the isolates. In FAM, bacteria were slowly acclimatized to higher substrate concentrations. By using FAM they isolated many previously uncultured bacteria which belonged to the group *Actinobacteria, Alpha-, Betaproteobacteria, Bacteroidetes,* and *Spirochaeta.*

VII. Ubiquity of oligotrophic bacteria

Several oligotrophic bacterial species from varied genera and sources have been isolated. Most of them were Gram negative bacteria. In few instances oligotrophic isolates were obligate oligotrophic, non-motile, Gram negative and oxidase positive rods (Ishida and Kadota 1981). Characterization of 162 oligotrophic bacteria isolated on low nutrient media from estuarine environment showed that 90% bacteria belonged to the genera, *Alcaligenes, Corynebacterium, Hyphomicrobium, Hyphomonas, Listeria, Nocardia, Pedomicrobium, Planococcus, Sphaerotilus, Streptothrix,* and *Streptomyces* (Mallory *et al.*, 1977). The remaining 10% were unidentified sheathed bacteria. In one thesis available on the internet, there was a mentioning of a work by West and Fry (1989) describing 421 isolates; of them, 94.8% were facultative and 1.9% obligates oligotrophs; the facultative oligotrophic bacteria mostly reported were from the genera *Acinetobacter, Bacillus, Brevibacterium, Corynebacterium, Escherichia, Klebsiella, Salmonella, Acidovorax, Comamonas, Serratia, Providencia, Enterobacter, Micrococcus, Proteus* and *Pseudomonas* (Al-Talhi, 2000). Katsunori and Masafumi (2006) isolated 538 oligotrophic bacteria from hospital tap water, of which 23.6% (108) were *Methylobacterium* followed by *Pseudomonas* (13.2%) and 60% bacteria could not be identified. A number of oligotrophic species, were isolated from various sources, have been compiled as follows: *Spirillum* sp. (freshwater pond), *Pseudomonas* sp.486 (coastal water), *Pseudomonas* sp. R.P 303 (coastal water), *Arthrobacter* spp. (soil), *Caulobacter cresentus* (freshwater, marine water), *Asticcacaulis biproshtecum* (unknown), *Aeromonas* sp. No.6 (Lake Biwa), *Flavobacterium* sp. M1 (Lake Mergozzo, Italy), *Pseudomonas flourescens* (drinking water), *Flabobacterium* sp. S12 (Tap water), *Spirillum* sp. NOX (slow sand filter), *Hypomicrobium* sp T37 (fresh water), *Acinetobacter* sp GO1 (sea water), *Agromonas* sp. (soil, rice roots), *Corynebacterium* sp. MC2 (canal water, UK), *Curtobacterium* sp. CF2 (taff feeder canal, UK), *Pseudomonas flourescens* (spring water, UK), *Bacillus pumilis* WF01 (Lianishen reservoir, UK), *Pseudomonas* sp. WO01 (Lianishen reservoir, UK) [Fry, 1990]. Oligotrophic strains like *Acintobacter johnsonii* MB52, and *Klebsiella pneumoniae* strain MB45 were reported from river water (Kumar *et al.*, 2010, and 2011). A new genus *Agromonas* with oligotrophic species *A. oligotrophica* was established by Ohta and Hattori (1983). The above said acetylene-reducing oligotrophic strain, *A. oligotrophica* was isolated from paddy soil. Similarly a halo- and organo-sensitive oligotrophic bacterium *Sphingomonas oligophenolica* was also isolated from paddy soil near Sendai in Japan (Ohta *et al.*, 2004). Two novel gram-negative oligotrophic strains, *Xanthobacter xylophilus and Ancylobacter abiegnus* were isolated from dystrophic humified waters formed by xylotrophic fungi grown on decaying spruce wood (Zaichikova *et al.*, 2010a, 2010b). Han *et al.* (2012) isolated 200 oligotrophic bacterial strains from rhizospheres of various soil samples in Korea, of them two oligotrophic bacterial strains, *Pseudomonas monteilii* B001 and *Bacillus cereus* C003 were found to provide a broad spectrum of induced systemic resistance to the plants.

Recently a gram-positive facultative oligotrophic stain, *Brevibacterium siliguriense* was isolated from river water sample (Kumar *et al.*, 2012).

VIII. Antibiotic resistance in oligotrophic bacteria

The age of antibiotics is usually traced back to 1928, the year of penicillin discovery by Alexander Fleming. Since then a vast number of antibiotics have been introduced in market for treatment of several fatal and non-fatal diseases. Besides fundamental application in human health, antibiotics (antimicrobials in broad) have also been used for preventing and treating animals and plants infections as well as for promoting growth in cattle farming (McManus *et al.*, 2002; Smith *et al.*, 2002; Singer *et al.*, 2003; Cabello, 2006). It is important to state that several antibiotics are produced by environmental microorganisms (Waksman and Woodruff, 1940) that exerts selective pressure in natural environment for bacteria growing in that habitat (Baya *et al.*, 1986). On the other hand, getting antibiotic resistance genes via Horizontal Gene Transfer (HGT) is another cause of spread of antibiotic-resistance in environmental and clinical bacteria (Davies, 1997). Antibiotic-resistance genes can also evolve under strong antibiotic selective pressure through natural selection (Martinez and Baquero, 2000; Martinez *et al.*, 2007). Additionally, mobile genetic elements like such as integrons, transposons, and plasmids contribute to the spread antibiotic resistance among the environmental bacteria (Recchia and Hall, 1995; Carattoli, 2001; Rowe-Magnus and Mazel, 2002). However, spent antibiotic residues in environment also contribute to a different type of pollution enriching the population of intrinsically resistant bacteria, and consequently leading to the reduction of the susceptible bacterial population. There are reports which illustrates that antibiotics themselves can promote targeted mutations (Cirz *et al.*, 2005; Kaufmann and Hung, 2010; Kohanski *et al.*, 2010; Thi *et al.*, 2011). Recently Li *et al.* (2010) performed a comparative study on impact of antibiotics in bacterial resistance. They collected samples from river surface water from 5 km upstream and 20 km downstream from the discharge point receiving effluent (waste water) from an oxytetracycline production plant. They found that almost all bacteria (97%) from the immediately discharged waste water samples, and downstream water samples, were multidrug-resistant while in upstream water samples, these were less frequent (28%). Evidence suggests that non-pathogenic environmental bacteria constitute the reservoir of antibiotic-resistance genes with potential to be transferred to pathogens (Riesenfeld *et al.*, 2004; Allen *et al.*, 2009; Donato *et al.*, 2010). Whole genome sequence analysis of an oligotrophic, water disinfection resistant bacterium, *Minibacterium massiliensis*, revealed the presence of a unique genomic island that encoded resistance to several antibiotics and heavy-metal ions and metalloids (Audic *et al.*, 2007).

Majority of studies on antibiotic-resistance, knowingly or unknowingly, were concentrated on copiotrophic bacteria. In comparison to copiotroph, little is known about antibiotic resistance in oligotrophic bacteria (Nikitin *et al.*, 1988; Zlatkin *et al.*, 1991; Kimura *et al.*, 1995; Oh *et al.*, 1995; Tada *et al.*, 1995; Riesenfeld *et al.*, 2004; Kumar *et al.*, 2011). Miyake *et al.* (2003) isolated bacteria on diluted nutrient broth agar medium from the sediment of oligotrophic Lake Biwa, where predominance of sulfamethoxazole resistant bacteria was noted. A recent study conducted on oligotrophic bacteria of a pristine cave, Lechuguilla Cave in New Mexico, has revealed that most of the bacteria were resistant to different antibiotics used in their study; even some were multiple-antibiotic-resistant strains resisting up to 14 different antibiotics (Bhullar *et al.*, 2012). Two large plasmids, pREV1 and pREV2 (about 150 and 250 kb, respectively) isolated from an oligotrophic bacterium, *Ancylobacter vacuolatus* , carrying resistance genes for chloramphenicol and trimethoprim in addition to genes coding functions related to oligotrophy have been reported very recently (Zlatkin *et al.*, 2012). Oligotrophic bacteria therefore can be a "potential reservoir of antibiotic resistance genes that can be acquired by pathogens through plasmid transfer".

IX. Recent advances in studies related to oligotrophic bacteria

Several oligotrophic bacteria isolated in recent times were previously unidentified and uncultured. Recent developments in the molecular methods, without the need of cultivation of bacteria enabled the breakthrough in the discovery of specific prokaryotic taxa which were not cultivated or cultured previously (Spring *et al.*, 2000; Handelsman, 2004; Lee *et al.*, 2007; Vaz-Moreira *et al.*, 2011). Epifluorescence microscopy (Porter *et al.*, 1980) and direct viable count (Kogure *et al.*, 1979) had shown that only 0.01 to 0.1% of all the microbial cells from marine environments formed colonies on standard agar plates (Ferguson *et al.*, 1984). Culture independent measurement of microbial diversity which is based on 16S rRNA gene sequencing explained that there is great discrepancy in the data of direct count and plate count methods (Giovannoni *et al.*, 1990; DeLong, 1992; Suzuki *et al.*, 1997). Deininger and Lee (2001) developed an ATP assay for rapid determination of bacteria in potable water. This test was fast and could determine the total bacterial populations in a very short time. The ATP bioluminescence assay allows an estimation of bacterial populations within minutes and it can be applied on-site. The present concern is that many microbial groups are still uncultivated and there is a need to cultivate these uncultivated groups to make possible genome-enabled physiology. This culturing of uncultured bacteria will possibly require new approaches other than standard plating methods (Cho and Giovannoni 2004). Recently a number of novel approaches were applied to cultivate the ones which were previously uncultured microorganisms, for example, high-throughput culturing (HTC) using dilution-to-extinction (Connon *et al.*, 2002), cultivation with a diffusion growth chamber (Kaeberlein *et al.*, 2002), encapsulation of cells in gel microdroplets (Zengler *et al.*, 2002), and modified plating methods (Eilers *et al.*, 2001; Janssen *et al.*, 2002) have enabled to reveal the reality. One significant achievement was the cultivation of members of the SAR11 clade, previously branded as unculturable (Rappé *et al.*, 2002). In addition to SAR11 clade, numerous novel strains belonging to *Proteobacteria*, *Planctomycetes*, *Bacteroidetes*, *Acidobacteria*, and *Verrucomicrobia* were also identified and cultivated in the laboratory. One reason for this success is thought to be the use of growth conditions which closely mimic the chemical composition of natural environments (Connon *et al.*, 2002; Kaeberlein *et al.*, 2002; Zengler *et al.*, 2002). Some of the strains obtained by high-throughput culturing have already been taxonomically classified as novel genera in a novel order or family (Cho and Giovannoni 2004).

The definitions of the oligotroph is polemic and profoundly confusing from several decades (Schut *et al.*, 1997a), it is widely accepted that a general characteristic of oligotrophic bacteria is the ability to grow in low-nutrient (0.5 to 15 mg of C/L) media, irrespective of whether they grow in rich nutrient media or not. Cho and Giovannoni (2004) observed that twelve representative isolates in the oligotrophic marine *Gamma-proteobacteria* (OMG) group grew well in carbon-unamended natural seawater medium and addition of mixed carbons (176 mg of C/L^1) significantly reduced specific growth rates. No isolates grew in the medium containing more than 351 mg of dissolved organic C/L. It implies by definition that obligately oligotrophic bacteria cannot grow at substrate concentrations above 0.3 g of C/L (Ishida *et al.*, 1986) or 6 g of C/L (Fry, 1990). The fact that failure of microorganism to grow in nutrient-rich media upon first cultivation from natural habitats (Button *et al.*, 1993; Schut *et al.*,1993; Schut *et al.*, 1997b) supports obligate oligotrophic character of the isolates obtained from high-throughput culture collection in the OMG group (Cho and Giovannoni, 2004). *Sphingopyxis alaskensis* strain $RB2256^T$, isolated from Resurrection Bay, Alaska (Button *et al.*, 1993; Schut *et al.*, 1993), is defined as a model oligotrophic marine ultra-micro-bacterium because of following reasons: (i) ability to grow in very low nutrient media (<1 mg of C/L) but not in 5 mg of dissolved organic C/L; (ii) ultramicrosize of <0.1 μm^3; (iii) relatively low growth rate (μ = <0.2 h^{-1}); (iv) and high-affinity substrate uptake systems (Button *et al.*, 1993; Schut *et al.*, 1993; Eguchi *et al.*, 1996; Schut *et al.*, 1997; Fegatella *et al.*, 1998; Fegatella and Cavicchioli, 2000; Eguchi *et al.*, 2001;

Ostrowski *et al.*, 2001). However prolong storage at 4 to 5 °C and under laboratory cultural conditions, the strains RB2256^T and AFO1 turned to be facultative oligotrophs (Schut *et al.*, 1993; Schut *et al.*, 1997b; Eguchi *et al.*, 2001). Antibodies for PstS (a periplasmic binding protein required for high affinity uptake of phosphate by marine *Synechoccus* and *Prochlorococcus* during phosphate) were used to demonstrate the seasonal phosphate stress in *Synechoccus* and *Prochlorococcus* populations (Scanlan *et al.*, 1997; Fuller *et al.*, 2005). Though number of recent techniques enabled the scientists to cultivate and understand diverse physiology of the oligotrophic bacteria, still large gap remains to understand completely the nature of the oligotrophic bacteria. Hence there is need of innovating new media and cultivation methodologies to cultivate the uncultivated bacterial species; and to understand their physiologies in detail.

X. Aim of this study

The proposed research was undertaken to excavate data based on phenotypic and genotypic analysis of the oligotrophic bacterial population of a city-waste polluted river of northern West Bengal, India, with special emphasis on the genomics of integrons. As integrons are the dynamic platform for acquiring and disseminating gene cassettes in an ecological niche, predicting structure and functions of the putative ORFs borne by the cassettes will throw possible light of adaptive gene-acquisition phenomenon.

XI. Significance of study

The present study of integrons in oligotrophic bacteria is significant in terms of novelty because such study has never been reported elsewhere (as far as the available literature). Profiling of antibiotic resistance and identification of genes harbored in integrons in oligotrophic bacterial genome will help to reveal the actual reservoirs of resistance-gene pool in the environment. The present study (like previous studies) demonstrated that oligotrophic bacteria required very low nutrient for their survival and could be cultured. Once culturable strains are available, the physiology and gene-expression studies are also possible. Since these bacteria can survive in very low nutrient and can sustain for long periods with extremely stressed conditions in the hospital settings and also on surgical instruments, the study implying the mechanisms to survive are very important to devise novel therapeutic measures. The oligotrophic bacteria are good source of industrially useful enzymes and other substances. As this study has revealed several genes captured within integrons, it has expanded the horizon to study novel ORFs besides those that encode antibiotic resistance genes. The Department of Biotechnology, Government of India, has been acknowledged in all occasions of publications that have resulted from this study.

XII. Objectives undertaken in this study

1. To provide detailed descriptive information about the nature of antibiotic resistance in culturable oligotrophic bacteria from the water samples of river Mahananda
2. To study the diversity of the oligotrophic bacteria of Mahananda river
3. To apply molecular systematics in ascertaining taxonomic status of the isolates
4. To explore the incidence of resistance integrons in oligotrophic bacterial population
5. To explore the molecular diversity of the antibiotic resistance gene cassettes

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Chapter-1

Isolation of oligotrophic bacteria from river Mahananda and determination of antibiotic susceptibility/Resistance pattern of the isolates

1.1. Background

The ability of oligotrophic bacteria to develop at low concentrations of organic substances and the absence of growth of many of them at high concentrations may be ascribed to a number of reasons. The most important peculiarity of oligotrophic bacteria is their ability to rapidly reproduce at the minimal concentrations of organic matter in the medium, which ensures their advantage in competition for the substrate under natural conditions (Rosjak and Cowell, 1987). The reasons behind growth inhibition of the oligotrophic bacteria on the rich nutrient substrates are not clear at present. It may be due to the action of toxic metabolites, in particular hydrogen peroxide, which forms in a number of metabolic reactions. Earlier authors have shown that the oligotrophic bacteria *Leptothrix pseudoochraceae, Siderocapsa eusphaera,* and *Metallogenium personaturn,* upon cultivation on rich nutrient media begin to lyse due to accumulation of sufficient amounts of hydrogen peroxide in the medium (Kuzenetov *et al.*, 1979). It is known that the catalase activity in microorganisms may sharply decrease in the presence of significant concentrations of glucose or on beef extract media (Kuzenetov *et al.*, 1979). Inhibition of growth in some oligotrophs may also occur in the presence of insignificant quantities of amino acids (Koch, 2001).

Oligotrophic bacteria have been shown to be abundant in a wide variety of habitats. They have been isolated from soil (Hattori, 1984), rivers (Yanagita *et al.*, 1978; Kumar *et al.*, 2010; Kumar *et al.*, 2011; Kumar *et al.*, 2012), lakes (Bahr *et al.*, 1996), oceans (Connon and Giovannoni, 2002; Kuzenetov *et al.*, 1979; Martin and Macleod, 1984, and Yanagita *et al.*, 1978) tap water (Ajitkumar *et al.*, 2003; Baida *et al.*, 2001) and ultra pure water lacking organic substances (Audic *et al.*, 2007). Some oligotrophic isolates can even grow in distilled water (Alexander *et al.*, 2005). Oligotrophic bacteria mostly have been isolated on several fold diluted traditional media or on agar plates without supplementing any extra organic nutrients. However, growth on nutrient rich medium do not exclude the oligotrophic organism since facultative oligotrophs have the capability to grow on nutrient-rich as well as on nutrient-poor media. To differentiate colonies of oligotrophs from copiotrophs, replica plating on nutrient-rich and nutrient-poor plates is preferred. The facultative oligotrophic bacterial colonies will appear on the imprints of both nutrient-poor and nutrient-rich agar medium. The colonies of obligate oligotrophs on nutrient-poor master plates will show growth only on nutrient-poor replica plates. The bacterial colonies on nutrient-rich master plates which would show growth only on nutrient-rich replica plates will be differentiated as copiotroph. The oligotrophic bacteria have received little attention and less is known about them. The diversity and biomass of the oligotrophic bacteria are dominant in biosphere, and thus, play an important role in biogeochemical cycles. The applied perspectives of oligotrophic bacteria in environmental science are gaining importance day by day. A previous study reported the role of oligotrophic bacteria in detection of heavy metals from river water samples by simple turbidimetric method (Tada *et al.*, 2001).

There is scarcity of information on antibiotic resistance in oligotrophic bacteria (Nikitin *et al.*, 1988;, Zlatkin *et al.*, 1991; Oh *et al.*, 2009; Kumar *et al.*, 2011; Bhullar *et al.*, 2012). An antibiotic is a compound or substance produced by micro-organisms that either kill or inhibit the growth of other micro-organisms i.e. by microorganism for microorganism (Levy, 1992). The term "antibiotic" is used to refer to a drug that cures infections caused by bacteria, whereas an antimicrobial agent is a

general term that refers to a group of drugs that includes antibiotics, antifungals, antiprotozoals, and antivirals. Antimicrobial agents may be naturally-occurring, semi-synthetic or synthetic compounds with antimicrobial activity that can be administered orally, parenterally or topically. They are widely used as medicine to treat and prevent infectious disease, and also for growth promotion in food animals (Phillip *et al.*, 2004). The first antibiotic, penicillin, was discovered by Noble laureate, Scottish bacteriologist, Alexander Fleming in 1928 when he observed that a common mold (*Penicillium notatum*) produced a substance that destroyed *Staphylococcus* bacteria in culture (Ligon, 2004).

In 1939 René Dubos, an American microbiologist isolated antibiotic tyrothricin from a soil bacterium, *Bacillus brevis* that was highly toxic to a broad range of bacteria as well as to red blood and reproductive cells in humans. This said antibiotic was more effective when applied topically. The first major development came up when penicillin introduced as ampicillin, this offered a broad spectrum activity than the original penicillins. Later on several new antibiotics with novel properties were discovered, including streptomycin, chloramphenicol, and tetracycline. Within the 18 years of the antibiotic era, approximate 30 antimicrobial agents come up into use (Swartz, 2000). In the following decades the discovery of manufactured antibiotics to control diseases revolutionized the medicine world. It has also greatly reduced the threat of many lethal diseases. The use of these marvelous drugs led to a dramatic drop in deaths from diseases that were previously widespread, untreatable and frequently fatal. Along with control of many infectious diseases, these drugs have also contributed to the major gains in life expectancy experienced during the latter part of the last century. These achievements are now seriously jeopardized by another recent development: the emergence and spread of resistant bacteria. Antibiotic resistance has been called one of the world's most pressing public health problems as it creates complications due to the propensity to distribute multiple antimicrobial resistance genes to susceptible bacterial genera and species. Rivers are important sources of drinking water for human and animals, irrigation, fishery and energy production. The quality of water is described by its physical, chemical and microbiological characteristics (Rajeshwari and Saraswathi, 2009). It is well known that rivers are used as the dumping grounds for the sewage of urban effluents, agricultural wastes, and industrial wastes that contain substances varying from simple nutrients to highly toxic chemicals (including heavy metals). The obvious consequence therefore would be that the river water received different types of chemicals, organic and inorganic compounds as it flowed through human settlements. These dissolved compounds changes the river water quality by inducing quantitative variation in certain minerals. In addition to heavy metals, the contamination of antimicrobial agents in river water bodies has become a major threat to public health. The presence of antibiotic residues and the occurrence of bacteria resistant to them in environment are swiftly changing the nature of commensal and nonclinical bacterial flora. Moreover, investigations on antimicrobial resistance of river microflora have led to a new dimension in water pollution studies. Several studies have demonstrated the wide spread occurrence of antibiotic resistant bacteria in many rivers and streams (Ash *et al.*, 2002). Antibiotic resistance is a property of bacteria being able to survive exposure to antibiotic to which they were once sensitive. Antibiotic resistance is as ancient as antibiotics, shielding antibiotic-producing organisms from their own products, and other originally susceptible organisms from their competitive assault in nature. Resistance may occur as a spontaneous, genetic mutation or due to acquisition of genetic elements like plasmids, transposons, integrons, or gene cassettes (Muto *et al.*, 2003) (Fig. 1.1). However, there is great variation in the development of resistance, some may develop rapid resistance in the individual treated, and others may remain susceptible to the exposed drug/antibiotic. Thus antibiotic resistance can be defined as a microbiological phenomenon, which may or may not have health/clinical implications depending on pharmacokinetic and pharmaco-dynamic parameters as they apply to specific antibiotics. Nevertheless, even low-level resistance is noteworthy since it may be a first sign towards bacterial

resistance (Phillip *et al.*, 2004). Since all bacteria do not possess/ or share same biochemical and physiological pathways hence, all antibiotics are not active against all bacteria and they are intrinsically resistant (resistance without chromosomal mutation or acquisition of plasmid bearing resistance factor) to one or more antibiotics. Inherent features of the bacterial cell prevent antimicrobial action, and these properties are typically species characteristics for example; Gram positive *Mycobacteria* produce an unusual bilayer outside the peptidoglycan layer that function as an efficient barrier (Nikaido, 1994) while the acquired resistance emerges through mutation of existing DNA or acquisition of new DNA by horizontal gene transfer (Thomas and Nielsen, 2005).

The present era of bacterial resistance recognized by multiple antibiotic resistant (MAR) bacterial (bacteria resistant to two or more antibiotics) species. The incidence of MAR bacteria in the environment is undeniably a well-known phenomenon (Cook, 1975; Sizemore and Colwell, 1977; Gonzal *et al.*, 1979). Report of UK House of Lords stated that "Resistance to antibiotics and other anti-infective agents constitutes a major threat to public health and ought to be recognized as such more widely than it is at present" (Kummerer, 2004). The percent occurrence of MAR bacteria in different environmental compartments for example wastewater, surface water, ground water, sediments and soils, has been a growing concern. The antibiotic contamination is the major issue for both medical and environmental components. Antibiotic resistant bacteria were detected in drinking water as early as the 1980s (Armstrong *et al.*, 1981) and later in the 1990s (Kolwzan *et al.*, 1991). They found that the percentage MAR bacteria considerably higher among isolates from treated water samples than that of bacteria in corresponding untreated source waters (Armstrong *et al.*, 1981). MAR gram-positive cocci (Staphylococcus) and MAR gram-negative, nonfermentative rods (*Pseudomonas*, *Alcaligenes*, Moraxella-like group M, and *Acinetobacter*) were dominant in drinking waters. Diab *et al.* (2000) reported the presence of antibiotic resistant gram-negative bacteria in several drinking water samples in Islamia city of Pakistan. In addition to these increased rates of bacterial resistance were also noted in the drinking water from different sampling points by Schwartz *et al.* (2003).

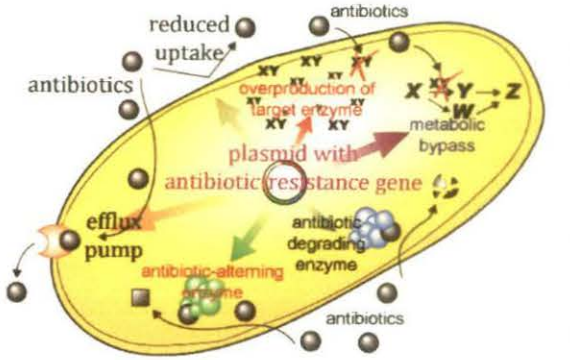

Fig. 1.1: Various mechanism of antibiotic resistance in bacteria. [Fig. adapted from Wicox S. CATIONIC PEPTIDES: A NEW HOPE. The Science Creative *Quarterly.* 2004]

Antibiotics are rarely found in ground water (Kummerer, 2004). However few workers reported antibiotic resistance in bacteria isolated from ground water (McKeon *et al.*, 1995). In India microbial contamination of ground water sources, revealed the presence of coliforms above the acceptable limits, but no attempts have been made to assess the antibiotic resistance profile of the isolates (Dayal, 1992; Sharma and Mathur, 1994; Mitra and Gupta, 1997). Presence of antibiotic resistant bacteria in the aquatic environment has been studied worldwide. The antibiotic resistances in gram-negative bacteria isolated from four tributaries which enter to Tillamook Bay, Oregon and the bay itself have been studied by Kelch and Lee (1978). Distribution of antimicrobial resistance and the pattern of resistance among fecal coliforms in sewage, surface waters and sea water were investigated by Niemi *et al.* (1983). Several other workers demonstrated the wide spread occurrence of such organisms in many rivers and streams (Jones 1986, Sokari *et al.*,. 1988, Magee and Quinn, 1991; Leff *et al.*, 1993; Ogan and Nwiika, 1993). The result of Polluted water samples collected from the River Tigris revealed a high incidence of antibiotic resistant bacteria in natural waters that could be related to the widespread use of antibiotics in that locality (Al-Jebouri, 1985).

Boon and Cattanach (1999) studied the antibiotic resistance of native and faecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, southeastern Australia. The occurrence of several representatives from the main group of antibiotics in wastewater treatment plant effluents and in river water was investigated by Hirsch *et al.* (1999). The study conducted by McArthur and Tuckfield (2000) have demonstrated the spatial distribution of antibiotic resistance in natural bacterial communities of two streams. The proportion of resistant bacteria was substantially higher in the mid reaches of an industrially perturbed stream but no such pattern was apparent in an undisturbed reference stream. The results of the said study implied that heavy metal pollution might contribute to increased antibiotic resistance through indirect selection.

According to a report (1999-2000) of the toxic substances hydrology program at the U. S. Geological Survey (USGS), antibiotics were found to be present in many fresh water sources throughout the United States. Four or five different antibiotic residues, out of 22 antibiotics assayed, were present in 139 water samples collected from different streams and rivers. The frequently detected antibiotics were erythromycin-H_2O (22%), lincomycin (19%), trimethoprim (27%) and sulfamethoxazole (19%). The other nine antibiotics detected were: tetracycline, chlortetracycline, oxytetracycline, ciprofloxacin, norfloxacin, roxithromycin, sulfadimethoxine, sulfamethazine and sulfamethizole (Kolpin *et al.,* 2002). It was found that antibiotics used in livestock production have made their way, via animal waste products, into the nation's waterways. Studies performed on 16 United State rivers revealed that rivers turning into the reservoirs of antibiotic resistance genes (Ash *et al.,* 2002). Antimicrobial resistance has also been reported in marine and estuarine bacteria (Cohen *et al.,* 1986; Barkay *et al.,* 1995). Microbiological analyses of coastal waters polluted with sewage showed the presence of gentamicin resistance genes in the members of *Enterobacteriaceae, Acinetobacter* spp., *Pseudomonas* spp. as well as in phylogenetically distant bacterial members of alpha and beta proteobacteria (Heuer *et al.,* 2002). The occurrence of strains that are resistant to oxolinic acid, oxytetracycline, sulfamethoxazole-trimethoprim and nitrofurantoin among heterotrophic bacteria, including human and fish pathogens, in two fresh water eel farms has been reported (Alcaide *et al.,* 2005). A study on an Indian River Mahananda has also revealed the abundance of MAR bacteria (Mukherjee *et al.,* 2005, Kumar *et al.,* 2010). Results of previous studies have shown that river waters are the main receptacle for antibiotic resistant bacteria, since they receive the sewage of urban effluents and the rivers all over the world have started becoming the reservoirs of antibiotic resistance genes to serve as media for the spread of antibiotic resistance genes (Biyela *et al.,* 2004; Kummerer, 2004; Mukherjee *et al.,* 2005).

River Mahananda is a trans-boundary river that flows through Indian states of West Bengal and Bihar; and Bangladesh. This originates from Himalayas, Mahaldiram Hill near Chimli an eastern part of Kurseong in district Darjeeling of West Bengal at an elevation of 2,100 meters. 11,530 sq. km out of 20,600 sq. Km (total drainage area of the Mahananda river) lies in India (Jain *et al.,* 2011; www.siligurionline.com/info/rivers). Siliguri and Malda are the two important towns situated alongside the Mahananda River. River Mahananda is the prinicipal river flowing through Siliguri. Siliguri, the fastest growing city of West Bengal, with a population of over 5, 00,000, is situated in the foothills of Himalayas on the banks of the River Mahananda. The city with forty seven municipal wards is a serious demographic spot where magnitude of water supply and sanitation problem has reached a critical state in the background of depleting ground water resources and environmental degradation. A huge quantity of community waste water and other kinds of wastes including animal wastes from disorganized cattle farms situated by the side of the river and hospital wastes pass through the estimated 250 km long open drains and ultimately discharged into the main Mahananda River (Fig. 1.2). The unregulated use of antibiotics including therapeutic and prophylactic prescribing in Siliguri was studied indirectly from a random survey conducted on retail medicine sellers at their counters (Mukherjee *et al.,* 2005). The results revealed the presence of fairly high

1.2.3. Study design

Sequence of the microbiological analysis of the river-water samples has been shown in Fig 1.4

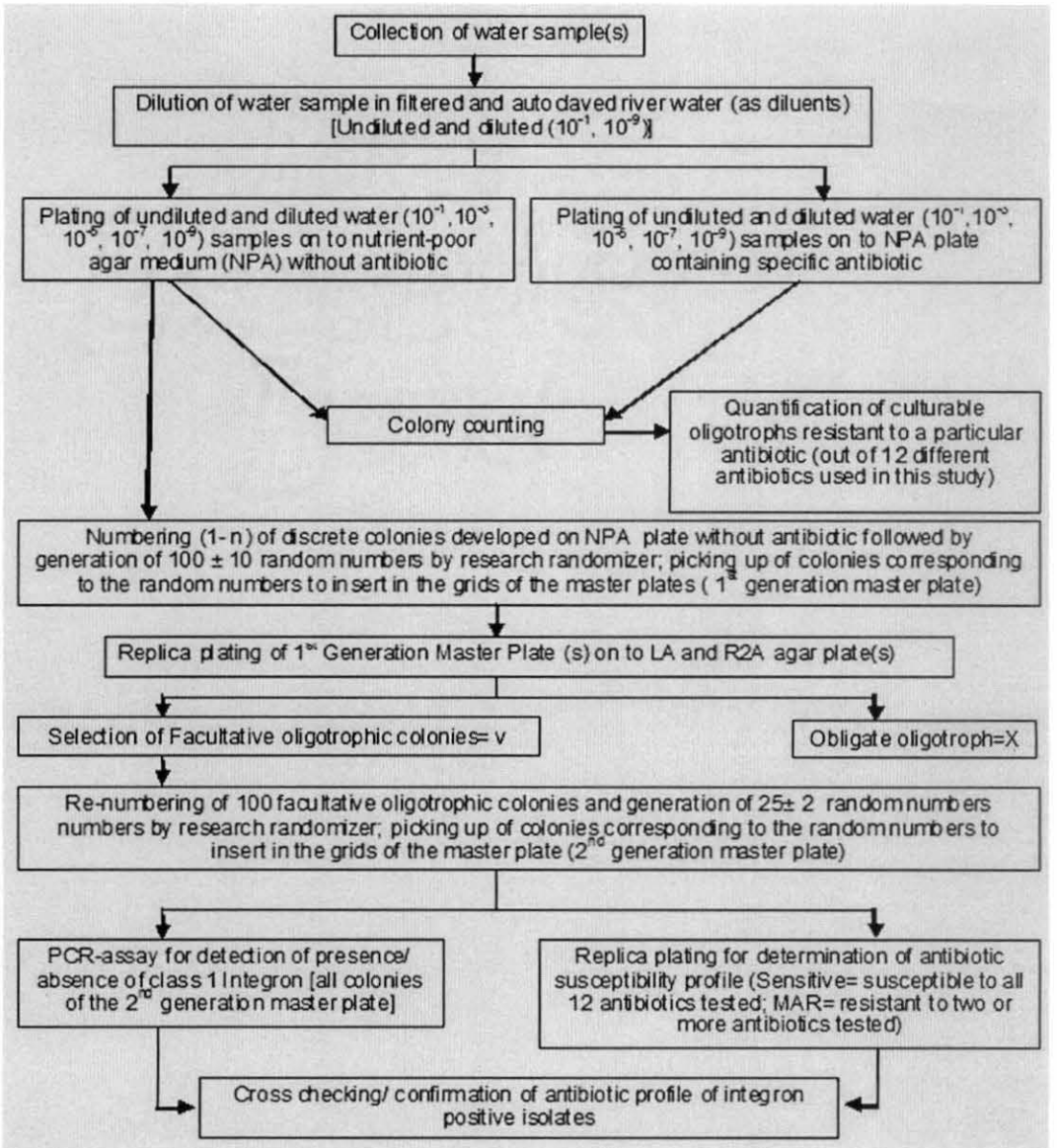

Fig 1.4: Flow diagram describing the sequence of the microbiological analysis of the river-water samples

1.2.4. Sample collection

Samples were obtained either by filling bottles from the river's edge, by wading, or by lowering a sterile glass bottles (lid opened at the time of sampling) from the bridge, depending on the conditions. In case of collecting water samples from the mid-point of the river at all locations, care was taken to obtain the samples following standard technique. From each sampling site, three grab samples were collected from left, right and middle of the river. Sterilized glass bottles (capacity, 500 ml) were used. The bottles were opened under water, rinsed thoroughly with the sample water even it was pre-cleaned and were half filled by opening and closing the bottles underneath flowing water. The samples were transported to the laboratory in icebox and analyses were done within 24 hours.

1.2.5. Preparation of sample and dilution series

Three samples, collected from middle and both banks of a particular sampling site, were mixed in equal proportion under aseptic condition to constitute a composite sample. Each composite water sample was distributed into two sterile containers; one of which was used for microbiological analysis and the other was filtered through 0.2 µm membrane filter. 9.0 mL aliquots of the filtered

composite water in culture tubes were autoclaved at 121 psi for 15 minutes. Sterilized 9 mL blanks were serially diluted (10^{-1} to 10^{-9}) where 1.0 mL of the crude composite water was transferred aseptically to first tube (designated as 10^{-1} dilution) containing 9 mL of filtered-autoclaved river water.

1.2.6. Preliminary isolation of antibiotic sensitive facultative oligotrophic bacterial strains from Mahananda river water and standardization of the protocols for determining antibiotic susceptibility/ resistance patterns of oligotrophic bacteria

Prior to the detailed analyses with water samples collected on monthly basis, a pilot experiment was run to isolate oligotrophic bacteria from a test water sample collected from river Mahananda. The diluted water samples were spread-plated on nutrient-poor agar (NPA) medium [Nutrient-poor broth (NPB) composition (g/L): peptone, 0.01; yeast extract, 0.005; sodium chloride, 0.005 amended with 1.5% agar; pH, 7.0 designated as NPA].

Discrete colonies on NPA plates were numbered serially (1, 2, 3 . . . n). Random numbers comprising a set of n/4 unique numbers were generated using Research Randomizer software (www.randomizer.org). Colonies corresponding to the numbers from the set of unique numbers were picked up with sterilized toothpicks and imprinted on the master plate made of R2A agar (a standard medium, has been used by several workers to isolate oligotrophic bacteria from the environmental samples) [R2A agar composition (g/L): peptone, 0.5; yeast extract, 0.5; casamino acids, 0.5; glucose, 0.5; soluble starch, 0.5; sodium pyruvate, 0.3; K_2HPO_4, 0.3; $MgSO_4.7H_2O$, 0.05; agar 15; pH, 7.2 pH was adjusted with crystalline K_2HPO_4 or KH_2PO_4] . Each master plate was replicated separately on nutrient-rich agar (NRA) [NRA composition (g/L): peptone, 10; yeast extract, 5; sodium chloride, 5 amended with 15 agar; pH, 7.0], NPA and R2A agar. After incubation of these plates for 72 h at 30 °C, colonies that had grown on NPA and R2A agar but not on NRA were termed as "obligate oligotrophs," whereas colonies that were able to grow on all three different plates were termed as "facultative oligotrophs."

Selection of antibiotic-sensitive oligotrophic isolates and determination of LD50 of each of 12 antibiotics used in this study

The master plates constructed with oligotrophic strains were replicated on antibiotic plates. For each antibiotic, three plates were used. The concentration of antibiotics in the plates was 10, 25, and 50 mg/L, respectively. The plates were incubated for 96 h at 30 °C. Colonies that failed to appear in all 12 different antibiotic plates of 25 mg/L concentration were tentatively chosen as sensitive isolates (n=5). For determination of LD_{50} (LD_{50} of each antibiotic is the dose required to kill half the members of the tested bacterial population), a duplicate set of 16 culture tubes each containing sterile 5 ml R2A broth was amended with 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 mg/L of antibiotic, respectively. Each tube was inoculated with pre-grown sensitive bacterial culture to an initial cell density of 10^6 CFU/mL. After 72 hr of incubation at 30 °C, optical density was recorded at 540 nm. The particular tube (with defined antibiotic concentration) wherein OD_{540} was recorded to be half the OD value of the tube without the antibiotic was taken as LD_{50}.

Antibiotic Resistance determination

Obligate oligotrophic isolates were tested for susceptibility (abbreviation: S=sensitive, R=resistant) to a panel of 12 antibiotics, representing 7 different classes. The concentrations of the antibiotics were chosen as five times the calculated LD_{50} values. Antibiotics and their concentrations employed in this investigation were as follows: aminoglycosides (azithromycin, <S/R≥ 5 mg/L; kanamycin, <S/R≥ 5 mg/L; netilmicin, <S/R≥ 3.75 mg/L; and streptomycin, <S/R≥ 2.5 mg/L); antifolates (trimethoprim, <S/R≥ 5 mg/L; co-trimoxazole, <S/R≥ 15 mg/L); cephalosporins (cefepime, <S/R≥ 7.5 mg/L; cefotaxime, <S/R≥ 7.5 mg/L); penicillin (ampicillin, <S/R≥ 25 mg/L); quinolones (ciprofloxacin, <S/R≥ 5.0 mg/L; livofloxacin, <S/R≥ 5.0 mg/L); others (chloramphenicol, <S/R≥

30mg/L; oxytetracycline, <S/R≥ 15mg/L). R2A agar was used as the basal medium. The desired concentrations of the antibiotics were stirred into the melted agar at approximately 45 °C and immediately poured into Petri plates to minimize the exposure of elevated temperatures.

On the other side, sensitivity and resistance of facultative oligotrophic isolates were also determined for above antibiotics following agar dilution method using Mueller Hinton (MH) agar as described in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) definitive document, E. Def 3.1 (2000) [http://www.escmid.org/fileadmin/src/media/PDFs]. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were taken as the quality control strains. All antibiotics were purchased from HiMedia (Mumbai, India). Antibiotic stock solutions were prepared by dissolving measured amounts of respective antibiotics to its suitable diluents. These concentrated stock solutions were made at least once a month and were stored at
-20 °C.

1.2.7. Detailed microbiological analysis of the water samples collected from three sampling site for three consecutive years

1.2.7.1. Routine Enumeration of oligotrophic bacteria on nutrient-poor agar (NPA) medium and determination of fractions resistant to different antibiotics

0.1 mL of water sample [undiluted and diluted water (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9})] was spread uniformly on NPA and plates were incubated at 30°C for 72 h. Total oligotrophic bacterial population within water sample, was quantified by counting bacterial colony forming units (CFUs) appeared on NPA medium.

Side-by-side an aliquot of 0.1 mL of bacterial suspensions [undiluted and diluted (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9})] of the same water sample were spread uniformly on NPA containing specific concentration of each antibiotic [aminoglycosides (azithromycin, 5 mg/L; kanamycin, 5 mg/L; netilmicin, 3.75 mg/L; and streptomycin, 2.5 mg/L); antifolates (trimethoprim, 5 mg/L; co-trimoxazole, 15 mg/L); cephalosporins (cefepime, 7.5 mg/L; cefotaxime, 7.5 mg/L); penicillin (ampicillin, 25 mg/L); quinolones (ciprofloxacin, 5.0 mg/L; livofloxacin, 5.0 mg/L); others (chloramphenicol, 30 mg/L; oxytetracycline, 15 mg/L)] to quantify total resistant oligotrophic population within water sample. The plates were incubated at 30 °C for 72 h. The bacterial colonies, appeared on NPA containing specific antibiotic, were counted. The fractions of resistant oligotrophic bacterial population were quantified against bacteria developed on NPA without antibiotic.

1.2.7.2. Screening of facultative oligotrophs from the population of oligotrophic bacteria (obligate + facultative oligotrophic bacteria)

Discrete colonies evident on NPA plates (plated with 0.1 mL water samples of different dilutions) were numbered serially (1, 2, 3...n) at the backside of the petri-plates corresponding to the colonies that manifested on the surface of solid medium. Approximately, 100 ±10 random numbers (out of > 300 discrete colonies) were generated using research randomizer tool (www.researchrandomizer.com). Colonies corresponding to the random numbers were then picked up with the help of sterile tooth pick and transferred to respective grids of the first generation master plate composed of R2A agar. The first generation master plates were then replicated separately on NRA, NPA and R2A agar plates and incubated for 72 h at 30 °C. Isolates showing growth on NRA, NPA and R2A agar plates were considered as facultative oligotrophs while colonies showing no growth on NRA but significant growth on NPA and R2A agar plates were considered as obligate oligotrophs. The obligate oligotrophs were not included in this study due to its slow growth and ambiguity in forming colonies. Facultatitve oligotrophic bacterial colonies identified from the master plates were further renumbered and randomized to obtain 25±1 random numbers. The colonies corresponding to each of the 25±1 random numbers were dilution streaked onto R2A agar

plate to get the pure cultures of the isolates. The second generation master plate was prepared on R2A agar with pure cultures of facultatively oligotrophic isolates. All the isolates of second generation master plates were subjected to CS-PCR assay for detection of class 1 integron (will be discussed in next chapter).

1.2.7.3. Determination of antibiotic susceptibility/ resistance of facultatively oligotrophic bacteria

Replica plating method was employed for determining the antibiotic resistance profile of individual isolates. The second generation master plate, prepared with pure cultures of facultatively oligotrophic isolates, was replica plated onto the R2A plate containing antibiotic of defined concentration of each antibiotic as mentioned above. Last impression of 2nd generation master plate was done onto R2A agar plate containing no antibiotic to confirm successful imprinting. All the replicated plates were incubated at 30 °C for 72 hours and drug resistance was determined.

The isolates were considered resistant to antibiotic, if their growth on antibiotic containing plate were as good as on the control plate (agar plate having no antibiotic). The isolates were considered MAR (multiple antibiotic resistant) if growth on at least two different antibiotic containing plates was equal to that on the control plate. The isolates which have failed to grow in all the twelve antibiotic plates but have shown growth only on the control plate were considered to be sensitive.

1.3. Results:

1.3.1. Data analyses of total oligotrophic bacteria enumerated on NPA and NPA amended with specific antibiotic used in this study

1.3.1.1. Enumeration of oligotrophic bacterial load in water samples collected from three sampling sites in three consecutive years (2007-2009) from river Mahananda and determination of oligotrophic bacterial fractions resistant to each antibiotic tested

Ninety water samples were collected from three sampling sites in three consecutive years (2007-2009) and studied on monthly basis. The density of oligotrophic bacteria in water samples collected every month (except July and August) from three sampling sites are shown in Table 1.1

Table 1.1: Oligotrophic bacterial density [CFU/mL ($\times 10^3$)] in water samples collected in different months (from Jan 2007-Dec 2009) from three sampling sites (SS).

Months	Sampling sites			Months	Sampling sites			Months	Sampling sites		
	SS I	SS II	SS III		SS I	SS II	SS III		SS I	SS II	SS III
2007 Jan	4	5.1	2.3	2008 Jan	6	8.8	6.7	2009 Jan	1.74	3.2	1.4
2007 Feb	1	7	4	2008 Feb	2.5	3.7	2.8	2009 Feb	5.6	11.84	1.9
2007 Mar	2	4	1.9	2008 Mar	1.86	9.4	1	2009 Mar	3.8	10.9	7.5
2007 Apr	5.6	16.4	3.06	2008 Apr	1.36	2.38	1.51	2009 Apr	2.4	9	7
2007 May	5	2.96	1.64	2008 May	12.4	17	8.4	2009 May	10	27.2	5
2007 Jun	3	10.6	2.2	2008 Jun	31.5	59	38.8	2009 Jun	3.7	9.6	2
2007 Sep	6	8.2	1.3	2008 Sep	9.2	27.6	11	2009 Sep	3.6	9.2	1.15
2007 Oct	1.4	5.3	11.2	2008 Oct	13.2	18.6	6.5	2009 Oct	5.2	8.8	8.4
2007 Nov	4.6	16.6	12.4	2008 Nov	5	14	2.2	2009 Nov	2.89	18.9	4.28
2007 Dec	7.36	8.86	1.22	2008 Dec	6.4	23.2	1.72	2009 Dec	1.76	22.4	6.1

Maximum (31.5×10^3 CFU/mL) and minimum (1×10^3 CFU/mL) occurrences of oligotrophic bacteria were recorded in the month of June 2008 and February 2007 respectively from SS I (Table 1.1). The maximum (59×10^3 CFU/mL) and minimum (2.38×10^3 CFU/mL) oligotrophic bacterial load at SS II

were recorded in June 2008 and April 2008 respectively (Table 1.1). For the SS III, the maximum (38.8×10^3 CFU/mL) occurrence of oligotrophic bacteria was recorded in the month of June 2008 and minimum (1×10^3 CFU/mL) in March 2008 (Table 1.1). The densities of culturable oligotrophic bacteria did not remain constant and have shown considerable variations in water samples collected throughout the successive sampling in three years from 2007-2009. However, high densities of oligotrophs were recorded in the month of June 2008 at each sampling site.

Determination of oligotrophic bacterial fractions resistant to each of the twelve antibiotics tested per sample per month per sampling site

The fraction(s) of oligotrophic bacteria resistant to each of the twelve antibiotics, in water samples collected from three sampling sites on monthly basis are shown in Table 1.2a, 1.2b 1.3a, 1.3b, 1.4a and 1.4b.

Table 1.2a: Fractions (%) of oligotrophic (facultative + obligate) bacteria resistant to each antibiotic per sample per month in three consecutive years (2007-2009) from SS I.

	Antibiotics[a]											
Months	**Amp**	**Azi**	**Cef**	**Cft**	**Chl**	**Cip**	**Cot**	**Kan**	**Lev**	**Net**	**Str**	**Tet**
2007 Jan	13.3	1.4	3.3	12.6	3.3	2.05	2.1	2.3	2.33	1.5	0.8	13.12
2007 Feb	3.6	0.21	0.5	1.3	8.6	1.15	3.43	2	6.25	6.2	2.25	9.31
2007 Mar	4.6	0.43	7.4	5.6	4.45	10.75	9.87	1	1.4	9.12	2.3	1.59
2007 Apr	12.8	2.5	0.17	3.1	19.1	1.44	32.6	0.12	1.47	4.3	1.7	23
2007 May	46	0	8.23	34	8.02	4	38	3.6	0.41	60	0	3
2007 Jun	53.33	0.52	13.33	17.33	31	0.14	9.33	1.5	1.35	3.2	0.66	2.42
2007 Sep	35.5	3.8	22.2	19.4	14	8.8	45.33	0.25	3.77	4.44	3.7	24.8
2007 Oct	80	1.4	8.57	4.28	78.57	3.04	6	1.88	24.28	7.85	34.28	2.2
2007 Nov	26.6	1.33	12	6	7.33	9.33	12	2.5	0	22.6	1.33	4.6
2007 Dec	3.47	0.33	0.67	1.42	2.45	4	3	1.75	0.06	0.57	0.51	12.28
2008 Jan	32.33	0	1.33	1.26	33	2.05	2.1	2.43	0.033	15.8	0.8	3.2
2008 Feb	16.65	0.21	0.25	11.53	8.46	11.15	3.3	0	1.23	6.92	6.65	9.1
2008 Mar	18.6	4.3	7.74	5.26	4.5	1.075	9.67	1	1.34	8.92	2.79	0.59
2008 Apr	28	0.25	1.7	3.35	19.1	0.44	32.6	5.12	14.7	48.3	2.147	2.35
2008 May	42.35	0.14	1.59	1.41	1.07	0.3	11.7	1.51	22	32.9	2.06	0.41
2008 Jun	73.33	5.2	1.33	7.3	43.1	24	93.3	7.15	15.3	23.05	6.6	37.2
2008 Sep	28.4	1.9	2.62	5.04	21.47	0.79	6.73	4	0.85	15.9	1.97	11.25
2008 Oct	66.66	0.37	20.6	34.84	2.06	2.65	8.33	1.66	0.22	5.45	30.3	23.1
2008 Nov	76	0.4	6.4	22	10.9	1.6	3	3.33	1.4	6	19	27
2008 Dec	15	20.45	9.5	21.87	0.57	7.84	1.71	1.81	3.6	26.25	12.9	4.37
2009 Jan	85	3.63	4	5.29	1.5	7.03	3.18	2.17	3.2	5.37	4.04	5.6
2009 Feb	29.4	2.21	6.5	7.43	6.9	3.04	5.53	1.25	8.6	9.1	6	2.3
2009 Mar	26.02	1.92	39.27	11.57	8.02	2.65	8.83	1.47	44.3	26.74	90.6	14.45
2009 Apr	22.83	6.87	12.33	10	0.84	5	12.33	4.37	15.83	27.66	8.66	3.41
2009 May	54.4	0.35	5.08	4.4	0.23	0.58	5.11	1.34	2.23	3.88	3.86	0.68
2009 Jun	37.51	3.35	10.91	11.67	5.4	0.48	8.86	4.1	8.34	34.05	29.72	4.1
2009 Sep	23.55	9.38	22.22	32.4	10.14	5.88	45.33	0.25	43.77	44.44	23.77	4.88
2009 Oct	14.12	17.77	20.63	17.46	7.7	15.55	10.31	3.78	33.96	26.34	15.87	25.39
2009 Nov	33.77	20.2	44.01	21.68	9.68	15.77	10.65	1.88	3.14	31.55	22.69	27.68
2009 Dec	47.27	8.65	23.87	12.27	16.32	6.13	22.04	0.98	4.82	30.9	27.5	22.27

[a]antibiotics: Amp, ampicillin; Azi, azithromycin; Cef, cefepime; Cft, cefotaxime; Chl, chloramphenicol; Cip, ciprofloxacin; Cot, cotrimoxazole; Kan, kanamycin; Lev, levofloxacin; Net, netilmicin; Str, streptomycin; Tet, oxytetracycline.

Table 1.2b: The average, maximum and minimum percentage(s) of oligotrophic (facultative+obligate) bacteria resistant to each antibiotic in water samples of SS I.

	Antibiotics[a]											
%	**Amp**	**Azi**	**Cef**	**Cft**	**Chl**	**Cip**	**Cot**	**Kan**	**Lev**	**Net**	**Str**	**Tet**
Average	35.01	3.98	10.60	11.76	12.92	5.29	15.54	2.21	9	18.31	12.18	10.85
Max	85	20.45	44.01	34.84	78.57	24	93.3	7.15	44.3	60	90.6	37.2
Min	3.47	0	0.17	1.26	0.23	0.14	1.71	0	0	0.57	0	0.41

[a]antibiotics: same described in Table 1.2a; Max, maximum; Min, minimum

The incidence of oligotrophic bacteria resistant to ampicillin in SS I was highest (85%) in January 2009, and the lowest occurrence (3.47%) of the said population was recorded in December 2007 (Table 1.2a). The average of all the values obtained from 30 samples (spread for three consecutive years) for the fraction of ampicillin-resistant oligotrophic bacteria in SSI was 35.01%; the maximum average value amongst all the resistant-fractions corresponding individually to twelve different antibiotics, while the least average value (2.22%) was obtained for kanamycin-resistant fraction followed by azithromycin-resistant fraction (3.98%), ciprofloxacin-resistant fraction (5.29%), and levofloxacin (9.0%). The average values corresponding to fractions resistant to rest of the antibiotics tested (cefepime, oxytetracycline, cefotaxime, streptomycin, chloramphenicol, cotrimoxazole, and netilmicin) fell in the range of 10.6 – 18.3% (Table 1.2b). The maximum occurance of azithromycin-resistant oligotrophic bacteria was documented in December 2008. Equal percentage (0.21%) of azithromycin resistant oligotrophic bacteria were observed in months of February 2007 and 2008 while no antibiotic-resistance were found in the samples collected in the months of May 2007 and January 2008. Similarly kanamycin-resistant, levofloxacin-resistant, and streptomycin- resistant oligotrophic bacteria were absent in the water sample collected in the month of February 2008, November 2007, May 2007 respectively. Data revealed that resistance to cefepime cefotaxime, and ciprofloxacin of the oligotrophic bacterial population was highest in the month of November 2009 (44%), October 2008 (38.84%), and June 2008 (24%) respectively. The lowest frequencies of resistance were documented in April 2007 (0.17%) for cefepime, January 2008 (1.26%) for cefotaxime and June 2007 (0.14%) for ciprofloxacin. The chloramphenicol-resistant bacteria occurred maximally (78.57%) in the water sample collected in the month of October 2007. Also in June 2008, the occurrence of chloramphenicol resistant population was recorded quite high (43%). Lowest occurrence (0.23%) for chloramphenicol resistant population was recorded in May 2009. Cotrimoxazole-resistant oligotrophic bacteria were found maximum in June 2008 and lowest in December 2008 (1.71%). At SS I, the percentage occurrence of cotrimoxazole-resistant oligotrophic bacteria was documented highest (93.3%) among all the antibiotics tested. The maximum occurrence (7.15%) of kanamycin resistance was observed in June 2008. The resistance towards levofloxacin and netilmicin was high (44.3% and 60%) in the month of March 2009 and May 2007 respectively. The lowest (0.57%) resistance of netilmicin was noticed in December 2007. The fraction of bacterial population exhibiting resistance to streptomycin was highest (90.6%) in March 2009. Maximum occurrences (37.2%) of oxytetracycline-resistant oligotrophic bacteria were recorded in June 2008 while minimum (0.41%) in the month of May 2008. Average percentages of oligotrophic isolates, resistant to each antibiotic at SSI in three consecutive years (2007-2009) are summarized in Fig. 1.5.

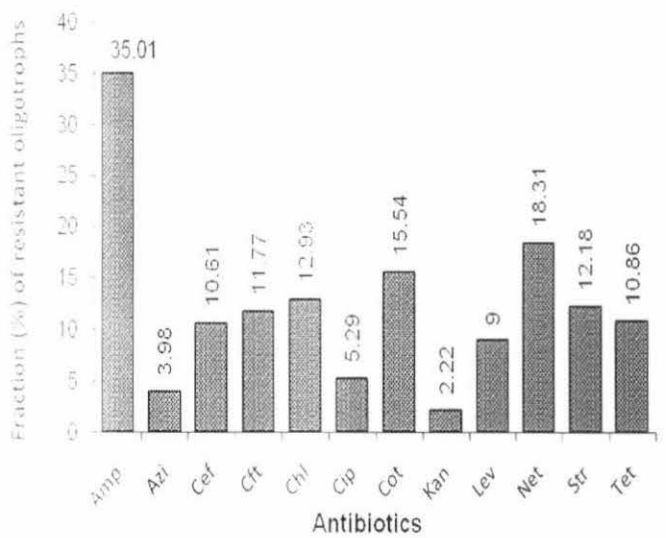

Fig. 1.5: Three years mean of fractions of oligotrophic bacteria resistant to each antibiotic in water samples of sampling site I.

The incidence of oligotrophic bacteria resistant to ampicillin in SS II was highest (70.45%) in February 2009, and the lowest occurrence (6.9%) of the said population was recorded in the same month of year 2007 (Table 1.3a).

Table 1.3a: Table 1.2a: Fractions (%) of oligotrophic (facultative + obligate) bacteria resistant to each antibiotic per sample per month in three consecutive years (2007-2009) from SS II.

	Antibiotics[a]											
Months	**Amp**	**Azi**	**Cef**	**Cft**	**Chl**	**Cip**	**Cot**	**Kan**	**Lev**	**Net**	**Str**	**Tet**
2007 Jan	9.5	1.7	2.32	10.2	9	9.93	2.17	3.34	7.61	12	3.7	3.1
2007 Feb	6.9	1.31	1.75	1.9	2.9	10.6	4.41	1.1	0.19	7	7.25	8.2
2007 Mar	54	3.7	27.2	17.66	3.1	2.02	15.37	3.7	7.9	7.47	4.95	33.88
2007 Apr	21	1.4	8.97	11.7	12	5.1	2.03	9.37	42.31	26.37	0.46	43.2
2007 May	15.4	0	24.47	36.97	43.75	16.66	52.6	6.2	11.97	38.54	0.52	78.12
2007 Jun	11.32	0.5	2.73	5.09	1.79	0.56	8.3	2.5	1.13	4.71	0.188	4.05
2007 Sep	53.2	11.2	0.45	0.77	27	0.762	8.77	5.4	8.5	3.7	7.07	1.73
2007 Oct	12.07	0.11	0.77	3.77	1.5	2.16	4.98	2.48	0.98	1.92	1.33	3.77
2007 Nov	17	5	21.6	2.17	36.45	28.38	32.25	4.5	6.9	29.6	13.46	50.32
2007 Dec	11.91	0.112	0.76	0.73	0.53	0.41	1.41	2.85	0.29	0.03	0.88	50.32
2008 Jan	51.95	1.75	0.22	42	90.9	1.93	22.57	1.43	7.1	1.27	2.27	23.17
2008 Feb	36.69	2.43	1.5	4.19	10.29	11.06	5.51	1	9.19	3.97	1.25	7.72
2008 Mar	18.4	1.8	11.06	17.6	11.1	0.57	1.32	2.1	6.21	1.01	0.79	1.29
2008 Apr	69.08	0.97	5.6	3.35	22.3	21.46	31.57	9.21	5.1	2.31	13.67	13.57
2008 May	31.02	0.44	6.2	4.23	6.8	4.6	35.51	3.15	0.25	6.24	3.26	0.76
2008 Jun	25	14	7.9	14.5	37.7	4.1	29.1	3.8	27.9	10.9	7.052	17.55
2008 Sep	9.3	1.25	3.5	20.77	41.22	7.6	18.57	7	0.45	0.47	0.77	1.69
2008 Oct	38.7	0.21	17.2	19.08	7.2	6.02	30.1	2.65	3.76	3.76	2.247	13.44
2008 Nov	31.42	1.21	7.85	4.4	3.1	2.14	22.42	4.36	1.92	8.57	3.57	16.42
2008 Dec	31.3	2.41	1.55	41.8	5.07	32	24.68	3.81	58	79.7	9.65	3.56
2009 Jan	65.46	2.41	2.72	4.6	0.05	37.7	3.6	2.27	2.65	3.75	5.07	3.6
2009 Feb	70.45	0.86	0.25	0.36	2.6	0.47	0.74	2.35	0.9	0.75	0.83	0.7
2009 Mar	54.31	13.57	27.52	37.6	3.11	13.02	51.37	3.47	71.19	47.7	45.5	31.83
2009 Apr	54.21	2.48	0.97	1.76	2.12	1.41	2.33	10.37	2.1	2.67	2.46	3.2
2009 May	13.66	1.61	2.35	6.07	1.21	20.5	0.22	0.74	11.1	13.77	0.66	1.05
2009 Jun	45.09	3.04	47.91	5.45	17.72	24.1	2.91	1.38	0.47	10.49	5.52	1.75
2009 Sep	36.41	4.49	3.93	5.95	13.1	4.54	13.93	4.02	9.34	15.65	11.31	6.23
2009 Oct	24.1	4.16	2.16	14.1	5.1	3.61	24.23	1.33	24.3	16.2	0.36	4.11
2009 Nov	11.42	59.35	9.87	33.86	0.35	14.39	32.16	3.88	53.69	24.97	19.04	35.97
2009 Dec	56.78	38.21	4.7	42.85	12.6	10.17	53.57	1.44	10.71	50.71	35.08	38.03

[a] antibiotics: same as described in Table 1.2a.

Table 1.3b: The average, maximum and minimum percentage(s) of oligotrophic (facultative+obligate) bacteria resistant to each antibiotic in water samples of SS II.

	Antibiotics[a]											
%	**Amp**	**Azi**	**Cef**	**Cft**	**Chl**	**Cip**	**Cot**	**Kan**	**Lev**	**Net**	**Str**	**Tet**
Average	32.9	6.05	8.53	14.11	14.38	9.93	17.95	3.70	13.13	14.54	7	16.74
Max	70.45	59.35	47.91	42.85	90.9	37.7	53.57	10.37	71.19	79.7	45.5	78.12
Min	6.9	0	0.22	0.36	0.05	0.41	0.22	0.74	0.19	0.03	0.188	0.7

[a]antibiotics, same as described in Table 1.2a; Max, maximum; Min, minimum

The average of all the values obtained from 30 samples (spread for three consecutive years) for the fraction of ampicillin-resistant oligotrophic bacteria in SSI was 32.9%.;the maximum average value amongst all the resistant-fractions corresponding individually to twelve different antibiotics, while the least average value (3.7%) was obtained for kanamycin-resistant fraction followed by azithromycin-resistant fraction (6.05%), streptomycin-resistant fraction (7%), cefepime (8.5%), and ciprofloxacin-resistant fraction (9.93%). The average values corresponding to fractions resistant to rest of the antibiotics tested (levofloxacin, cefotaxime,

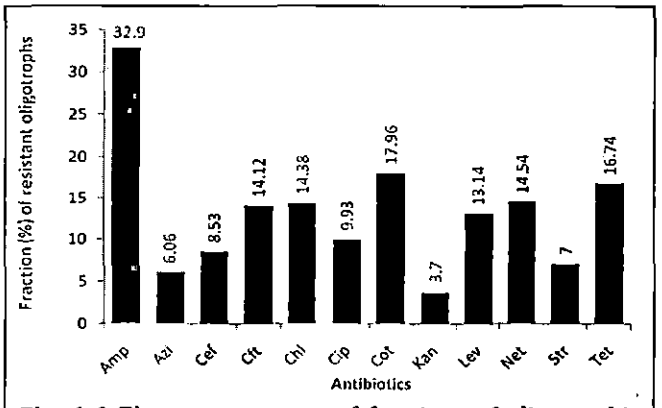

Fig. 1.6 Three years mean of fractions of oligotrophic bacteria resistant to each antibiotic in water samples of sampling site II.

chloramphenicol, netilmycin, oxytetracycline, and cotrimoxazole) fell in the range of 13.13 – 17.95% (Table 1.3b). Among all the antibiotics tested, the incidence of chloramphenicol-resistant oligotrophic bacteria was highest (90.9%). The maximum occurance of chloramphenicol-resistance was observed in the month of January 2008, and the lowest (3.47%) of the said population was recorded in January 2009 (Table 1.3a). In October and December 2007, equal frequency of azithromycin-resistance was found at SS II while no resistance was observed in the water sample collected in the month of May 2007 (Table 1.3a). The azithromycin resistance was highest (59.35%) in November 2009. Among the recovered oligotrophic bacterial population, the highest fraction resistant to Cefepime (47.9%), cefotaxime (42.85%) and ciprofloxacin (37.7%) appeared in June 2009, December 2009 and January 2009 correspondingly. Highest percentage of the kanamycin (10.37%), levofloxacin (71.2%) and netilmicin (79.7%) resistant oligotrophic bacterial population was found in April 2009, March 2009 and December 2008 respectively; and lowest were found in the month of May 2009 for Kanamycin, February 2007 for levofloxacin, and December 2007 for netilmicin. The highest occurrence of streptomycin (45.5%), co-trimoxazole (53.57%) and tetracycline (78.2%) resisting population were recorded in March 2009, December 2009, and May 2007respectively. In order to above said antibiotics the lowest resistant percentage were documented during June 2007, May 2009 and February 2009. The percentage of oxytetracycline resistant bacteria remained same in November and December 2007. Average frequency of isolates, resistant to each antibiotic at SS II in three consecutive years are reviewed in Fig. 1.6

The incidence of oligotrophic bacteria resistant to ampicillin in SS III was highest (92.45%) in December 2009 followed by November 2008 (86.36%). The lowest occurrence (0.98%) of the said population was recorded in May 2008 (Table 1.4a).

Table 1.4a: Fractions (%) of oligotrophic (facultative + obligate) bacteria resistant to each antibiotic per sample per month in three consecutive years (2007-2009) from SS III

	Antibiotics[a]											
Months	**Amp**	**Azi**	**Cef**	**Cft**	**Chl**	**Cip**	**Cot**	**Kan**	**Lev**	**Net**	**Str**	**Tet**
2007 Jan	9	3.5	8.6	3.3	25.1	7.9	15.16	3.21	23.12	7.1	5.15	7.44
2007 Feb	14	1.81	2.71	4.47	7	1.25	3.15	5.215	10.78	4.32	0.53	2.37
2007 Mar	6.74	0.4	2.23	4.1	2.5	7.13	11.2	1.9	10.3	1.57	2.03	10.4
2007 Apr	43.6	12.4	13.2	3.53	4.16	3.15	12.05	8.02	22.7	8.27	24.12	3.72
2007 May	10.36	0	0.87	10.9	2.6	0.4	2.43	1.16	0.03	4.26	0	0.6
2007 Jun	40.9	1.81	5.45	11.7	10.45	0.45	35.9	0.95	1.36	13.63	1.81	6.81
2007 Sep	7.2	10	2.4	1.74	13.13	6.05	5.09	4.435	7.6	15.4	1.91	4.32
2007 Oct	30.2	0.3	2.08	4.54	3.9	1.86	2.35	1.11	0.29	5.6	0.9	5.6
2007 Nov	4.93	0.23	1.024	1.2	0.48	0.54	3.37	2.3	0.12	2.71	0.96	1.8
2007 Dec	6.8	1	3.36	10.24	3.27	0.98	2.37	0.75	0.4	0.24	2.95	29.5
2008 Jan	7.89	0.5	7.67	1.34	5.12	1.31	5.6	1.23	3.2	0.71	2.35	0.74
2008 Feb	2.14	0.81	0.71	1.42	27	0.5	0.35	0.25	0.78	1.42	0	0
2008 Mar	6	2.4	2.3	1	2.5	1.3	1.4	1.1	0.3	1.7	2.4	0.4
2008 Apr	2.36	2.4	3.2	2.33	48.6	3.5	2.5	3.02	2.75	8.33	23.2	7.2
2008 May	0.98	0.1	2.06	1.31	4.3	0.29	5.29	0.51	0.07	0.29	1.37	0.35
2008 Jun	41.6	11.16	15.2	31	17.2	9.12	5.72	6.12	3.71	4.28	0.92	3.51
2008 Sep	1.72	1.03	1.41	1.45	13.63	0.65	8	3	0.6	0.54	12.21	0.42
2008 Oct	8	1.57	56.61	1.23	8.13	0.3	56.61	2.16	8.3	8.61	8.46	98.46
2008 Nov	86.36	13.63	54.54	45.4	0.45	0	40.9	1.31	13.63	27.27	0	36.36
2008 Dec	12.2	7.09	4.65	7.9	17.2	7.67	5.34	0.17	8.13	22.32	15.34	1.02
2009 Jan	1.07	1.32	0.71	0.89	1.4	0.51	0.37	1.61	0.45	2.58	1.94	0.04
2009 Feb	5.91	12.09	12.47	12.25	2.5	4.95	8.92	0.25	34.94	27.09	28.49	8.65
2009 Mar	20.26	3.46	12	3.86	5.29	1.6	5.6	2.047	57.33	18.4	14.66	4.8
2009 Apr	7.37	1.27	2.48	1.86	12.21	3.63	2.35	3.327	8	7.77	3.87	1.1
2009 May	32	0.6	0.8	12.6	11.45	1.6	3.8	0.31	11.9	19.33	7.4	1.8
2009 Jun	10.6	1.6	15.8	10	7.2	0.95	5.2	0.12	5.31	24.8	10.2	4.15
2009 Sept	13.56	19.04	18.95	66.08	11.4	6	16.17	1.01	71.65	46.6	30.26	10.69
2009 Oct	11.73	9.23	6.52	18.47	21.5	5.1	20.87	1.421	31.3	16.95	15.65	7.33
2009 Nov	27.11	26.16	89.71	24.29	15.29	24.53	30.37	7.047	83.17	97.19	53.73	39.25
2009 Dec	92.45	72.13	14.42	70.81	2.23	15.57	59.01	1.307	10.32	85.24	65.57	34.42

[a]antibiotics, same as described in Table 1.2a

Table 1.4b: The average, maximum and minimum percentage(s) of oligotrophic (facultative+obligate) bacteria resistant to each antibiotic in water samples of SS III.

	Antibiotics[a]											
%	Amp	Azi	Cef	Cft	Chl	Cip	Cot	Kan	Lev	Net	Str	Tet
Average	18.83	7.3	12.14	12.37	10.24	3.96	12.58	2.21	14.42	16.15	11.28	11.1
Max	92.45	72.13	89.71	70.81	48.6	24.53	59.01	8.02	83.17	97.19	65.57	98.46
Min	0.98	0	0.71	0.89	0.45	0	0.35	0.12	0.03	0.24	0	0

[a]antibiotics, same as described in Table 1.2a; Max, maximum; Min, minimum

The average of all the values obtained from 30 samples (spread for three consecutive years) for the fraction of ampicillin-resistant oligotrophic bacteria in SS III was 18.83%.;the maximum average value amongst all the resistant-fractions corresponding individually to twelve different antibiotics, while the least average value (2.2%) was obtained for kanamycin-resistant fraction followed by ciprofloxacin-resistant fraction (3.96%), and azithromycin (7.3%). The average values corresponding to fractions resistant to rest of the antibiotics tested (chloramphenicol, oxytetracycline, streptomycin, cefepime, cefotaxime, cotrimoxazole, levofloxacin, and netilmycin) fell in the range of 10.24 - 16.15% (Table 1.4b). There were no azithromycin-resistant oligotrophic bacteria in the water sample collected in the month of May 2007. Similarly no resistance towards ciprofloxacin and oxytetracycline were observed in the month of November 2008 and February 2008 respectively. Streptomycin-resistant oligotrophic bacteria were absent in the water sample collected in the months of May 2007, February 2008, and November 2008. The occurance of oligotrophic bacteria resistant to azithromycin was maximum (72.13%) in the month of December 2009 while lowest (0.1%) were observed in May 2008. The proportion of chloramphenicol-resistant oligotrophic bacteria was highest (48.6%) in month of April 2008 while lowest occurrence was observed in November 2008. Maximum occurances of antibiotic-resistant-oligotrophic bacteria were documented in November 2009 for cefepime (89.7%) and ciprofloxacin (24.53%); and in December 2009 for cefotaxime (70.81%). The percentage resistance of ciprofloxacin in compare to cefepime and cefotaxime was quite low throughout the sampling. The highest percentage of kanamycin-resistant (8.02%), levofloxacin-resistant (83.17%), and netilmicin-resistant (97.2%) oligotrophic bacterial population were recorded in the month April 2007 and November 2009 respectively. The lowest occurrences of resistance for the said antibiotics were recorded in June 2009, May 2007 and December 2007 correspondingly. The highest occurrence (65.57%) of streptomycin and cotrimoxazole-resistance among oligotrophic bacteria were recorded in December 2009 and lowest were recorded in October 2007 and February 2008 respectively.

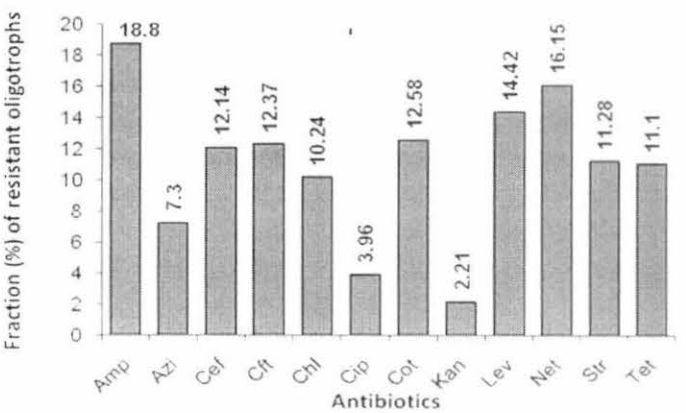

Fig. 1.7: Three years mean of fractions of oligotrophic bacteria resistant to each antibiotic in water samples of sampling site III.

The occurrence of oligotrophic bacteria resistant to oxytetracyline was found highest (98.46%) amongst all the tested antibiotics in the water samples collected in all three years. The lowest incidence (0.04%) of the said population was recorded in May 2008 (Table 1.4a). Average frequencies of antibiotic-resistant oligotrophic bacteria collected from three sampling sites in three consecutive years are shown in Fig. 1.7.

1.3.1.2. Comparative analyses of annual average percentages of twelve different antibiotic-resistant oligotrophic bacteria and their trends of increase or decrease

Comparative analyses of annual average percentages of twelve different antibiotic-resistant oligotrophic bacteria and their trends of increase or decrease have been represented in the Fig. 1.8 (a, b, c) and 1.9 (a, b, c). The average percentage occurrences of azithromycin, cefotaxime, ciprofloxacin, levofloxacin netilmicin, and streptomycin resistant bacteria in water samples collected during three consecutive years (January 2007 to December 2009) from SS I, have shown an upward trend but downward trend was noted for chloramphenicol resistant bacteria. Variable trend of occurrences of chloramphenicol, cefepime, cefotaxime netilmicin cotrimoxazole and tetracycline resistant bacteria were recorded from January 2007 to December 2009 at SS II. Analysis of Site II and Site III samples revealed an upward trend for ampicillin, azithromycin, ciprofloxacin, levofloxacin, streptomycin resistance while uneven trend of chloramphenicol and tetracycline resistance were observed at both sampling sites in all three years. An upward trend of cefipime resistance was found in water samples collected month-wise from all three years at SS III but was variable at SS II. During January 2007 to December 2009 oligotrophic bacteria, resistant to cefotaxime, and netilmicin have shown to be fluctuating at sampling site SS II. Kanamycin resistant bacteria showed an approximate constant level of occurrences in all sampling sites.

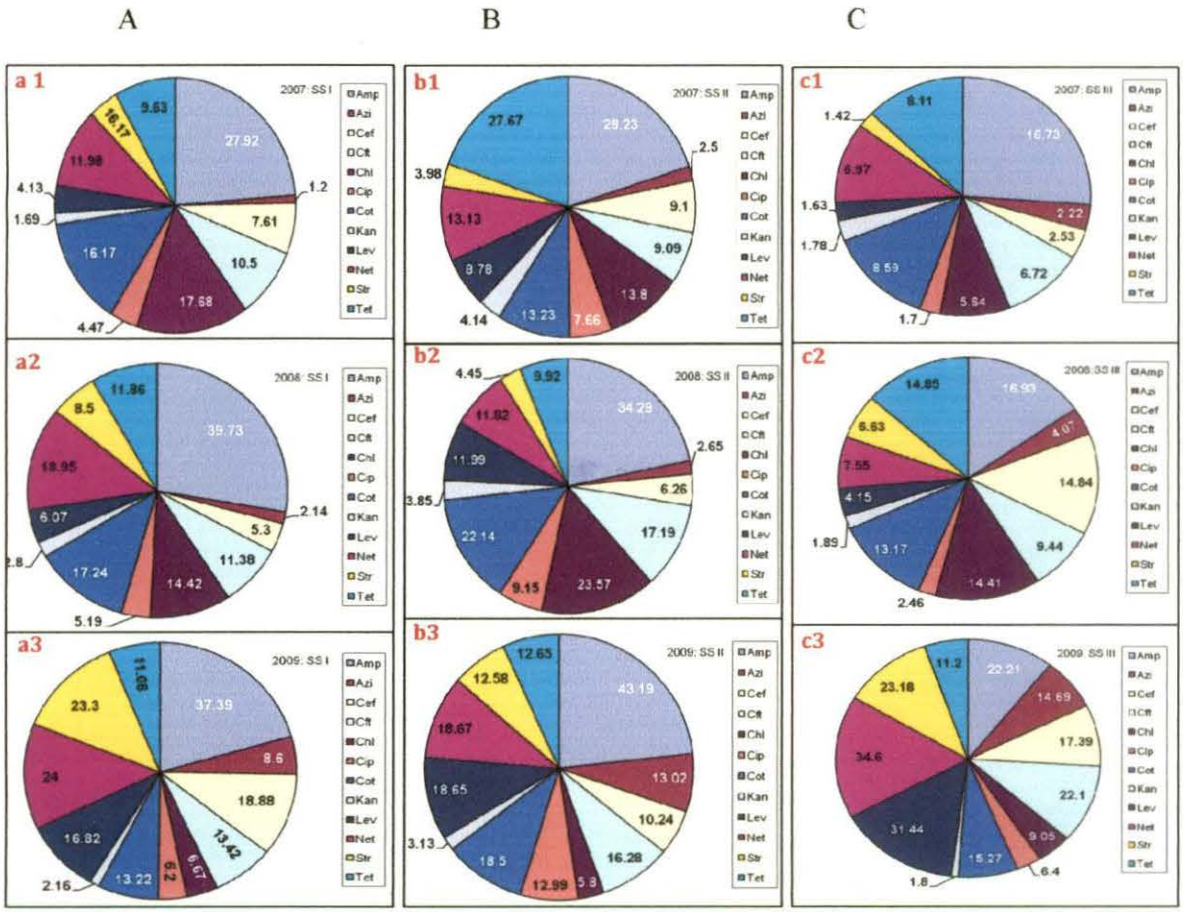

Fig. 1.8: Pie diagram showing fraction(s) of oligotrophic bacteria resistant to each antibiotic in water samples collected from three different sampling sites in three consecutive years (Jan 2007- Dec 2009).
A, sampling site I (a1, 2007; a2, 2008; a3, 2009); B, sampling site II (b1- b3: same as a1-3); and C, sampling site III (c1-c3: same as a1-3).

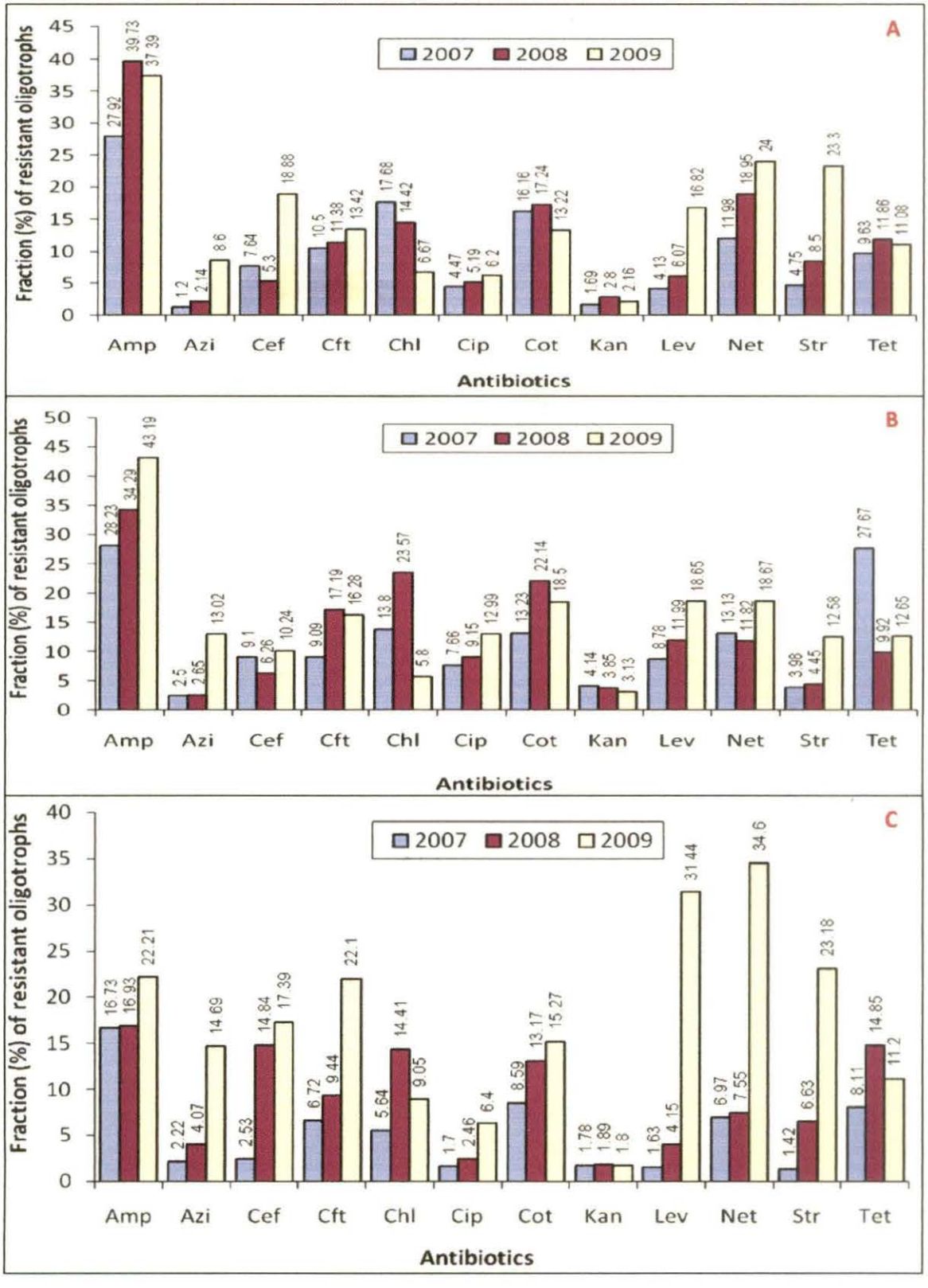

Fig. 1.9: Bar diagram showing comparative analyses of the fraction(s) of oligotrophic bacteria (present in water samples) resistant to each of the 12 different antibiotics at a particular sampling site in three consecutive years (2007-2009). A, sampling site I; B, sampling site II; C, sampling site III.

1.3.2 Occurrence(s) of sensitive, single and multiple-antibiotic-resistant bacteria among randomly selected facultatively oligotrophic (from second generation master plates) population

25±1 randomized colonies of facultatively oligotrophic isolates of first generation master plate (for detail please see section 1.2.7.2) were picked and transferred to R2A plate which were designated as second generation master plates. A total of 2188 isolates were picked in three consecutive years (2007-2009) from all three sampling sites and screening was performed by the replica-plating method (described in the section 1.2.7.2) for differentiation of sensitive, single-antibiotic-resistant, and multiple-antibiotic-resistant isolates.

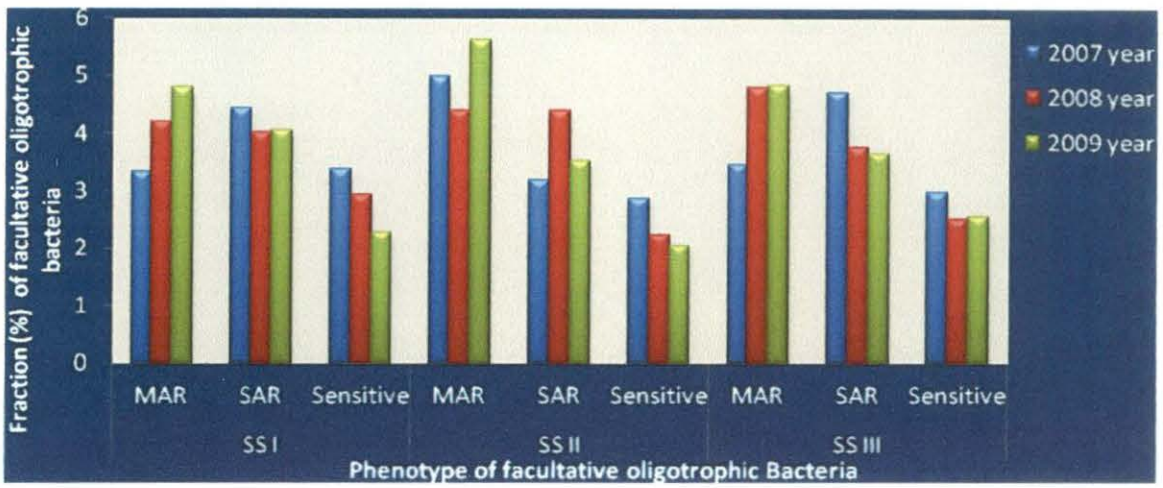

Fig 1.10: Comparative analyses of fraction(s) of MAR, SAR and sensitive facultative oligotrophic isolates out of entire oligotrophic (facultative + obligate) bacterial population in water samples from three different sampling sites (SS) in three consecutive years

The percentage occurrences of multiple-antibiotic-resistant facultatively oligotrophic bacteria have shown increasing trends in SS I and SS III, while fluctuation has been observed among SS II isolates. The occurrences of single-antibiotic-resistant facultatively oligotrophic bacteria were found to be decreasing from 2007 to 2009 at SS III. Approximately similar frequencies of single-antibiotic-resistant ones were observed during year 2008 and 2009 at SS I. Maximum frequency of single-antibiotic-resistant facultatively oligotrophic bacteria was observed in isolates collected from samples of the year 2008 at SS II. A gradual decrease in sensitive and concomitant increase in multiple-antibiotic- bacteria was observed from 2007- 2009 in randomly selected facultatively oligotrophic isolates from SS I and SS III. However for SS II isolates, multiple-antibiotic-resistant bacteria were most frequent ones than sensitive or single-antibiotic-resistant isolates; but gradual decrease in sensitive isolates has been noted (Fig. 1.10).

Table 1.5: Annual distribution of sensitive, SAR and MAR isolates at different sampling sites in different years.

Year	Fraction (actual number in parentheses) of sensitive and antibiotic-resistant (SAR and MAR) facultative oligotrophic isolates out of entire oligotrophic bacteria (obligate + facultative) at different sampling sites								
	SS I			SS II			SS III		
	MAR	SAR	Sensitive	MAR	SAR	Sensitive	MAR	SAR	Sensitive
2007	3.33 (73)	4.43 (97)	3.38 (74)	4.98 (109)	3.2 (70)	2.88 (63)	3.47 (76)	4.7 (103)	2.97 (65)
2008	4.2 (92)	4.02 (88)	2.93 (64)	4.39 (96)	4.39 (96)	2.24 (49)	4.8 (105)	3.75 (82)	2.51 (55)
2009	4.79 (105)	4.06 (89)	2.29 (50)	5.62 (123)	3.52 (77)	2.06 (45)	4.84 (106)	3.65 (80)	2.56 (56)

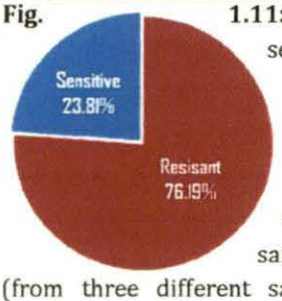

Fig. 1.11: Over all fraction(s) of sensitive and antibiotic-resistant Facultative oligotrophic bacterial population of Mahananda river water [data of 90 water samples taken together (from three different sampling sites in three years)].

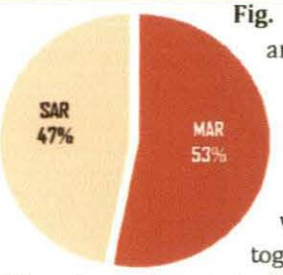

Fig. 1.12: Distribution of MAR and SAR population within antibiotic-resistant facultative oligotrophic bacteria of Mahananda river water [data of 90 water samples taken together (from three different sampling site in three years)].

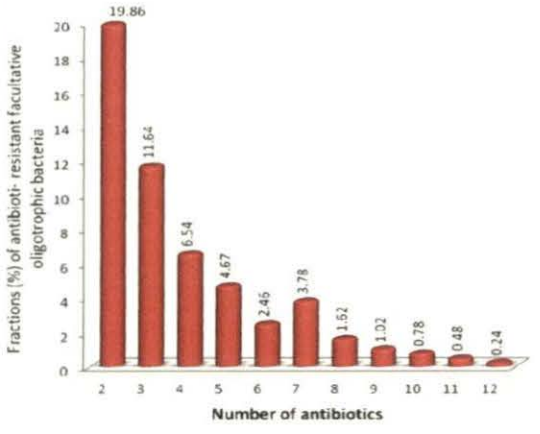

Fig. 1.13: Fraction of antibiotic resistant isolates, resisting 2, 3, 4, 5, 6, 7, 8, 9 , 10, 11, and 12 number of antibiotics within MAR facultative oligotrophic bacteria

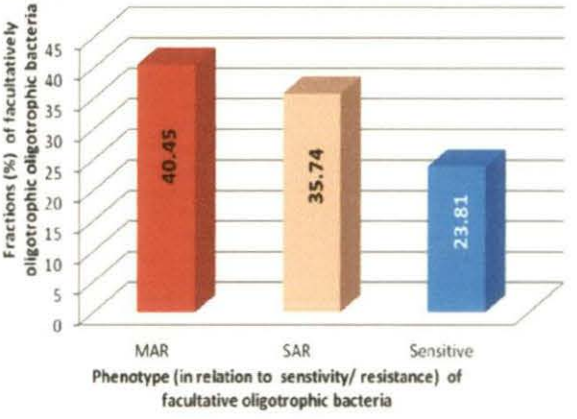

Fig. 1.14: Over all distribution of isolates exhibiting different phenotypes [in relation to antibiotic sensitivity/resistance] among facultative oligotrophic bacteria [data of 90 water samples taken together (from three different sampling site in three years)].

Out of 2188 facultatively oligotrophic bacterial isolates, 76.19% (1667) were antibiotic-resistant and 23.81% (521) were sensitive to all antibiotics used in this study (Fig. 1.11). Annual distributions of sensitive, single and multiple-antibiotic-resistant isolates at different sampling sites are presented in Table 1.5. Amongst antibiotic-resistant isolates, 47% (782) exhibited resistance to single antibiotic, designated as SAR (single-antibiotic-resistant) and 53% (885) were resistant to two or more than two antibiotics, designated as MAR (multiple-antibiotic-resistant) (Fig. 1.12). Among the multiple-antibiotic-resistant group, 19.86% (331) were resistant to two, 11.64% (194) to three, 6.54% (109) to four, 4.67% (78) to five, 2.46% (41) to six, 3.78% (63) to seven, 1.62% (27) to eight, 1.02% (17) to nine, 0.78% (13) to ten, 0.48% (8) and 0.24%(4) to all the 12 antibiotics tested(Fig. 1.13). Fig. 1.14 showed an average percentage of fractions of multiple-antibiotic-resistant, single-antibiotic-resistant and the bacteria sensitive to all antibiotic used. A gradual drop was observed in the numbers of sensitive facultative bacteria from year 2007 to 2009 in the pool of randomly selected isolates.

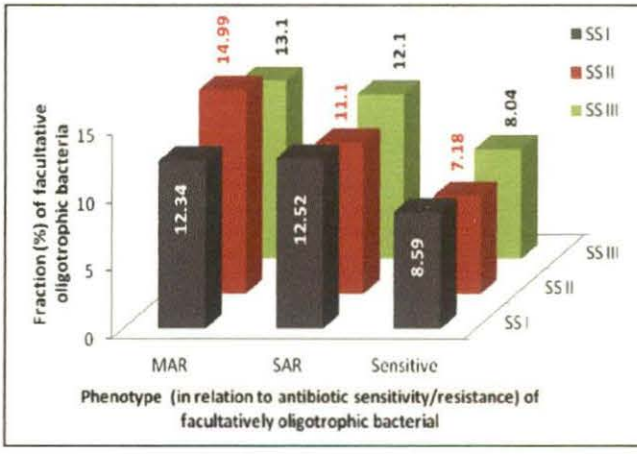

Fig. 1.15: Relative abundance (%) of sensitive, single-antibiotic (SAR) and multiple-antibiotic resistant (MAR) among facultative oligotrophic bacteria [data of 90 water samples taken together (from three different sampling site in three years)].

The relative abundance of sensitive, SAR and MAR bacteria in the pool of selected facultative oligotrophic bacterial population in three successive years, 2007, 2008 and 2009, has been depicted in Fig. 1.15. It was found that MAR facultatively oligotrophic bacteria (in total) were dominant in SS II samples compared to SS I and SS III. The SAR ones were more frequent amongst isolates from SSI and SSIII; similar observation was also noted in case of sensitive oligotrophic bacteria.

1.4 Discussion

For successful monitoring of river water quality it is important to have sufficient knowledge about the morphometric details of the river to be investigated, selection of particular sampling site(s), sample collection methods, and preservation & maintenance of samples for the types of analyses to be made. The study of morphometry, measurement of morphological features of the river basin, always provides valuable information in selecting sample collection site(s). Water quality of the river water also depends on physiographical factors, such as basin, bank, catchments area, and settlement around the river, as well as annual sedimentation load, water volume, width, and depth of the river.

Water samples collected from the river are of two main types depending on the collection principle: grab-samples, and composite samples. Grab samples are collected at a specific spot in a site over a short period of time, on the other hand when multiple grab samples are combined and treated as a single sample, it is called composite sample. Samplers and containers should always be thoroughly cleaned before use, and should be rinsed with the sample water before collection. Preferably the amber colored glass containers with polypropylene cap should be used for the collection and preservation of samples. For microbiological study collected samples should be transported and kept until use in ice container (4°C) and should be processed within 24 hrs.

Much of the current concern with regards to environmental quality is focused on water because of its importance in maintaining the human health and health of the ecosystem. The earth is almost flooded with water reservoirs but most of them are oceanic i.e salty which cannot be used in drinking or in other purposes e.g. irrigation etc. Freshwater is the fundamental requirement to sustain terrestrial life. It is finite resource, essential for agriculture, industry and even human existence; without fresh water of adequate quantity and quality, sustainable development will not be possible. The amount of fresh water is large as well but its distribution over the globe is uneven. With the increase in population, there is growing demand for fresh water supplies all over the world. In an effort to spur action to meet the impending crisis, the UN General Assembly has proclaimed the period from 2005 to 2015 as the International Decade for Action, "Water for Life" (www.peopleandplanet.net). In theory, some 34,000 cubic kilometres of freshwater are available globally for human use every year. If evenly distributed this would provide each person with roughly 8,000 cubic metres of water per year. Even this amount would be enough to meet human needs, if fresh water resources were evenly distributed. But available fresh water supplies are not distributed evenly around the globe throughout the seasons or from year to year. Throughout much of the developing world the freshwater supply comes in the form of seasonal rains. Such rains run off too quickly for efficient use, as during the monsoons in Asia. India, for example, gets 90 per cent of its annual rainfall during the summer monsoon season, which lasts from June to September. For the other eight months the country gets barely a drop (www.peopleandplanet.net).

Rivers are the main sources of fresh water. There is an extensive literature, which stresses deterioration of water quality (Tiwari and Mishra, 1986; Reddy and Venkateswar, 1987). The addition of various kinds of pollutants and nutrients through the agency sewage, industrial effluents, agricultural runoff etc. in to the water bodies brings a series of changes in the physicochemical and other characteristics of water, which have been the subject of several investigations (Vollenweidre, 1986; Milway, 1987; Olimax and Sikorska, 1975; Piecznska *et al.*, 1975). Deterioration of the fresh water quality is now a global problem (Mahananda *et al.*, 2005). Discharge of toxic chemicals, over pumping of aquifer and contamination of water bodies, demographic explosion, urbanization,

unplanned development, land degradation and lack of infrastructure for waste disposal are leading to a rapid deterioration in water quality in the majority of rivers all over the world. This poses a threat both to the environment and to the health of the people in the region. Pollution of rivers and lakes reduces accessible fresh water supplies. In developing countries, 90% of the sewage is being discharged directly into rivers, lakes, coastal waters without any treatment (World Resource Institute, 1996) (www.wri.org). Each year roughly 450 cubic meters of wastewater are discharged into rivers, streams and lakes. To dilute and transport this dirty water before it can be used again, another 6000 cubic kilometers of clean water are needed – an amount equal to about two thirds of the world's total annual usable fresh water runoff (www.peopleandplanet.net).

The trends, in the contamination of river water worldwide, have changed greatly over time. The fecal and organic pollution from untreated wastewater was the major contamination problem 100 years ago. In most industrialized countries, fecal contamination of water has been largely eliminated, however, in much of the world especially in cities in developing countries, organic pollution is still a problem. New pollution problems, particularly from agricultural runoff and industrial effluents are increasing in both industrialized and developing countries. In rapidly industrializing countries like China, India, Mexico, Brazil untreated sewage and industrial waste create substantial pressures on water quality that are much greater than the problems of the past.

In the recent years, emphasis is on the so-called emerging contaminants including pharmaceuticals such as antibiotics, endocrine disrupters and on various additives. Human use pharmaceuticals enter sewage effluents through improper disposal from private households and from hospitals. Direct inputs into natural waters are also possible during rain events and this normally occurs in less industrialized countries. In wastewater treatment plants the antibiotics are only partially eliminated and residual amounts can reach ambient waters and ground water. Antibiotics are of main interest because we do not know currently whether their presence in natural waters contributes to the spread of antibiotic resistance in organisms. Successive knowledge regarding the effect of sub-inhibitory concentrations of the antimicrobials on the survival of bacteria in environment is insufficient and contradictory. But on the other face voluminous evidences are there which revealed the existence of antibiotic resistant bacteria in nature and horizontal transfer of antibiotic resistance determinants between them.

In environmental settings polluted by human and animal waste or both, high frequencies of MAR isolates exist in the coliform and fecal coliform population. These environments include surface waters receiving runoff from lands occupied by livestock, polluted estuaries and contaminated water supplies. Fluvial waters receive human and animal wastewater discharges, which are expected to contain antimicrobial agents likely to exert a selective pressure, and commensal resistant bacteria, capable of transferring their resistances to autochthonous bacteria. Consequently, the fresh water bacteria may become a reservoir for antimicrobial resistance genes, and the reuse of these waters for humans and animals may contribute to the limitation of antimicrobials efficiency. Any body of water that receives human waste products can be studied for its content of antibiotic resistant bacteria. Beyond human use of antibiotics, there are a number of other sources that may shoulder part of the blame for high resistance levels. Resistance can come from the natural production of antibiotics by organisms in the soil. It may also result from antibiotic-contaminated runoff from animal feed or crops, or wastes from farm animals (Ash *et al.,* 2002). It was shown in Greece that some resistant bacteria came from the feces of seabirds or warm-blooded mammals that live near the coastal waters (Arvanitidou *et al.,* 2001; Bennani *et al.,* 2012). Studies have also been performed within the animal production industry to show the impact of antibiotic resistance. For example, fish farms routinely treat bacterial infections in the fish with the use of antibiotics. These antibiotics are released into the water. They then can move downstream, unfiltered and untreated by the fish farms (Schmidt *et al.,* 2000). Occurrences such as this allow for an increased ability of bacteria to develop a resistance.

The distribution of antibiotic-resistant strains in the aquatic environment has been studied in different parts of the world. Majority of the investigations focused on the antibiotic resistance patterns of the fecal coliform bacteria because of their use as pollution indicators and association with disease causing genera of importance to public health and hygiene. However, in many freshwater systems, fecal bacteria are of little numerical significance in spite of the fact that they are released into almost all inland waters. It is also not uncommon to find standard plate count bacteria (SPC) in drinking water at frequencies more than 10,000 times the frequency of coliforms. Earlier studies have reported the occurrence of high frequencies of antibiotic resistant organisms within the SPC populations (Armstrong *et al.*,. 1982). There is evidence that SPC bacteria in marine and freshwater environments can possess the same kinds of antibiotic resistance patterns as total and fecal coliform populations.

A substantial portion of bacteria previously thought to be unculturable were recently shown to be oligotrophic. Oligotrophic bacteria have been isolated from diverse sources including clinical sample and distilled water (Mallory *et al.*, 1977; Kuznetsov *et al.*, 1979; Tada *et al.*, 1995; Watve *et al.*, 2000; Nagarkar *et al.*, 2001; Miyake *et al.*, 2003; Pramanik *et al.*, 2003; Alexander *et al.*, 2005; Katsunori and Masafumi, 2006; Hu *et al.*, 2007; Ishii *et al.*, 2011, Bhullar *et al.*, 2012). Studies showed that a number of oligotrophic bacteria exhibit antibiotic resistance (Nikitin *et al.*, 1988; Zlatkin *et al.*, 1991; Kimura *et al.*, 1995; Oh *et al.*, 1995, Tada *et al.*, 1995; Miyake *et al.*, 2003; Kumar *et al.*, 2010, Kumar *et al.*, 2011, Bhullar *et al.*, 2012; Kumar *et al.*, 2012, Mandal *et al.*, 2012), most of them were plasmid borne (Tada *et al.*, 1995, Zlatkin *et al.*, 2012). On the basis of existing data it was hypothesized that oligotrophic bacteria can therefore be a potential reservoir of novel antibiotic resistance genes that can be acquired by pathogens through plasmid transfer. The nutrient content in river water is indeed far less than that in microbiological media conventionally used for the cultivation of bacteria from a natural water environment. Nutrients in free flowing river water system are heterogeneously distributed in an environment; therefore, based on this consideration, we could assume that there must be bacteria that are able to grow in the presence of low level of nutrients (oligotrophs). Oligotrophic bacteria are defined (though no uniformly acceptable definition formulated yet) as heterotrophic bacteria that able to multiply in very low nutrient condition (media containing a minimal content of organic matter of either 1 mg or 1 to 15 mg C/L) usually supplied as complex mixtures of peptone, trypticase, and other nutrients (Ishida and Kadota, 1981; Kuzenetsov *et al.*, 1979). Eutrophic or copiotrophic bacteria, on contrary, have been considered as the bacteria able to grow on similar nutrients but at levels supplying 2 or more than 2 g C/L (Akagi *et al.*, 1977). In India there are few reports available on oligotrophic bacteria (Watve *et al.*, 2000; Pramanik *et al.*, 2003) but not on incidences of antibiotic-resistant oligotrophs. Recently class-I integrons bearing MAR oligotrophic bacteria from an Indian River was reported by Kumar *et al.* (2010, 2011).

The present study validated the capability of nutrient-poor medium (NPA/NPB) and its amendment with antibiotics to quantify proportions of antibiotic-resistant oligotrophic bacteria in river water. In fact, standardization of the assay conditions for assessing fractions of antibiotic resistant oligotrophic bacteria, especially the type and concentration of antibiotic exposure by determining LD_{50} concentration for each antibiotic using sensitive oligotrophic strains, marked a major advancement in this area of water research. With the set of standards for determining susceptibility/ resistance, bacteriological investigations on the prevalence and abundance of antibiotic-resistant oligotrophic bacteria in water samples of the river Mahananda (collected month-wise from three sampling site for three consecutive years) at Siliguri city of the northern West Bengal, were done. Ninety water samples were collected and analyzed in between January 2007 to December 2009 from the three sampling sites from River Mahananda. The SS I is situated at the entry point of city Siliguri near Champasari (26°44′22.62″ N, 88° 25′21.92″E) (Fig. 1.3a). The location of SS II is under Mahananda bridge (26°44'23.20"N, 88°25'22.89"E) at the heart of city Siliguri (Fig. 1.3b).The SS III is chosen at the exit point of river from city Siliguri near Fulbari dam (26°38′42.44″

N, 88°24'19.67"E) [this sampling site supposedly has weak anthropogenic activity](Fig. 1.3c). Plate count on nutrient-poor medium was made to assess the oligotrophic bacterial content of the water samples collected from the three sampling sites. For the cultivation of oligotrophic bacteria, NPA agar plates were used. The recovered bacteria may not represent all the bacterium present in water but only those able to grow and form visible colonies on the NPA media under given condition of temperature and incubation were considered to assess the situation. The cultivable oligotrophic bacteria developed on agar plate did not reflect the actual number; the number that can be enumerated may be higher than the cultivated. It was observed that the numbers of colonies developed on low nutrient Luria agar were quite high in compared to the numbers that grew on rich nutrient Luria agar (this study).

Maximum recovery of the culturable oligotrophic bacterial populations recorded in month of June 2008 at all three sampling sites. The oligotrophic bacterial density in collected water samples of river Mahananda ranged 1×10^3 to 5.9×10^4 CFU/mL. In an earlier study, those bacteria which grew at the lower nutrient level but failed to grow when transferred to the higher nutrient concentration were considered to be oligotrophic bacteria (Yanagita, *et al.*, 1978); however report supports that on subsequent subculture of the isolates, many of them regained their ability to grow on rich medium (Kuznetsov *et al.*, 1979). Somehow a clear-cut distinction has been accepted that bacteria having the ability to grow only at lower concentration of nutrients were called obligate oligotrophs, whereas those which grew at both low and high concentrations of nutrients were termed facultative oligotrophs (Ishida *et al.*, 1980; Ishida *et al.*, 1982). In this study the obligate oligotrophs were omitted due to their slow growth and ambiguity in forming colonies on NPA medium used. Facultatitve oligotrophic bacterial colonies identified from the first generation master plates were further renumbered and randomized to obtain 25±1 random numbers. The colonies corresponding to each of the 25 random numbers were purified onto R2A agar plate and pure colonies were then imprinted onto the fresh R2A plate, designated as second generation master plate. The second generation master plate was replica plated on to the R2A plate containing antibiotic of defined concentration to determine the susceptibility profile (antibiogram) of the isolates.

The overall distribution of resistant oligotrophs was achieved by plating 0.1 mL aliquot of composite water sample on NPA agar amended with defined concentration of each antibiotic. The percentages of resistant oligotrophic bacteria were estimated throughout the study during each sampling in the same manner. The antibiotic resistance profile (ARP)/antibiogram/susceptibility profile/resistance profile of the facultative oligotrophic bacteria (obtained from first generation master plate through replica plating method) were determined by replica plate method as described elsewhere in this chapter. All the colonies picked up randomly to screen sensitive and resistant bacterial strains were used for the screening of class 1 integron (detail description in chapter 2).

The quantification of antibiotic-resistant oligotrophic bacteria showed that the maximum average-percentage of oligotrophic bacteria resistant to an antibiotic, in all three consecutive years (2007-2009), was documented for ampicillin and minimum for kanamycin amongst the 12 different antibiotics tested (Fig. 1.5, 1.6, and 1.7). In addition to ampicillin, the annual average percent of resistant-oligotrophic bacteria in the year 2009 at SS I was recorded maximum for netilmicin (24%) (Fig. 1.9a). The average annual frequency resistant oligotrophs at SS II were observed quite high for oxyteteracycline (27.67%) in the year 2007 (Fig. 1.9b). Similarly maximum frequency of antibiotic-resistance at SS III, were observed for netilmicin (34.6%) in year 2009 (Fig. 1.9c).

It has been a general observation that SS II [compared to SS I (upstream) and SS III (downstream)] receives high influx of household, hospital effluents, cattle slurry, and industrial wastes and have high anthropogenic activities on both banks. Since this site was highly polluted with different kinds of wastes, it was assumed that probability of resistant bacteria will be high. In a previous study conducted on Yarra river of Australia, it was predicted that the incidence of antibiotic resistance in bacteria isolated from polluted sites would be greater than the incidence in bacteria

isolated from pristine sites and upstream reaches or flowing through areas of low intensity agriculture (Boon and Cattanach, 1999). The results of this study supported the hypothesis of Boon and Cattanach (1999); i.e high probability of finding resistant bacteria in the sampling site II. Since, oligotrophic bacteria constitutes a sub-set of the universal set of bacteria present in an ecological niche, high occurrences of oligotrophic bacteria resistant to single and multiple antibiotics were obtained from samples of SS II [the profile(s) of MAR facultatively oligotrophic isolates from SS II having class 1 integrons have been detailed in chapter 2] . The study on facultatively oligotrophic bacteria showed that the average percentage of multiple-antibiotic resistant (MAR) was greater than the single-antibiotic resistant (SAR) at SS II, however approximate similar load of MAR and SAR facultatively oligotrophic bacteria were observed at SS I and SS II (Fig. 1.9, 1.10 and 1.15). In an anthropogenically affected site, like sites where the river flows through a populous city, the chances of horizontal transfer of antibiotic resistance genes amongst diverse bacteria following dissemination into the environment are often very high. Many antibiotics of industrial origin also circulate in aquatic-environments which also act as the selective agents for amplification of antibiotic-resistance genes vis-à-vis enrichment of antibiotic-resistance gene pool of the microbial ecosystems. Previous studies concerned with rivers of northern West Bengal, dealt with copiotrophic bacteria (Mukherjee *et al.*, 2005). As such, the studies on antibiotic-resistance phenomenon in oligotrophic bacterial isolates are very limited. The results of the present study on facultatively oligotrophic bacteria have shown that 76.2% of them were antibiotic resistant and 23.8% were sensitive (subject to the limitation that the study was of random type) of an Indian river is resistant to one or more than one antibiotics. Among resistant facultatively oligotrophic bacteria 47% were SAR and 53% were MAR. The frequency of multiple-antibiotic-resistant facultatively oligotrophic bacteria was 6% greater than the bacteria resistant to one antibiotic. Within the MAR population of facultatively oligotrophic bacteria, 19.86% were resistant to two, 11.64% to three, 6.54% to four, 4.67% to five, 2.46% to six, 3.78% to seven, 1.62% to eight, 1.02% to nine, 0.78% to ten, 0.48% and 0.24% to all the 12 antibiotics tested (Fig. 1.13). An inverse relation was observed in average frequency of resistant and sensitive facultatively oligotrophic bacteria examined in three consecutive years (2007-2009) or in other words we can say that with progress of time (2007 to 2009), there is a gradual decrease observed in susceptible oligotrophic population in Mahananda River water. Furthermore a progressive decrease in facultatively oligotrophic bacterial population resisting single antibiotics associated with increase in MAR oligotrophic bacteria resisting more than two antibiotics was noted. Hence, the trend analyses (responses to antibiotics according to time) of total oligotrophic (obligate + facultative) and selected facultative oligotrophic bacterial population showed that proportion of oligotrophic bacteria was found to be increasing and percentage of sensitive oligotrophic bacteria decreasing with passage of time. This is an alarming situation; where immediate intervention is necessary. These bacteria were found to carry several antibiotic resistance genes as cassettes in their class 1 integron platforms; many of these cassettes are yet to be predicted for a functional protein (codes for hypothetical protein). These hypothetical proteins may in course of time develop into resistance genes against yet-to-develop chemotherapeutic agents. Hence it was really thought-provoking and tempting to assume that the oligotrophic bacteria may serve as potential reservoir of novel genes (antibiotic resistance and other stress-combating genes).

1.5 Conclusion

The antibiotic resistance in bacteria is a world-wide problem. It is therefore essential to assess the gravity of the problem and also to concentrate on types of resistances not only in the commensal and pathogenic copiotrophic bacteria but also in oligotrophic bacteria which are largely uncultured; and we are ignorant about the resistance-gene carriage within oligotrophic bacterial genomes. Earlier studies have shown that rivers are major reservoirs of antibiotic resistance genes (Park *et al.*, 2003; Biyela *et al.*, 2004; Xi *et al.*, 2012). Finding antibiotic resistance in copiotrophic bacteria is not novel

but probing antibiotic-resistance in oligotrophic bacteria of a dynamic fluvial system certainly deserves an element of novelty. Most of the reports on antibiotic resistance have been done on copiotrophic bacteria (standard plate count bacteria; retrieved on nutrient-rich media) but not on oligotrophic ones. Since oligotrophic bacteria (which can sustain long period of low nutrient conditions) have been reported from clinical sample (Tada *et al.*, 1995), hospital tap water (Katsunori and Masafumi, 2006), and filtered water used for patients in the hospital (Audic *et al.*, 2007) which may cause opportunistic infections in susceptible patients, the present study on antibiotic-resistance in oligotrophic bacteria present in environmental samples has become immensely important. A noteworthy paper by Chee *et al.* (2001) addressed the critical area of antibiotic resistance and dissemination of resistance genes in the environment. Surface waters, like lakes and rivers, could promote mobilization of resistance genes through the aquatic food chain with consequent exposures to humans. Therefore surveillance programmes are needed to understand the size of the problem and also for the better control of antibiotic resistance. The results of this study revealing high frequency of antibiotic-resistant oligotrophic bacteria, in a major river of northern West Bengal draining one of most populous cities, have broadened the complexity of the problem.

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Detection of class 1 integrons and molecular characterization of their gene cassettes in facultative oligotrophic bacteria of River Mahananda

2.1. Background

The localization of antibiotic-resistance determinants onto mobile entities such as plasmids and transposons readily explained the phenomenon of lateral gene transfer. On further examination of these resistance determinants located on mobile plasmids and transposons, a novel type of genetic element which later on termed as "INTEGRON" was discovered (Stokes and Hall 1989; Hall and Collis, 1995). Integrons are one of the genetic elements involved in the adaptation of bacteria. Integrons are found in plasmids, chromosomes and transposons. It consists of a gene encoding a site specific recombinase along with a specific site for recombination capable of recruiting gene cassettes. However, the element differed from transposons in two important characteristics: (i) Transposons have direct or indirect repeat sequences at their ends, but the regions surrounding the antibiotic resistance genes in the integrons were not repeats, and (ii) the integrons contained a site-specific integrase gene of the same family as those found in phage but lacked many gene products associated with transposition. Due to these differences, the elements were not grouped with transposons (Martinez and de la Cruz, 1990; Ouellette and Roy 1987, Stokes and Hall 1989, Sundstrom *et al.* 1988).

Integrons, a natural gene expression system: The present day definition of integrons was formulated by Hall and Collis (1995). Integron units are naturally occurring gene expression systems that can potentially take into custody one or more circularized open reading frames (termed as gene cassettes) and convert them into functionally expressed genes (Martinez and de la Cruz, 1990; Collis and Hall, 1992; Collis *et al.* 1993, Hall and Stokes 1993, Hall and Collis, 1995; Hall and Collis, 98, Rowe-Magnus *et al.* 1999, Rowe-Magnus *et al.*, 2002). It is these gene cassettes that encode mostly (but not always) the resistance determinants to several antimicrobial agents (Fluit and Schmitz 2004). The integrons themselves are defective for self-transposition but this defect is often complemented through their association with IS, transposons and/ or conjugative plasmids that can serve as vehicles for the intra and inter-species transmission of these genetic structures. These novel DNA elements are frequently found as part of chromosomally located or plasmid residing transposons, including *Tn21, Tn1403, Tn1404, Tn1696, Tn1412* and *Tn 2000* (Carattoli *et al.*, 2001; Naas *et al.*, 2001; Partridge *et al.*, 2001; Patridge *et al.*, 2002; Sundin, 2002; Villa *et al.* 2002). The plasmids that harbor integrons are often large (>100kb) conjugative plasmids belonging to groups IncFI, IncFII or IncL/M. Class 1 integrons are found associated with a variety of insertion sequence elements, including *IS*26, *IS*1999, *IS*2000 and *IS*6100. Most frequently occurring IS element at the 3' - end of integrons is *IS*6100.

2.1.1. Types and structural organization of Integrons

Integrons have been categorized in two major groups: "chromosomal integrons" and "mobile integrons". Chromosomal integrons are located on the chromosome of bacterial species; *insilico* study demonstrates that 17% of the bacterial genomes exhibit such genetic arrangements (Cambray *et al.*, 2010). Chromosomal integrons are frequently described in bacteria isolated from marine or terrestrial ecosystems, for example *Vibrio* spp. and *Xanthomonas* spp. The chromosomal integrons have also been termed "super-integrons" (SIs) as they can carry large numbers (>100) of cassettes

that mainly encode proteins with unknown functions. While mobile integrons are located on mobile genetic elements such as transposons and plasmids, which promote their dissemination among bacteria but they are not self-movable. Mobile integrons contain a very limited numbers of gene cassettes (Naas *et al.*, 2001).

On the basis of amino-acid sequence identity of the IntI protein mobile integrons have been classified into several classes and of them classes 1-3 are more commonly studied (Hochhut *et al.*, 2001; Cambray *et al.*, 2010; Xu *et al.*, 2011; Stalder *et al.*, 2012). The gene cassettes (mobile DNA elements which is found in free circular form when not incorporated in integron platform) within these mobile integrons often encode antibiotic-resistance determinants. Integrase gene (*intI*) is responsible for the synthesis of the enzyme, integrase, which helps in the integration and excision of gene cassettes in the integron. The enzyme recognizes site-specific recombination sites for integration. Approximately 45-58% homology exists between the three-integrase classes suggesting that their evolutionary divergence has extended over a longer period than the 50 years of the antibiotic era (Rowe-Magnus and Mazel, 1999). The class 1 integron platform is the most ubiquitous among multi-drug resistant bacterial populations and is found associated with *Tn21* transposon family (Hall, 1997) while class 2 integron are found in association of *Tn7* transposon family (Hall and Stokes, 1993). Arakawa *et al.* (1995) identified class 3 integron platform on a large transferable plasmid in a *Serratia marcescens* strain. Correia *et al.* (2003) revealed the presence of a new class 3 integron on p22K9. The integron, which was previously designated class 4, is now named *Vibrio cholerae* SI (Fluit *et al.*, 2004). This distinct type of integron is now known to be an integral component of many Gammaproteobacterial genomes (Rowe-Magnus *et al.*, 2001).

Integrons possess two conserved segments separated by a variable region that includes different combinations of inserted gene cassettes. The essential components found within the 5' conserved segment include the *intI* gene of tyrosine recombinase family (Nunes-Duby *et al.*, 1998), which encodes a polypeptide of 337 amino acids, *attI* site which is recognized by the integrase and acts as a receptor for gene cassettes and on the opposite strand, a common promoter region (P_{ANT}) from which integrated gene cassettes are expressed (Collis and Hall 1995). The integrase mediates a site-specific recombination between the *attI* site in the integron and a secondary target called *attC* (59 base element) found in the downstream of the integron associated gene cassettes. Boucher *et al.* (2007) statistically analyzed about 603 completely or partially sequenced genomes, and revealed that 9% of them contained integrons, indicating that integrons are much more widely distributed than previously expected.

2.1.2. Class 1 integron

Class 1, the best-characterized integrons, has been frequently reported in clinical (Martinez Freijo *et al.*, 1998; Martinez Freijo *et al.*, 1999; Chang *et al.*, 2000; Schmitz *et al.*, 2001; White *et al.*, 2001; Thungapathra *et al.*, 2002; Jones *et al.*, 2003; Lindstedt *et al.*, 2003; Betteridge *et al.*, 2011) and environmental isolates (Gebreyes *et al.*, 2002; Chen *et al.*, 2004; Nandi *et al.*, 2004; Nardelli *et al.*, 2012; Stalder *et al.*, 2012). Like the other classes of integrons, their 5' CS contains the *intI*1, and *attI* loci, while their 3' CS is specific and usually contain a truncated antiseptic resistance gene (*qacEΔ1*), a sulfonamide resistance gene (*sul1*) and an open reading frame

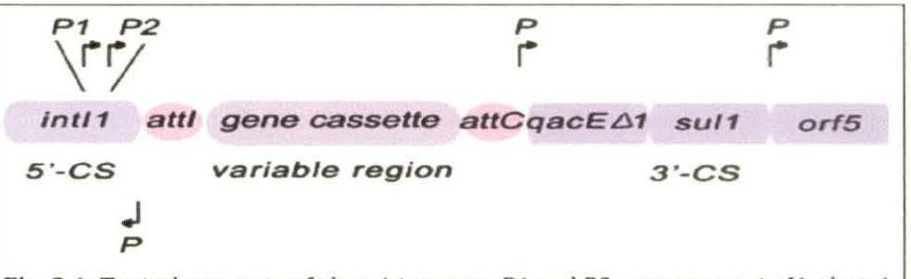

Fig. 2.1: Typical structure of class 1 integron. P1 and P2 , promoters; *intI1*, class 1 integron integrase gene; *attI*, integron associated attachment site; *attC*, cassette associated attachment site; *qacEΔ1*, Quaternary ammonium sulphate resistance gene; *sul1*, sulfonamide resistance gene, *orf5*, unknown function; CS, conserved segment. [Fig. adapted from Xu *et al.*, 2011].

(ORF5) of unknown function (Fig 2.1). Integron integrases, as described earlier, being members of tyrosine recombinase family, possesses four invariant amino-acid residues (RHRY) in conserved motifs (termed as boxes I and II) and mediates recombination between the *attI* site and secondary target called an *attC* site (also known as 59 base element or 59be site). Multiple alignments of integron integrases with tyrosine recombinases have revealed that the DNA binding and recombination properties of class 1 integron integrase variants carried mutations at residues that are well conserved among the tyrosine recombinases and at some residues from the additional motif that are conserved among the integron integrases. The well conserved residues studied were H277 (histidine) from conserved tetrad RHRY (about 90% conserved), E121 (glutamate) found in patch I motif (about 80% conserved in prokaryotic recombinases), K171 from the patch II motif (nearly 100% conserved), W229 (Tryptophan) and F233 (Phenylalanine) from the patch III motif, and G302 (Glycine) of box II (about 80% conserved in prokaryotic recombinases). Additional *intI* mutated residues were K219 and a deletion of the sequence ALER 215. It was observed that E121, K171 and G302 play a role in the recombination activity but can be mutated without disturbing binding to DNA. W229, F233 and the conserved histidine (H277) may be implicated in problem folding or DNA binding. Some of the extra residues of *intI* seem to play a role in DNA binding (K219) while others are implicated in the recombination activity (Reechia *et al.*, 1994).

The *att*C (59 be) is the recocmbination site, present in the gene cassette, recognized by the integron integrase (Stokes *et al.*, 1997). The *attC* sites comprise a family of diverse sequences which are not highly conserved and vary considerably in size from 57 to 141 bp (Collis *et al.*, 1993; Collis and Hall, 1995; Recchia and Hall, 1995; Mazel *et al.*, 2000). The *attC* region consists of four essential sites called 1R, 2R, 1L and 2L, with 1R and 2R as part of the RH consensus sequence, and 1L and 2L as part of the LH consensus sequence (Francia *et al.*, 1999; Fluit and Schmitz, 2004). The similarities of the *attC* sites are primarily restricted to their boundaries, which correspond to the inverse core site as RYYYAAC (R = Purine, Y = Pyrimidine) and the core site as GTTRRRY (Collis and Hall, 1995; Stokes *et al.*, 1997). The *attC* sites are generally associated with a single ORF in a structure termed gene cassettes, which are not necessarily observed in integrons, but once integrated they become part of the integron (Fluit and Schmitz, 1999). Cassettes are inserted at *attI*, a unique integrase recombination site located in the 5' conserved region of integron adjacent to the integrase gene with a consensus sequence, GTTRRRY (core site). Recombination process occurs between G and first T. The 59 be which occurs at 3' end consists of an inverted imperfect repeat between 50 and 150 bp which has an inverse core site at the 5' end of the inverted repeat and a core site at the 3' end. The insertion of gene cassette into *attI*1 site results in the formation of a secondary site (*attC*) downstream of the cassette.

Three formally distinct reactions can be catalyzed by the class 1 integrase, *intI1*, which involve recombination either between *attI1* and a 59 base element (be), two 59 be or between two *attI1* sites. Experimental evidences have shown that events involving two *attI1* sites are less efficient than the reactions in which a 59-be participates (Partridge *et al.*, 2000). Actually an unusual reaction between the *attI*1 site and a 59-be appears to be responsible for the loss of the central region of a 59-be to create a potential fusion of two adjacent gene cassettes. The full *attI*1 site, 65 bp in length, is required for high efficiency recombination with a 59-be site. Each integron structure carries only one *attI*1 site located at the 5' border of the cassette closest to the promoter. All sites further downstream belong to the *attC* type (Hanson *et al.*, 1997). The structural difference between the *attI*1 sites from that of the 59-be site plays a vital role in ensuring that cassettes are preferentially integrated adjacent to the *attI*1 site of a class 1 integron. However, the only common feature between *attI*1 and 59 be is the 7 bp core site (GTTAGGC or GTTRRRY). Both an identifiable 7 bp inverse core site and the extensive inverted repeats associated with 59 be is absent in *attI1* (Reechia *et al.*, 1994). The degrees of conservation in 59-base elements are not high and their lengths vary

from 57 to 141 bp. They can be identified by their location and the relationship of over 20 bp at their outer ends to consensus sequences that are imperfect inverted repeats of one another.

2.1.3. Gene cassette

The antibiotic resistance genes that captured by integrons, are located on the mobile circular DNA element called as gene cassette. The cassettes usually consist of a promoter less coding gene sequence (most commonly an antibiotic resistance gene but may carry non-antibiotic resistance genes) and at the 3' end of this sequence, a so-called 59 base element (*attC* site). Hence, gene cassettes are not necessarily part of the integron. In nature they exist in a free circularized form but cannot be replicated or transcribed in this form. A recombination event that occurs between *attI*1 and *attC* follows integration of the cassettes into the integron variable region. The gene on the cassette is then bound by the *attI*1 site on the 5'-side and by *attC* on the 3'-side. The *attC* sites comprise a family of diverse sequences which are not highly conserved and vary considerably in size from 57 to 141 bp (Collis *et al.*, 1993; Collis and Hall, 1995; Recchia and Hall, 1995; Mazel *et al.*, 2000). The *attC* region consists of four essential sites called 1R, 2R, 1L and 2L, with 1R and 2R as part of the RH consensus sequence, and 1L and 2L as part of the LH consensus sequence (Francia *et al.*, 1999; Fluit and Schmitz, 2004). The similarities of the *attC* sites are primarily restricted to their boundaries, which correspond to the inverse core site as RYYYAAC and the core site as GTTRRRY (Collis and Hall, 1995; Stokes *et al.*, 1997). The integrase, apart from catalyzing the recombination between *attI*1 and *attC* sites, also facilitates excision events that can lead to loss of cassettes from an integron and generate free circular cassettes. In general, Gene cassettes consist of one coding sequence but may also contain a variable number of non-translated nucleotides (Fluit and Schmitz, 1999). The gene cassettes in an integron are all inserted in the same orientation with respect to their coding regions and are expressed from a common promoter (Pc) region located 214 bases from the inner boundary of the 5' conserved segment (Collis and Hall, 1995) of integron. In fact, the Pc of class 1 integrons potentially contains two promoters, P1 and P2. Four different P1 and two different P2 promoters have been described (Stokes and Hall, 1989, Bunny *et al.*, 1995). The start codons of many gene cassettes largely remained undetermined, but the first in frame start codon is generally assumed to function as such. Few gene cassettes appear to carry their own promoter sequences but most gene cassettes are transcribed from a common promoter region.,The first gene cassette with its own promoter described was the chloramphenicol resistance determinant *cmlA* (Stokes *et al.*, 1991, Bissonette *et al.*, 1991). The *qacE* and *qacG* gene cassettes carry their own promoter sequences as well (Paulsen *et al.*, 1993, Laraki *et al.*, 1999, Guerineau *et al.*, 1990). Summarily, the gene cassettes may be characterized as follows (i) Gene cassettes are discrete genetic elements that may exist as free, circular, non- replicating DNA molecules when moving from one genetic location to other, but are normally found as linear sequence that contribute part of a larger DNA molecule such as plasmid or bacterial chromosome, (ii) Gene cassettes normally contain only a single gene and an additional short sequence, called 59 be, that functions as a specific recombination site, (iii) The cassettes are small, normally ranging from 500-1000 bp, (iv) The genes carried on gene cassettes usually lack promoters and are expressed from a common promoter of integron, (v) A cassette may carry two genes; these are generated by the fusion of two individual cassettes, which at one time were side by side. The double gene cassette being generated by a deletion that recovered sequences on either side of the joint boundary, including the 59 be that was located at the end of the first gene, i.e, the one that reads towards where the joint boundary was.

In naturally occurring integrons there appear to be no restrictions on the number or order of inserted cassettes. Arrays of several different antibiotic resistance genes can be created following successive integration or deletion of the gene cassettes from the respective integron structures (Fig. 2.2 and Fig. 2.3). As an effect, the cassette containing the gene that encodes resistance to the antibiotic in the environment will be closest to the promoter. In this manner, the resistance gene

necessary for the survival will be maximally expressed. Due to their ability to acquire new genes, integrons have a clear role in the evolution of the genomes of the plasmids and transposons that contain them. Mostly gene cassettes identified between the resistance integrons are those conferring resistance to antibiotics. The list is of antibiotic resistance genes, covering most classes of antimicrobials structured as gene cassettes are growing day by day (Fluit *et al.*, 2004; Gillings *et al.*, 2008a).

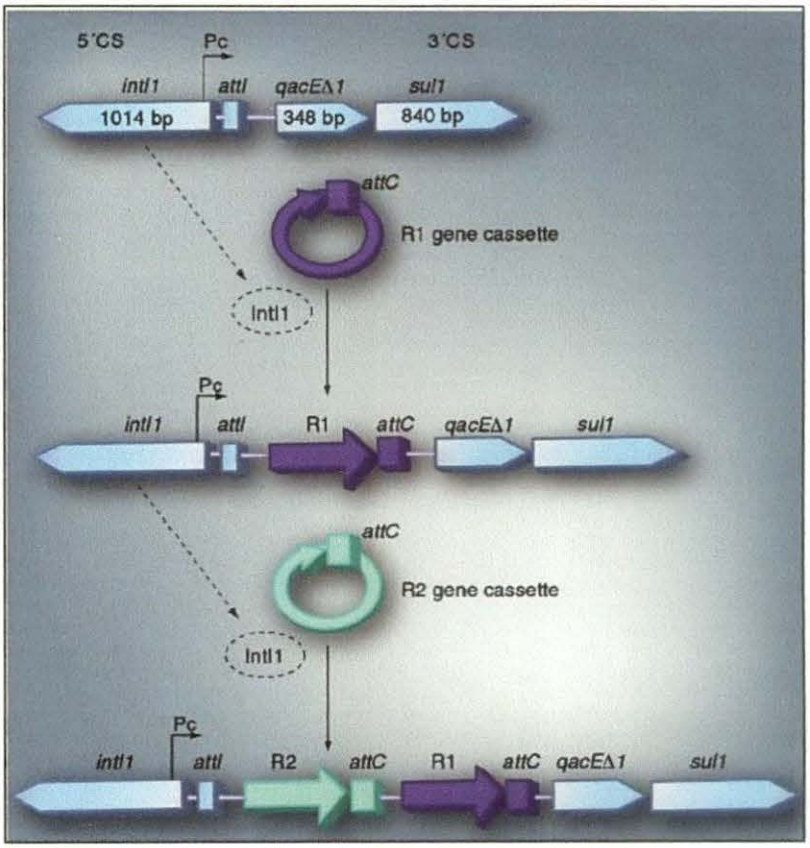

Fig. 2.2: The process of sequential integration of gene cassettes within integron platform following site-specific recombination between *attI* site on integron and *attC* site on gene cassettes. (i) Acquirement of the first gene cassette by using *attI* site. (ii) Acquirement of the second gene cassette by using the same *attI* site. (iii) Integron structure with two inserted gene cassettes. Symbols, IntI: Integrase; *attC* (59be): Recognition site for the integrase; Pc: Promoter. [Fig. adapted from Zhao and Hu, 2011].

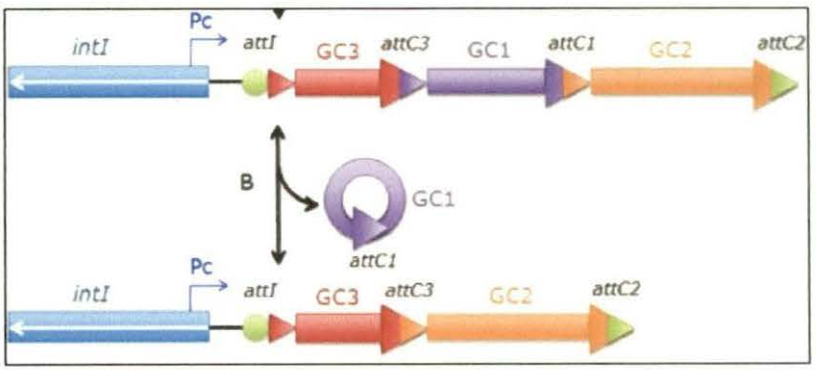

Fig. 2.3: The process of excision of gene cassette (GC) by site-specific recombination mechanism. The IntI1 protein catalyzes the insertion and excision (ii) of the GC in the integron. GC excision preferentially occurs between two *attC* sites. The GC1 is excised following the recombination between the two *attC1* and *attC3* sites. Symbols Pc: gene cassette promoter; *attI*: integron recombination site; *attC1*, *attC2*, and *attC3*: *attC* GC recombination sites; *intI*: the integrase gene; GC1, GC2, GC3 are the gene cassettes, and arrows indicate the direction of coding sequences. [Figure adapted from Stalder *et al.*, 2012].

Dissemination of gene cassettes: Spread of antibiotic resistance genes by horizontal transfer has led to the rapid emergence of bacterial multi-drug resistance (MDR), especially among the gram-negative enteric species (Chen *et al.*, 2004). The spread of resistance is greatly enhanced when the resistance genes form part of a mobile gene cassette and reside themselves on resistance determinants like plasmids and transposons. The horizontal transfer of these gene cassettes occur by several mechanisms which include (i) Mobilization of individual cassette by the integron encoded integrase. (ii) Movement taking place when the integron containing the cassette relocates-probably by targeted

transposition. (iii) Dissemination of large transposons such as *Tn21* carrying integrons and (iv) Movement of conjugative plasmids containing integrons among different bacterial species.

2.1.4. Integron Epidemiology

Integrons in clinical samples: Several groups have made systematic surveys on integron distribution in different environmental settings. A large proportion of such studies have dealt with the isolates from clinical settings where they have contributed significantly to the prevalence and dissemination of antibiotic resistance genes. Several studies have demonstrated broad distribution of class 1 integrons and their significant association with resistance to multiple classes of antibacterial compounds in European hospitals. Schmitz *et al.* (1999) tested 278 consecutive blood isolates belonging to 11 different gram-negative species and detected dominance of Class 1 integrons among them. 13% of these, belonging to six species, were shown to carry an integron. It was found that 42% of 163 strains (representing 13 species of Gram-negative bacteria), isolated from European countries, carried an integron. Another study on 900 blood culture isolates, representing five enterobacterial species, had revealed a gradual increase in the rate of occurrences of class 1 integrons among them over a period of seven years. The results, of this study indicated that prevalence increased from 4.7% in 1993 to 9.7% in 1996 and finally to 17.4% in 1999 (Schmitz *et al.,* 2001). A Chilean study investigated *Acinetobacter baumannii* isolates in which 17 isolates were found carrying integron (Gonzalez *et al.,* 1998). In Taiwan, 52% of the tested clinical isolates of *Escherichia coli* showed presence of class 1 integrons (Chang *et al.,* 2000). Ploy *et al.* (2000) analyzed twenty *Acinetobacter baumannii* strains containing integron. The results indicated that integrons play a major role in development of multi drug resistance in *Acinetobacter.* The presence and spread of class 1 integrons among epidemiologically unrelated nontyphoidal *Salmonella* strains, isolated between 1989 and 1998 in a Spanish region had been noticed by Guerra *et al.* (2000). White *et al.* (2001) revealed the incidence of class 1 integrons in a collection of 120 urinary isolates collected from nine clinical settings in Sydney, Australia. A study by Lindstedt *et al.* (2003), reported presence of class 1 integrons in clinical strains of *Salmonella enterica* subsp. *enterica* serovars *Typhimurium* and *Enteritidis,* from Norwegian hospitals. Heir *et al.* (2004) demonstrated an overall low and stable prevalence of class 1 integron gene cassettes in clinical *Enterobacteriaceae* and *E. coli* isolates in Norway. Put latest data. Human pathogenic samples are not the only reservoirs of integrons. Apart from their abundance in clinical samples, the distributions of class 1 integrons have also been evidenced from samples other than clinical interest. Integrons were found in gram-negative isolates from primates (Fluit *et al.,* 1999). Integrons have even been detected in a *Pseudomonas* spp. from an apple orchard (Schnabel *et al.,* 1999). Mazel *et al.* (2000) conducted a study on clinically unselected Enterobacteria to find out the prevalence of class 1 integrons among them. For this, 72 *Escherichia coli* strains isolated from a variety of animal hosts and a variety of geographic locations, were analyzed and only four of them were found to harbor the respective structure. In the United States, Goldstein *et al.* (2001) reported for the first time the distribution of class 1 to 4 integrases in veterinary Enterobacterial members isolated from livestock, companion animals, and exotics. Class 1 integrons have been identified in isolates obtained from cattle (Morabito *et al.,* 2002), swine (Sunde *et al.,* 2001, Gebreyes *et al.,* 2002), chickens (Bass *et al.,* 1999), fish (L'Abée-Lund and Sørum, 2001; Nawaz *et al.,* 2010). Incidence of class 1 integrons had also been evidenced in *Salmonella* isolates recovered from retail meats purchased in United States and Peoples Republic of China (Chen *et al.,* 2004). A large collection of Irish thermophilic *Campylobacter* isolates, from human and poultry sources, was investigated for the presence of integrons by O'Halloran *et al.* (2004). Their presences have also been described in isolates obtained from farm and nonfarm animals such as swine, dogs, cats, horses, deers (Kadlec and Schwarz, 2008; Yang *et al.,* 2010), zoo animals (Mazel *et al.,* 2000; Ahmed *et al.,* 2007; Sato *et al.,* 2009) and Birds (Nebbia *et al.,* 2008). Recently class 1 integron detected in 67% of the total Gram-negative bacteria collected from animal samples like caecum,

faeces, intestine, oviduct, skin or vaginal fluid of cats, dogs, jennies, deer, eagles, emus, fox, owls, snake, squirrels, chickens, gilthead breams, rabbits and trouts (Domingues *et al.*, 2010). Recently in Portugal the *intI1* gene was identified in 72.5% of *S. Enterica* isolates recovered from Bísaro pigs and wild boars (Caleja *et al.*, 2011). Integron sequences have also been detected from soils (Neild *et al.*, 2001; Heringa *et al.*, 2010). Using culture independent methods, they detected several novel integrase genes.

Integrons in aquatic environment: Predominance of class 1 integrons have also been evidenced in various environmental samples including fish farms, lakes, estuary, irrigation water sources, and other aquatic environments. 109 gram-negative bacteria out of 3000 bacteria isolated from an estuarine environment analyzed, showed presence of *inti1* gene of class 1 integrons. Characterization of the integrons revealed that majority lacked integrated gene cassettes in the variable region. The frequent identification of empty integrons led to support the view that antibiotic selective pressure might play a significant role in promoting the incorporation and maintenance of gene cassettes in integron variable regions (Rosser and Young, 1999). A collection of 313 motile Aeromonads isolated at Danish rainbow trout farms were analyzed to identify some of the genes involved in high levels of antimicrobial resistance. In 135 isolates combined sulfonamide/trimethoprim resistance appeared closely related to the presence of a class 1 integron (Schmidt *et al.*, 2001). Study related to coliform bacteria isolated from the aquatic environment led the detection of class 1 integrons, and 24% of the isolates were found to carry *intI1* gene. Some of the isolates had the incomplete or nonfunctional class 1 integrons. The results indicated the possible role of antibiotic selective pressure for the maintenance of gene cassettes within the class 1 integron structure. In the absence of sustained antibiotic pressures, such as the aquatic environment, coliform bacteria may carry empty or non-functional class 1 integrons (Park *et al.*, 2003). Irrigation water and sediments contaminated with fecal bacteria could serve as the source of class 1 and class 2 integron bearing *Escherichia coli* (Roe *et al.*, 2003). Another study reported the incidence of enteric bacteria isolated from Mhlathuze River and the distribution of genetic elements that might be responsible for the observed antibiotic resistance. PCR based methods demonstrated the presence of class 1 integrons in more than 50% of those environmental bacteria that were multiple antibiotic resistant (MAR).

Integrons in Gram-positive bacteria: Furthermore, functional multi-resistance integrons are no longer restricted to the gram-negative bacteria. A survey by Kazama *et al.* (1998) demonstrated the presence of *qacEΔ1* in both Staphylococcal and enterococcal isolates. A truncated integron in *M. fortuitum* and a complete and functional integron in another gram-positive bacterium, *Corynebacterium glumaticum* have also been found. Antibiotic resistance genes and integrons in poultry house litter from commercial poultry farms have been quantified by Nandi *et al.* (2004). The authors found that integrons and associated resistance genes abound in several genera of gram-positive bacteria that constituted more than 85% of the litter community. In Guangzhou, China, Shi *et al.* (2006) isolated 46 gram positive bacteria from clinical specimen. PCR analyses of said isolated revealed the presence of class 1 integron in all the isolates. Very recently the consequences of surveillance and investigation on antibiotic resistance resulted in identification of class 1 integron in Methicillin-resistant staphylococci (MRS), the most prevalent pathogens that cause nosocomial infections throughout the world and labeled as a "super bug" in antibiotic resistance (Xu *et al.*, 2011).

2.1.5. Integron study in India

Above data suggested that integrons are evenly distributed among all members of bacterial families, and usually contribute antimicrobial resistance to them. Few studies in India have shown the prevalence and distribution of integrons from distinct clinical settings. Investigation of multidrug

resistance in *Vibrio cholerae* strains isolated from Calcutta, India, has established the association of antibiotic resistance phenotype with the presence of integrons (Thungapathra *et al.* 2002, Amita *et al.* 2003; Shi *et al.*, 2006). The gene cassettes identified in *V. cholerae* strains were *dfrA1, dfrA15, dfrA5, dfrA12, aac (6')-Ib, aadA1, aadA2* and *ereA2*. A new antibiotic resistance gene *aac(3)-Id* (aminoglycoside acetyltransferase gene) harbored in class 1 integron was reported in 2004 from a clinical isolate, *Vibrio fluvalis* H-O8942 (Ahmed *et al.* 2004). The isolate was also found to carry a class 1 integron borne aminoglycoside adenyltransferase gene, *aadA7*. In the same year a study conducted on *E. coli* isolates obtained from UTI (urinary tract infection) of pregnant woman at the Christian Medical College Hospital, Vellore, India, showed that all the tested isolates carried integrons (Mathai *et al.*, 2004). The β-lactamase content of a multi-resistant strain of *Pseudomonas aeruginosa* clinical isolate from the Indian subcontinent was analyzed by Aubert *et al.* 2004 which further underlined its spread in Asia. A study conducted on copiotrophic bacteria isolated from river Torsa, India, showed that out of 100 MAR copiotrophic isolate, 40 were bearing the class 1 integron and the gene cassattes detected within class 1 integron were: *dfrA1, dfrA5, dfrA7, dfrA17,* a variant of *dfrA12, aac(6')-Ib, aadA1* and *aadA6* along with one novel ORF (Mukherjee and Chakraborty, 2006). Shahid (2010) reported *sul1* type integron in 32.5% *Citrobacter* spp. isolated clinical samples. A study on clinical *Klebsiella pneumoniae* strains revealed 92% carriage of integrons (Bhattacharjee *et al.*, 2010). The bla_{CTX-M} and bla_{TEM} resistance markers associated with the class 1 integron in ESBL-producing uropathogenic *E. coli* isolates have been reported from India (Akram *et al.*, 2011). The most prevalent gene cassette array detected in the above said study was "*aadA5-dfrA17-dfrA7*". In a recent investigation dfrA7 and *dfrA1-orfC* cassettes along with an adjacent dihydropteroate synthetase gene were detected in class 1 integron of nontyphoidal *Salmonella* isolates recovered from 40 seafood samples (Deekshit *et al.*, 2012). The same study has also demonstrated the presence of silent *catA1* gene in phenotypically sensitive bacteria.

In the present study, a total of 2188 purified facultative oligotrophic bacterial isolates were examined for the presence of class 1 integrons, using a highly reproducible PCR strategy. Selection of 2188 facultatively oligotrophic bacterial colonies/isolates was random (without any human biasness), because research randomizer tool was used which generated a random set from non-selective plates serving as an universal set of numbers (each isolated colonies bearing a number) irrespective of the knowledge of the phenotype (sensitive, single or multiple-antibiotic-resistant). Presence of class 1 integrons was confirmed in only 89 Gram negative isolates and one Gram positive isolate. The cloning and sequencing of amplicons enabled to characterize the nature of gene cassettes present in ninety integron-positive oligotrophic bacterial strains. The descriptions of ORFs encoding proteins for antibiotic resistance and other unrelated functions have been presented in this chapter.

2.2. Materials and Methods

2.2.1. Random selection of facultatively oligotrophic isolates and detection of class 1 integrons in them

2188 discrete unique numbered (generated using research randomizer; www.researchrandomizer.org) facultatively oligotrophic bacterial colony were selected from the oligotrophic bacterial population cultured on NPA (nutrient-poor agar) medium from 90 water samples, collected in three consecutive years (2007-2009). Each selected bacterial colony was purified and master plate (containing R2A agar) was constructed. Master plate constructed with purified facultative oligotrophic bacterial colonies were replicated on R2A agar plate amended with defined concentration of each antibiotic tested (please see chapter 1), and antibiotic-resistance-profiles were prepared on the basis of their responses to 12 different antibiotics. All the facultative isolates (2188 isolates) of second generation master plate were screened for the presence of class 1 integron by using polymerase chain reaction (PCR) method. The antibiotic resistance profile (ARP)

of integron positive isolates were cross checked after conducting PCR-based screening for detection of class 1 integrons. The isolates bearing class 1 integrons were then subjected for phenotypic/ and or genotypic analyses for identification of tentative genus.

2.2.2. Antibiotic resistance determination of oligotrophic bacteria

Antibiotic resistance was determined according to the method described in chapter 1.

2.2.3. Polymerase chain reaction (PCR)-based screening for detection of class 1 integrons

To identify the presence of class 1 integrons, a CS-PCR (conserved segment polymerase chain reaction) was performed according to the method described earlier (Levesque *et al.*, 1995). Since primers 5′ CS (5′-GGCATCCAAGCAGCAAG -3′) and 3′ CS (5′-AAGCAGACTTGACCTGA-3′) used in this PCR anneal specifically in the 5′ and 3′ CS regions of class 1 integrons, the amplicons contained inserted gene cassettes flanked on both sides by small parts of the CSs. Primer Int_2F (5′-TCTCGGGTAACATCAAGG-3′), specific for the 3′ region of the integrase gene was also used in combination with the 3′ CS primer to show the proximity of the inserted gene cassettes to *intI* and to confirm the general structure of integron (Martinez-Freijo *et al.*, 1998, Schmitz *et al.*, 2001). The location of primer Int2F is ~ 600 bp upstream to the position of 5′ CS primer (as it is expected to generate an additional length of ~ 600 bp compared to the corresponding product length of the CS-PCR)

The DNA templates for PCR were prepared as described by Levesque *et al.* (1995). Bacterial isolates were grown in 5 mL R2A broth in absence (for sensitive isolates)/ or presence of a selective antibiotic (in case of resistant ones) at 30 °C overnight, then 0.2 mL of the overnight grown culture was added to 0.8 mL of double distilled/or de-ionized water and boiled for 10 minutes. The bacterial suspension was then centrifuged at 6000 rpm for 5 minutes to remove cell debris and the supernatant obtained was used as the template.

Another method (this study) was also developed which was equally efficient to method described above for DNA preparation. In this method, 2-4 colonies grown for 24-72 hrs (depending on bacterial growth) on solid agar medium (R2A /or Luria agar) were scraped and transferred to the 1.5 mL microcentrifuge tube containing 0.2 mL sterile distilled water. The bacterial cells were suspended by tapping or by vortexing. The tubes containing cell suspension were closed tightly and placed on floater. The whole set was then placed in a beaker or plate containing double distilled water. The external water served as medium to transfer heat (conduction) to cell suspension present inside tubes it also prevent the bumping of bacterial cell suspension inside microcentrifuge tube. The cell suspension was then exposed to microwave for 90 seconds at 800 watt. The lysed cells were cooled to room temperature and centrifuged at 8000 rpm for 2 minutes. The supernatant used as DNA template in PCR reaction to amplify integron or 16S rRNA gene sequences.

PCR amplification was performed in 25 µL reaction volume. Each 25 µL PCR mix contains; 1.5 µL of 10mM dNTP mix, 2.5 µL of 10X buffer containing 15 mM $MgCl_2$, 6.0 pmol of each forward and reverse primer, 3 µL of template DNA and 0.8U *Taq* DNA Polymerase. Taq DNA polymerase was added after 12 minutes at 94°C (hot start method). However addition of Taq DNA polymerase along with the reaction mixture was equally efficient as in hot start method. The PCR was done in a GenAmp PCR system (Applied Biosystems) and 96 well DNA engine (BioRed, USA). The thermal programme followed for the amplification: denaturation for 1 minute at 94 °C, annealing at 55 °C for 1 minute, and 2-3 minutes of extension at 72 °C for a total of 30 cycles with final extension of 7 minutes at 72 °C. In all reactions, PCR set up containing whole cell DNA of *Morgnella* sp. TR90 (integron positive from our laboratory) was used positive control. The genomic DNA of *Escherichia coli* JM109 and sterile distilled water were used as negative controls. The chemical used in PCR reaction were purchased from Genei, India and Sigma Aldrich.

2.2.4. Gel electrophoresis and size determination of the CS-PCR products

After PCR amplification, 5-7 µL of the reaction mix containing amplified product were electrophoresed on 1% (w/v) agarose (SRL, India) gel containing 0.5 µg/mL ethidium bromide [1% (w/v) agarose in 1X Tris-acetate-EDTA (TAE) buffer, pH 7.8 (Sambrook and Russell, 2001)]. Electrophoresis was performed in 1X TAE buffer. A 500 bp ladder (Genei, India) was used as the molecular size marker. DNA bands were visualized under UV-transilluminator (Gibco-BRL, USA) and size of amplicons determined.

2.2.5. Cloning of CS-PCR products

2.2.5.1. Purification and Elution of the PCR products

The CS-PCR products which shows clear single prominent band were directly purified using GenElute™ PCR Clean-up kit (Sigma-Aldrich, St. Louis, MO) following manufacture's instruction. The CS-PCR products which shows two or more bands, first precipitated by dehydrated ethanol and then suspended in 25 µL TE (10mM Tris-Cl : 1 mM EDTA, pH 7.5). 20 µL of the suspended DNA was loaded in an 'Low Melting Point agarose' gel and were subjected to electrophoresis at 40–45 mV for at least 8 –10 h. Setting and running of the gel was performed at 4 ºC in 1X TAE. The required band (s) were sliced and taken in a 2 mL microcentrifuge tube, and were melted at 65 ºC for 5 min. To the melted agarose equal volume of TAE buffer was added and agarose particles were removed by repeated phenol extraction. Finally DNA was precipitated, suspended in TE (10 mM Tris-Cl: 0.1 mM EDTA, pH 7.5) and quantified.

2.2.5.2. Preparation of competent *E. coli* by $CaCl_2$ method

Competent cells were prepared as described in Molecular cloning (Sambrook and Russell, 2001) volume 2.

2.2.5.3. Cloning of the purified PCR product

The pGEM–T Easy Vector System II (Promega Corporation, Madison, USA) was used for the cloning purpose of PCR products. For cloning ligation mixture were prepared according to the manufacturer's instructions and then transformed in competent *E. coli* (JM109, XL1 Blue and JM101) cells following heat shock method (Sambrook and Russell, 2001)

2.2.5.4. Selection of recombinants

The recombinants were selected on LB agar plate containing IPTG (isopropylthio-β-galactoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and amicillin (100 mg/L) as selection marker. Blue-white screening enabled to discriminate recombinant plasmids (white colored colonies) from non-recombinant (blue colored colonies). Recombinants for the presence of the desired insert were confirmed by PCR reaction using 5′ CS and 3′ CS primers. Recombinant-plasmids were isolated by alkaline lysis method as described in Molecular cloning (Sambrook and Russell, 2001). Recombinant plasmid (clone) containing gene of interest was further confirmed by restriction digestion using *Eco*RI restriction enzyme. The fragments were separated on 1% agarose gel and visualized under UV trans-illuminator after staining with EtBr (ethidium bromide) dye.

Reagents used for plasmid isolation (alkaline lysis method)

Solution I	**Solution II**	**Solution III**
Glucose: 50 mM	NaOH: 0.2 N	5 M CH_3COOK: 60.0 mL
Tris-Cl (pH-8.0): 25 mM	SDS: 1% (w/v)	CH_3COOH: 11.5 mL
EDTA (pH-8.0): 10 mM		H_2O: 28.5 mL

Sol^{n} Ist : Solution was prepared and sterilized by autoclaving at 121 °C for 15 min, and stored at 4 °C.
Sol^{n} IInd: freshly prepared at the time use
Sol^{n} IIIrd: Prepared stock solution was filter sterilized and stored at 4 °C

2.2.6. DNA sequencing and computer analysis of the sequence data

The recombinant plasmids were used for sequencing of the inserts using primers for T7 and SP6 promoters. For large PCR product sequencing, primer walking method was used. Sequencing was performed at DBT-supported DNA sequencing facility at University of Delhi, South Campus, India.

2.2.7. Bioinformatic analyses

Analysis of nucleotide sequences were performed with several bioinformatics tools.

I. ***NEB CUTTER v. 2.0***: For determination of restriction map of the sequence, the software package hosted by New England Biolabs Inc (http://tools.neb.com/NEBcutter2) was used.

II. ***ORF finder***: was used for detection of open reading frame in the nucleotide sequence. This software package is freely available at www.ncbi.nlm.nih. Another package, 'Fast PCR v 3.3.67' of the Institute of Biotechnology, University of Helsinki, Finland, was also used for finding *orfs* in a given sequence.

III. ***BlastN and BlastP suite***: are the freely available software packages (http://www.ncbi.nlm.nih.) which were used for similarity search of nucleic acid and amino acid sequences (Altschul *et al.*, 1997).

IV. ***Conserved domain search (CDD)***: The domain analysis of the amino acid sequence was done using the freely available program of Marchler-Bauer and Bryant (2004) at the website http://www.ncbi.nlm.nih.

V. ***Structure and function analysis***: For determination of probable secondary and three-dimensional structure of the peptides, the software packages, 'PredictProtein' from the website http://www.expasy.org/tools/ and 3D modeling was performed by automated mode 'SWISS-MODEL' (http://swissmodel.expasy.org/): a fully automated protein structure homology modeling server also accessible via ExPaSy web server (http://www.expasy.org). The function of the peptides was analyzed using the ProtFun 2.1 software of the website http://www.cbs.dtu.dk.

VI. ***CELLO v.2.5 and Psortb v3.0.2***: for detection of cellular localization of bacterial protein (available at http://cello.life.nctu.edu.tw; and http://www.psort.org/psortb/ respectively), used for bacterial protein localization prediction.

VII. ***ProtParam analyses***: a tool (available at http://web.expasy.org/protparam/) which allows the computation of various physical and chemical parameters for a given protein stored in Swiss-Prot or TrEMBL or for a user entered sequence. The computed parameters include the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY).

VIII. ***TMMOD tool***: a free server for trans-membrane proteins topology prediction using a hidden Markov model. TMMOD uses TMHMM (Sonnhammer *et al.*, 1998) as a prototype, but differs from TMHMM by the architecture of the submodels for loops on both sides of the membrane. This tool is available at website, http://liao.cis.udel.edu/website/servers/TMMOD/. Another membrane protein secondary structure prediction server (http://split4.pmfst.hr/split/4/), SPLIT 4.0, was used for predicting and comparing the data obtained from one server.

IX. ***Sequence Alignment tools:*** To determine percentage homology between two sequences (DNA or protein) the tool, pairwise alignment (http://www.ebi.ac.uk) was used. For multiple sequence alignment (MSA) following tools were used: ClustalW (http://www.ebi.ac.uk, offline available with phylip package and Mega package), ClustalX, T-COFFEE

(http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee), multiple align show (http:/bioinformatics.org/sms/).

X. ***Ident and Sim:*** this tool was used to calculate similarity and identity ratio of two or more than two protein sequence by pairwise comparisons (http:/bioinformatics.org/sms/)

XI. ***InterProScan:*** InterPro is a resource that provides functional analysis of protein sequences by classifying them into families and predicting the presence of domains and important sites. To classify proteins in this way, InterPro uses predictive models, known as signatures, provided by several different databases that make up the InterPro consortium. For this purpose InterProScan software package (available at http://www.ebi.ac.uk) that allows sequences to be scanned against InterPro's signatures was run.

XII. ***ProQ - Protein Quality Predictor:*** a neural network based predictor working on a number of structural features predicts the quality of a protein model. It is optimized to find correct models in contrast to other methods which are optimized to find native structures. Two quality measures are predicted *LGscore* and *MaxSub*. This is free server available at http://www.sbc.su.se/~bjornw/ProQ/ProQ.cgi
LGscore is -log of a P-value and
MaxSub ranges from 0-1, [0 is insignificant and 1 very significant]

Different ranges of quality based on ProQ server (http://www.sbc.su.se/~bjornw/ProQ/ProQ.cgi):

Correct	Good	Very good
LGscore > 1.5	LGscore > 3	LGscore > 5
MaxSub > 0.1	MaxSub > 0.5	MaxSub > 0.8

2.2.9. Homology modeling of DfrA1 and AadA1 Proteins derived from bacterium MB47:

A homology model of DfrA1 and AadA was built with SWISS-MODEL using default parameters. In generating homology model, SWISS MODEL utilized the PDB file 3e0bB (crystal structure at resolution2.25 Å of dihydrofolate reductase from *Haemophilus influenzae*) showing 30.57% identity and possessing 0.00e-1 E-value with the query DfrA1 protein of MB47 and PDB file 1no5B (crystal structure at resolution 1.80 Å of HI0073, the nucleotide binding domain of the Hi0073/Hi0074 two protein nucleotidyl transferase from *Haemophilus influenzae*) showing 21.84% identity and 2.00e-9 E-value with the query AadA1. The template was automatically selected by the software for building 3D model of DfrA1 and AadA1 protein of MB47.

2.2.10. Accession numbers

The nucleotide sequences of gene cassettes were assigned EMBL and GenBank accession numbers (Table 2.3 A and B).

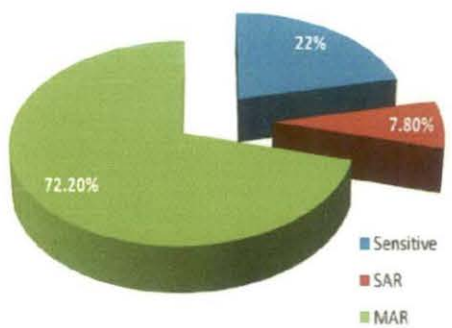

Fig. 2.4A: Frequency of sensitive, SAR (single-antibiotic resistant) and MAR (multiple-antibiotic resistant) among facultatively oligotrophic bacteria those yielded class 1 integron.

2.3. Results

2.3.1. Antibiotic resistance profile of integron positive isolates

The antibiotics-resistance-profile(s) of the ninety class 1 integron bearing facultative oligotrophic bacteria has been presented in Table 2.1. Of the 90 integron positive isolates, 18 (22%) were sensitive to all the twelve antibiotics, 07 (7.8%) were SAR (resistant to only one antibiotic), and 65 (72.2%) were MAR (resistant to two or more antibiotics) (Fig 2.4A).

Among multiple-antibiotic-resistant (65) integron positive isolates, 7 (10.77%) were resistant to two antibiotics, 8 (12.3%) were resistant to three, 5 (7.7%) were resistant to four, 9 (13.84%) were resistant to five, 12 (18.46%) were resistant to six, 9 (13.84%) were resistant to seven, 10 (15.4%) were resistant to eight, 1 (1.53%) was resistant to nine antibiotics, 3 (4.6%) were resistant to ten, 1 (1.53%) isolate was resistant to eleven antibiotics tested (Fig 2.4B). The facultative oligotrophic bacteria resistant to all twelve antibiotics used in this study did not yield any amplicon of class 1 integron. On the basis of resistance index (RI), the facultative oligotrophic bacteria bearing class 1 integrons can be categorized into 11 groups (Table 2.1 and 2.2).

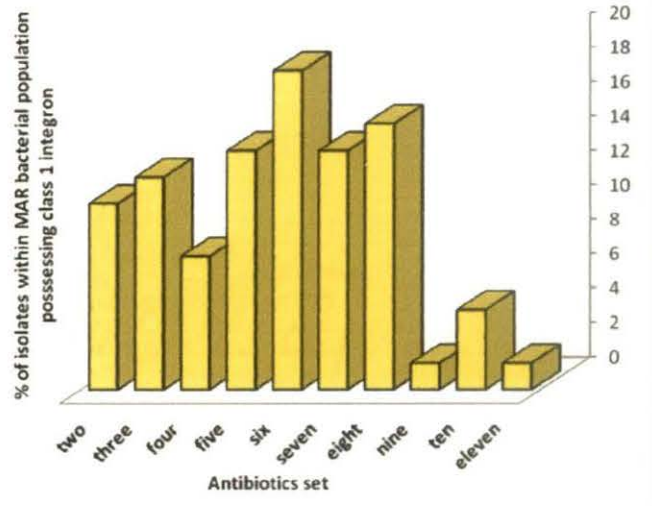

Fig 2.4B: Fractions of class 1 integron positive isolates exhibiting resistance to two and more than two antibiotics tested.

2.3.2. Detection of class 1 integrons

A total of 2188 facultative oligotrophic bacterial isolates, randomly selected (research randomizer tool was used to prevent any human biasness in random selection process) from the pool of oligotrophic bacteria which developed on NPA agar (for detail please see chapter 1), were screened for the presence of class 1 integron by specific PCR (CS-PCR) methodology. Of them, 90 isolates (4.1%) produced amplicon of variable lengths (as the methodology itself enables to amplify the variable region of the class 1 integrons; varied from 0.15 to 3.45 kb) (Fig. 2.5 and Table 2.3A, B, and C). Sequence analyses showed that majority of the amplicons of size >0.7 kb were found to carry antibiotic gene cassettes inserted in class 1 integron platform while in cases where the sizes of the amplicons varied between 0.1 to 0.7 kb, they were either related to empty class 1 integron or were coding for hypothetical proteins. The amplicon lengths ranging from >0.1 to < 0.5 kb of class 1 integron were recorded in five six oligotrophs (OB05, MB05, MB62, MB63, MB70, and MB83). The amplicons ranging between >0.5 kb and <0.7 kb were detected in seven bacteria (MR02, MB41, MB44, MB45, MB54, MB80, and MB81). Amplicons of lengths ranging from >0.7 to <1.0 kb of class 1 integron were recorded in sixteen oligotrophic bacteria (MR01, MR03, SR19, MB31, MB40B, MB42, MB43, MB49, MB51, MB55, MB56, MB57B, MB64, MB76, MB72, and MB77). Amplicon of lengths, ranging between ≥1.0 to ≤1.5 kb were noted in thirty three (OB12, MB12, MB16, MB22, MB48, MR04, MB19, MB39, MB46, MB20, MB24, MB50, MB57A, MB23, MB36, MB32, MB33, MB18, MB66, MB74, MB75, OD05, OD08, OC78, MB79A, MB79B, OD10, OD21, OD24, OC74, MB38, OC75, and NV66) of the 90 class 1 integron positive bacterial isolates. Twenty four isolates (MB52, MB03, MB21, MB26, MB67, MB47, MB40A, MB59, MB27, MB30, MB60, MB61, MB69, MB78, ºC16, MB29, MB34B, MB37A, MB53, MB73, MB25, MB65, OC24, and MB68) were found to carry amplicon of lengths, corresponding to the size which varied between >1.5 but ≤ 2.0 kb. A 2.0 kb and ~ 3.5 kb amplicon was recorded in MB82 and MB35 respectively (Table 2.3B). A very short variable region of 153 bp were amplified in two strains, MB62 and MB63, while 223bp amplicon was generated from isolates MB05 and MB70 (Table 2.3A). The amplicon of size ~1.0 kb was predominating and was detected in 24.4% (22/90) of the total integron positive isolates (Table 2.3 A and B).

2.3.3. Characterization of integron borne gene cassettes

Purified CS-PCR products cloned in pGEM-T Easy vector-II (Fig. 2.6) were subjected to DNA sequencing. Gene cassettes were identified by sequence analysis. The identity of the gene cassettes and their predicted functions are presented in Table 2.3 A and B. Facultative oligotrophic bacteria were found to carry both types of ORFs (i) not related to antibiotic resistance (Table 2.3A) and (ii) antibiotic resistance (Table 2.3B) gene cassettes.

2.3.3.1. Gene cassettes revealing ORFs unrelated to any known antibiotic resistance gene function

The cassettes, yielding ORFs unrelated to any known antibiotic resistance gene function, have been identified in 26 (~29%) of the total 90 isolates (Fig. 2.7). The features of such gene cassettes were analyzed and compared with previously existing data. The sequences derived from amplicons of OB05 and MB83 were found 75% identical (nucleotide-nucleotide) to the part of the genome of *Acidovorax avenae* subsp. *citrulli* (Ac. No. CP000512). The feature present in that part of *Acidovorax* genome included a portion of ribosomal large subunit pseudouridine synthase B. However the predicted polypeptide of the ORF generated from OB05 DNA sequence did not produce any significant similarity with existing sequences of the protein database. Sequence analysis of the amplicons of MB05 and MB70 revealed the presence of a truncated ORF of 64 amino acids that showed 73% identity with the methyl transferase protein from *Ralstonia eutropha* (Ac no. AAZ62060). A translated polypeptide from the sequence derived from bacterium MB09, signifying type I site-specific deoxyribonuclease, HsdR family, exhibited nearly 42% identity with a protein of the same family, found in *Thiomicrospira crunogena* (Ac. No. YP390604). Partial sequencing of the CS-PCR product (Ac. No. AM997273) obtained from MB12 showed the presence of a single ORF (*livM1*) of length 154 amino acids (Fig 2.8A). BlastP analysis (Table 2.4) of this putative polypeptide product exhibited best scores with ABC type branched chain amino acid transport system with 72% identity at amino acid level to the ABC transporter permease of bacterium *Aromatoleum aromaticum* EbN1 (Ac. No. NC_006513). The multiple sequence alignment of LivM1 derived from amplicon sequence of bacterium MB12 with its neighbouring ABC transporter proteins is shown in Fig. 2.8B. The transmemebrane (TM) regions predicted within the ABC transporter protein of MB12 (Protein ID: CAQ53856) are shown in Fig. 2.8C and 2.8D. Analysis revealed the presence of four transmembrane regions in the predicted LiVM1 protein of MB12. The predicted transmembrane (TM) regions (AA position 2-20, 50-74, 87-110, and 119-138) in ABC transporter protein of bacterium MB12 are presented in table 2.5. The conserved domain (CDD: conserved domain database tool available at www.ncbi.nlm.nih.gov) search for putative translated product obtained from translated ORF of MB12 integron sequence revealed that the predicted protein was one of the member of two TM subunits which play role in uptake of branched chain amino acids (Fig 2.8E). Mapping of signature motifs (Fig 2.8F) showed that 19 of 19 [TM_PBP1_LivM_like, a type of transporters which consist a PBP (periplasmic binding protein), two TMs (trans-membrane subunits) and two cytoplasmic ABCs (ATP-binding cassette)] residues which were recognized on the TM-ABC transporter protein sequences of other bacterial species were also found conserved on ABC protein derived of MB12. The theoretical pI and instability index of the putative ABC transporter of MB12 was computed 9.3 and 19.13. The protein was predicted as stable protein with an estimated half life of 10 h (*in vivo* in respect to *E coli*) with a high aliphatic index of 127.21. MB16 was found to carry gene cassette carrying *appA* gene encoding bacterial extracellular solute binding protein. ProtParam computing showed that the extracellular binding protein derived from bacterium was stable and its instability index was computed 18.94. The GRAVY (grand average hydropathicity), aliphatic index and theoretical pI was computed -0.087, 78.5, and 9.26 respectively. Psortb, a tool for subcellular localization prediction showed that the translated product of *appA* gene was periplasmic with localization score of 9.44. Sequence analysis of amplicon obtained from MB 19 has shown the

presence of a unique hybrid DNA sequence. In the 867 nucleotide long sequence, a continuous stretch of 242 nucleotides (from 3 to 244) produced 96% identity with a vertebrate (*Lepilemur dorsalis)* genomic fragment (Ac. No. AJ244007) and residual 623 nucleotide stretch produced (from nucleotides 245 to 867) 74% identities with *Burkholderia xenovorans* LB400 genomic DNA. This genomic DNA region of *B. xenovorans* coded for the putative reverse transcriptase maturase protein. MB 19 sequence yielded an ORF of 227 amino acids that has shown 65% identity with reverse transcriptase maturase protein from *Burkholderia cenocepacia* HI2424 (Ac. No. YP833935). An ORF of 164 amino acid residues (495 nucleotides) obtained following sequence analysis of 1010 bp amplicon of MB 22. The putative polypeptide shared 81% amino acid identity with phosphoribosylformylglycinamidine synthase (FGAM synthase) from *Acinetobacter baumannii* (Ac. No. YP001712860). The partial sequence of MB 28 (408 nucleotide) was 86% identical with *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 (Ac. No.CP000647) genomic DNA. The amino acid sequence (truncated protein) derived from amplicon of bacterium MB28 was encoding a bifunctional putative transcriptional regulator protein, this truncated protein of 84 amino acid shared homology of 96.3% with the similar protein present in *Klebsiella pneumoniae* (Ac. No.CP000647). The Complete sequence of 704 bp amplicon of MB 40B (Table 2.3B) yielded no significant homology with any nucleotide sequence available in the database. The same sequence yielded a complete ORF of 102 amino acids and BlastP analysis of this protein sequence revealed 58% identity with helicase domain protein of *Verminephrobacter eiseniae* EF01 (Ac. No. ABM585806). The sequences derived from amplicons of OB 12, MB 08, MB41, MB 44, MB 48, MB 51, MB 54, MB55, MB80 and MB81 did not produce any significant homology with any protein sequences available in the database. All of them were characterized as hypothetical proteins. All these polypeptides shared very low level of identity (2.03 to 25.19%) among either with each other or to pre-deposited hypothetical protein sequences. The translated product (Fig. 2.9A) of bacterium MB54 revealed a conserved domain belonging to the UPF0153 superfamily (Fig. 2.9B) when subjected to the BlastP search. However the function of proteins of this family is still unknown; since the proteins of this family contains 8 conserved cysteines hence it was expected that they may constitute a metal binding site and therefore might play a role in metal sequestering like Fe-S cluster as part of oxido-reductase complex. Protein homology and CDD search of putative polypeptide of 178 amino acid residues derived from the CS-PCR product of MB 56 isolate was found to carry a conserved protein domain, encoding hemolysin activator/ secretion protein that was involved in intracellular trafficking and secretion. This hemolysin activator protein of MB56 produced 55% identity with the hemolysin activator protein of *Acinetobacter* sp. (Ac. No. YP045656). Translation of DNA sequence obtained from MB 58 and MB71 yielded a truncated ORF of polypeptide of 181 amino acids. The BlastP analysis of the said truncated polypeptide yielded best score (97% identity) with transposase protein of *Acidovorax* sp. (Ac. No. YP987142).

2.3.3.2. Antibiotic resistance gene cassettes

A total of 2188 facultatively oligotrophic bacteria were selected for the detection of class 1 integron in their genome. To fulfill this target, a highly reproducible PCR methodology using specific primer pair (5′ CS and 3′ CS) was used. The class 1 integron was detected only in 90 isolates (4.1%) of the total oligotrophic isolates. Remaining 2098 bacterial strains failed to produce any amplicon with the primer set 5′ CS and 3′ CS.

A strong association between the nature of the gene cassettes and phenotype of the isolates was observed (Table 2.2 and 2.3 B and C). However, exception was noted in some cases of *aadA* gene cassettes where correspondence with the phenotype were not observed. About 71% (64/90) gene cassettes were found to carry antibiotic-resistance genes. The most common carriages were aminoglycoside adenyltransferase gene cassettes such as *aadA, aadA1, aadA2, aadA4,* and *aadA5* which encode aminoglycoside adenyltransferase enzyme conferring resistance to streptomycin/

spectinomycin antibiotics. Two types of dihydrofolate reductases, type-A (*dfrA1, dfrA5, dfrA7, dfrA12, dfrA16, dfrA17,* two novel *dfrA* genes, *dfrA28* and *dfrA30*) and type-B (*dfr-IIe*), conferring resistance to trimethoprim, were found.

Thirty three bacterial strains carried only one antibiotic-resistance gene cassette and twenty five were found to carry an array of two gene cassettes [Table 2.3 B and C]. An array of three gene cassette (*dfrA12-orf40A-aadA2*) was detected in three integron positive isolates (MB40A, MB61, and MB67) [Table 2.3 B]. One bacterium MB79A was having a gene cassette array of type B (also called as typeII) *dfr-IIe (dfrB)* dihydrofolate reductase, and *arr2* gene, responsible for resistance to rifampicin (a semi-synthetic antibiotic agent) [Table 2.3 C]. A gene cassette array of *Oxa1-aadA1* was detected from bacterium MB82 [Table 2.3 C]. This array was responsible for resistance towards β-lactam antibiotics such as ampicillin and aminoglycosides such as streptomycin/spectinomycin [Table 2.3 C]. The bacterium, MB35, revealed the largest array of gene cassettes, *blaIMP-9-aacA4-oxa10-aadA2,* among the all class 1 integron positive isolates. Maximum percentage (10%, of the total resistance cassettes) of two-gene-cassette array was observed for *dfrA-aadA1* type followed by *dfrA17-aadA5* (~8%). Several resistance phenotypes observed with the isolates (MB19, MB20, MB29, MB31, MB41, MB62, MB68, MB76 etc) for antibiotics like azithromycin, cefipime, cefotaxime, chloramphenicol, ciprofloxacin, levofloxacin, netilmicin, and oxytetracycline did not correspond to sequences of the gene cassettes amplified from them.

The details of gene sequences and types of resistance gene cassettes found within the variable region of class 1 integrons (amplicon generated though CS-PCR) from different bacterial isolates are shown in Table 2.3B. The sequence derived from the 1009 bp amplicon from bacterial strains MB20, MB50, MB23, MB36, MB32, MB33, MB66, OD05, OD08, showed single gene cassette bearing *aadA1* gene [Table 2.3B]. The *aadA2* gene cassette was seen alone in four isolates. Similarly aadA2 cassette was found alone in two isolates. The *aadA5* gene cassette was found singly in two isolates, MB18 and SR19 (Table 2.3.B and C). The sequence derived from MB 18 showed 99% identity at nucleotide level with *Riemerella anatipestifer* class 1 integron *aadA*5 gene (Ac. No. EF105289) and 98% identity at the amino acid level with aminoglycoside adenyltransferase from *Escherichia coli* (Ac. No. YP190214), *Klebsiella pneumoniae* (Ac. No. AF180469), *Salmonella enteritidis* (Ac. No. BAD02332). The isolate MB62 and MB63 produced amplicon of size 153 bp, sequence analysis revealed that these were empty class 1 integrons devoid of any gene cassette. Isolates, MB03 and MB52, were found to carry identical cassette array of two gene, *dfrA28* and *aadA1*. The gene dfrA28 (Ac. No. FN263373) was novel having 519-bp long ORF with shared homology (identity) of 76.4% at amino acid level to the *dfrA1* of *E. coli* (Ac. No. AJ419168). The details of this novel gene will be discussed in chapter 4. The 1913 bp amplicon carried two known genes, *dfrA12* and aadA2, as well as an ORF and was present in MB40A, MB61, and MB67 isolates. Aminoglycoside acetyltransferase gene cassette (*aac-6′-Ib*) was detected in only two isolates, OC74 and OC78 (Table 2.3B). The *aacA4* gene cassettes conferring resistance to kanamycin was detected from MB42, MB43, MB49, and MB35 isolates. In MB35, *aacA4* gene cassette was flanked by two gene cassettes conferring resistance to beta-lactam and aminoglycosides. A sequence of length 737bp from MR03 showed a single gene cassette, *dfrA16* coding for dihydrofolate reductase enzyme. The sequence derived from the 769 bp amplicon from three bacterial strains MB31, MB64, and MB76 showed single *dfrA7* gene cassette (Table 2.3B). The sequence derived from the 1543, 1556, and 1569 bp amplicons from seven isolates (MB26, MB47, MB59, MB60, MB69, MB65, and MB68) showed *dfrA1-aadA1* gene cassette array (Table 2.3B). The detailed sequence analyses (ORF features, *Insilico* restriction digestion pattern of nucleotide sequence, multiple alignment of translated ORF with the nearest sequence, CDD search, physic-chemical parameters, InterPro's signature search for DfrA1, protein secondary structure prediction, and homology modeling of DfrA1 and AadA1 proteins) derived from MB47 isolate are shown in Fig. 2.10A to 2.10Mz. Based on similarity of MB47 dihydrofolate reductase to the dihydrofolate reductase 3e0bB, the homology model was built. The

sequence identity between the query sequence and template sequence was found to be 30.57% with an expected value of 0.00e-1. The region of the enzyme from residue 1-151 was successfully modeled using SWISS-MODEL automated mode tool (Fig 2.10H). An alignment of query (MB47 DfrA1) and template sequence [dihydrofolate reductase of *Haemophilus influenzae* (PDB ID: 3e0bB)] is shown in Fig. 2.10H. The comparison of generated homology 3D model (Fig 2.10Hy) of MB47 DfrA1 protein (NCBI protein ID: CAQ76923) and 3e0bB three dimensional model (Fig 2.10Hx) of *Haemophilus influenzae* showed that the model of putative DfrA1 protein was similar to the dihydrofolate reductase of *Haemophilus influenzae.* The estimated Swiss-model quality showed that built model was reliable with Qmean score 0.7 (reliable range: 0-1) (Fig 2.10Hz). The model quality was also evaluated by ProQ tool. For homology modeling of AadA1 protein of MB47, template, 1no5B (crystal structure at resolution 1.80 Å of HI0073, the nucleotide binding domain of the Hi0073/Hi0074 two protein nucleotidyl transferase from *Haemophilus influenzae)* showing 21.84% identity and 2.00e-9 Evalue with query AadA1 was selected automatically by the software. The region of the enzyme from residue 10-96 (comprising nucleotide binding domain) was modeled as described above. Pairwise alignment between query (MB47 AadA1) and template sequence [nucleotidyl transferase from *Haemophilus influenzae* (PDB ID: 1no5B)] is shown in Fig. 2.10M. The comparative analysis between produced model (Fig 2.10My) of MB47 AadA1 protein (NCBI protein ID: CAQ76924) and 1no5B three dimensional model (Fig 2.10Mx) of *Haemophilus influenzae* showed that the model for nucleotide binding domain of AadA1 protein was similar to the nucleotide binding domain of *Haemophilus influenzae.* The obtained QMEAN score 0.51 exhibited that the generated homology model of AadA1 was reliable (Fig 2.10Mz). Further model quality was assessed by ProQ tool.

The sequence derived from the 1647 and 1694 bp amplicons from three bacterial isolates MB03, MB52 and MB78 respectively showed *dfrA28-aadA1* gene cassette array (Table 2.3B). The integron sequence derived from five bacterial isolates MB27, OC16, MB37A, MB53, and OC24 showed *dfrA17-aadA5* gene cassette array. Sequence features of variable region derived from MB53 are shown in Fig. 2.11. The *aad*A5 gene cassette was the second cassette identified from MB 53 and consisted of an ORF of 224 amino acids. AadA5 protein from MB 53 showed 100% identity with the same protein from a Gram positive bacterium *Staphylococcus epidermidis* (Ac. No. AB291061) and Gram negative bacterium *Enterobacter cloacae* (Ac. No. EF571855). The 1606 and 1663 bp amplicons of bacterial isolates (MB25 and MB34B) carried two tandemly inserted gene cassettes consisting of the *dfrA17* and the *aadA4* genes in class 1 integron sequence. The integron sequence derived from three isolates MB29, MB30, and MB73 showed two genes, *dfrA7* and *aadA5* in their gene cassette array. The sequence features (bearing *dfrA7* and *aadA5* ORFs) derived from MB29 are detailed in Fig. 2.12A - 2.12D. The gene cassette array, *dfrA1-orf* was detected in four bacteria, MB38, MB74, MB75, and MB57A (Table 2.3B). The sequence analyses of variable region derived from MB38 bacterium are detailed in Fig. 2.13A and 2.13B. Protein-protein homology of ORF38 derived from MB38 showed that it was 99% identical to the hypothetical protein of *Escherichia coli* (Protein id BAD08521) however, no conserved domain was observed in BlastP analysis. A single gene cassette, *dfrA30*, was observed in three isolates, MB45, MB72, and MB77. The gene, dfrA30 containing 471 bp long ORF, which shared maximum 93% identity at amino acid level with the closest known dfr (*dfrA5*) sequence of *E. coli* (Ac. No. AJ419169) was recognized as a novel (for reporting novel gene protein-protein identity should be <95%) gene cassette. The features of *dfrA30* gene and its source will be discussed in detail in chapter 5. The PROSITE motif search has revealed that the dihydrofolate reductase signature sequence, VIGngpdIPWsakg.EqllFkaiT, was intact in most of the Dfr protein sequences.

2.3.3.3. Integron carriages in samples collected from three different sampling sites (SS I, SS II, and SS III); and occurrences of class 1 integrons in different resistance index groups

The annual study of integron fishing in facultative-oligotrophic (%) isolates from each sampling site per year (2007-2009) is shown in Table Fig. 2.14. The study revealed that the frequency of class 1 integron was highest at SS II in all three years (Table 2.10; Fig 2.14).The maximum carriage of class 1 integron in isolates of SS III was in the year 2007 and at SS I, it was in year 2008. There was similar occurrence of class 1 integrons (7.7%) were observed at SS I, SS III and SS II during year 2007, 2008 and 2009 respectively (Table 2.10).

A thorough study of occurrences of integrons in different resistance index [Resistance index (RI) =number of antibiotics to which isolate exposed/ total number of antibiotics taken in study] ranges 0.08-1.0, were analyzed and results were interpreted. Results showed that incidence of integrons in resistant bacterial population were lowest at 0.08 RI and maximum at 0.66RI. A gradual increase of the presence of class 1 integron was noticed from 0.08RI to 0.5 and then an irregular pattern was observed (Fig. 2.15). It was found that incidence of class 1 integrons remained quite high between 0.5- 0.9; meaning that this category of resistance index is best for fishing integrons from oligotrophic bacteria.

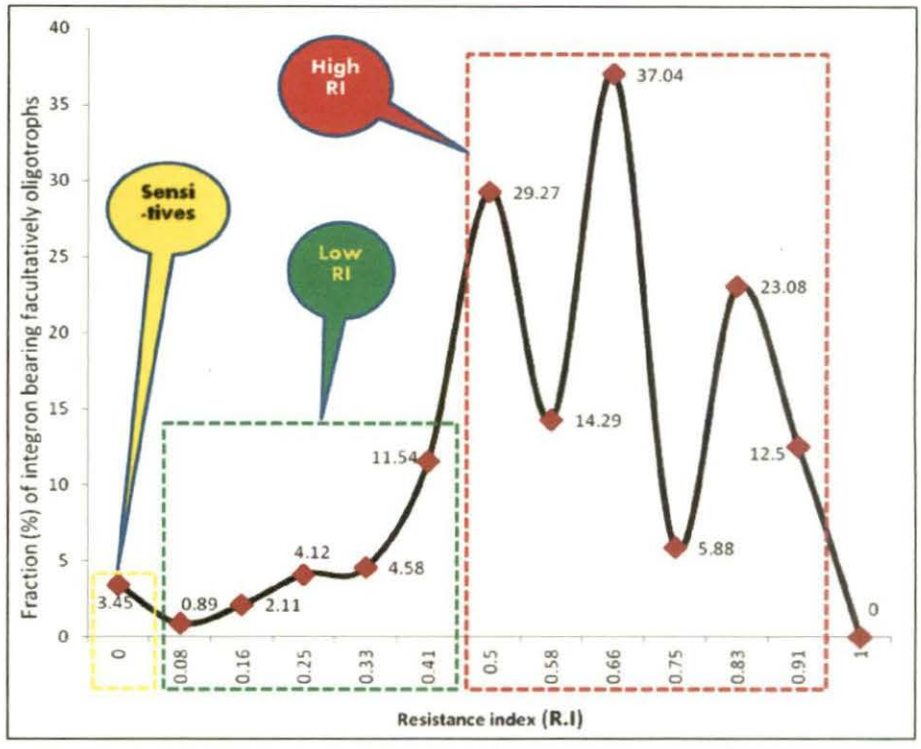

Fig. 2.15: Incidence of class 1 integrons in different resistance index groups

2.4. Discussion

The notion that the majority of bacteria existing in nature are yet to be discovered has gained its support from the large-scale employment of new technologies in microbiological research of the past decades. A marked discrepancy between the relatively low number of microorganisms cultivated under laboratory conditions and the number and diversity of organisms actually existing in the environment has been revealed from the analysis of environmental specimens conducted by molecular biological methods or microscopy. Genetic diversity of this majority and unidentified gene families remains still obscure. Bacteria are differentiated from other living systems by a striking plasticity which allows them to adapt easily to various frequently changing environmental conditions. Specific mechanisms by which changes are brought about in the amount or order of genes in bacterial genomes contribute to the high adaptability of bacteria (Ilyina, 2006). Bacteria undergo selection pressures in the presence of various toxic substances; hence developments of resistance against the inhibitory agents, commonly the resistance to metal and antibiotics, provide selective advantages (Hideomi *et al.*, 1977; Timoney *et al.*, 1978; Watkinson *et al.*, 2007). Resistant bacteria are ubiquitous as they are isolated from diverse geographical locations (Mudryk *et al.* 2000; DeSouza *et al.*, 2006; Xi *et al.*, 2009; Chattopadhyay and Grossart, 2011; Nageswaran *et al.*, 2012). With increasing bacterial resistance to almost all the discovered antibiotics, the phenomenon has been recognized by microbiologists from all over the world. A sensitive bacterium can become

resistant either through mutation or by acquiring resistance genes by means of horizontal transfer through transformation, transduction, or conjugation. It is assumable that amongst three modes of gene transfer mechanisms, conjugation may be the most common mechanism of transferring antibiotic resistance genes. By dint of conjugal gene transfer, plasmids and transposons carrying antibiotic resistance genes can easily mobilized from one cell to another. From the last quarter of the 20th century, another class of mobile DNA elements bearing antibiotic resistance gene came into prominence of research.The discovery of integrons came into light during 1986, when researchers meticulously analyzed the DNA sequence of numerous apparently unrelated antibiotic resistance genes and found that the regions flanking various antibiotic resistance genes were conserved. Furthermore these common regions were observed in different places on various plasmids, this suggested that, like transposons, these elements were also mobile. However, these elements differed from transposons in two important ways: (i) the regions flanking the antibiotic resistance genes in the new elements were not direct or indirect repeats, as found in transposons and (ii) the elements were holding a site-specific integrase gene of the same family (tyrosine recombinase) as found in λ phages but deficient in many gene products associated with transpositon. Due to these dissimilarities, the new elements were separated from transposons and were called integrons.

Integrons are the genetic elements with an ability to take into custody one or more non-functional genetic determinants [called as gene cassettes, a promoter-less (leaving exception) non-replicating mobile DNA elements which can also exist in free-circular form] often encoding antibiotic resistance genes by site-specific recombination and convert them in functional (Hall and Collis, 1995). The locations of these determinants have been traced in the chromosome as well as in different plasmids and transposons by several researchers (Hall and Stokes, 1993; Ozgumus *et al.*, 2009). On comparing the amino acid sequences of the integrases, encoded by *int*I genes, integrons have been classified into several classes and class 1 to 3 are termed as mobile integrons. Of these, the well studied class, class 1 integron is now ubiquitous and playing important role in the dissemination of antibiotic resistance (often) genes in both Gram-negative (Hall and Stokes, 1993; Mukherjee and Chakraborty, 2006) and Gram-positive bacteria (Nandi *et al.*, 2004; Shi *et al.*, 2006; Xu *et al.*, 2008; Xu *et al.*, 2011). Typically the integrons have been identified on plasmids or on transposable elements such as mercury resistance transposon *Tn21* (Grinsted *et al.*, 1990; Lindstedt *et al.*, 2003; Rodríguez *et al.*, 2006). Researchers have shown that most of the class1 integrons are derivative of defective transposon (Brown *et al.*, 1996; Xu *et al.*, 2009). Analyses of whole genome sequences resulting from genome sequencing projects (Mazel, 2006; Rowe-Magnus *et al.*, 2001; Boucher *et al.*, 2007) have revealed that about 10% of the sequenced genome of distantly related lineage possess a variety of *intI* genes (Boucher *et al.*, 2007). To date integrons in the Eucarya or Archaea have not been reported (Nemergut *et al.*, 2008)

Class 1 integrons, the commonly studied class of integrons consists of three DNA segments, (i) 5''-conserved segment, (ii) variable region which contain gene cassette (mostly coding for antibiotic resistance, but not always) of different lengths and sequences and the last (iii) 3'-conserved segment (Fig. 2.1). The 5'-conserved segment (5'-CS) includes the *intI1* gene (class 1 integrase) encoding the integrase (as discussed above) belonging to the tyrosine recombinase family which catalyzes site-specific recombination between *attI1* and *attC* (also called as 59 base element as the first described *attC* was of 59 bp long) sites with site preferences. The protein, IntI1, in bacteria can bring about recombination between, either two *attC*, one attI1 and one *attC*, or two attI1 sites (Collis *et al.*, 2001). Integration and excision reactions, involving attI1xattI1 or *attCxattC* sites, appear to be the most relevant biological events (Stokes *et al.*, 1997). This 5'CS also contains a common promoter (Pc) region. In fact Pc potentially contains two promoters, P1 and P2 (Bissonnette *et al.*, 1991; Stokes and Hall, 1989) which play role in expression of captured promoter-less gene cassettes. Thus, integrons are "natural cloning and expression systems" that are able to capture

cassettes with open reading frames (ORFs) and convert them into actively functioning genes (Rowe-Magnus and Mazel, 1999, 2001).

The 3′-conserved segment (3′-CS) is generally composed of a truncated *qacE* (*qacED1*) gene encoding low-level resistance to disinfectants, the *sul1* gene encoding sulfonamide resistance, and in some occasions two open reading frames (ORFs), ORF4 and ORF5 (Stokes and Hall, 1989)of unknown function. Integrons cannot be mobilized by itself but contain gene cassettes that can be mobilized to other integrons or to secondary sites in the bacterial genome. The class 1 integron system, being an effective gene-capture and assembling system, renders bacteria to incorporate gene cassettes and transforms them to functional entities by modulating their correct expression. Therefore, it has been seen as key player in the propagation and dissemination of resistance genes leading to simplistic spread of resistance genes and an agent for rapid evolution of resistance to a wide range of unrelated antibiotics among diverse bacteria (Mazel, 2006; Nemergut *et al.*, 2008).

The gene cassettes are important in the swapping of antibiotic resistance genes. The origin of these cassettes is still unclear but it is expected that they might have originated from chromosomal or super integrons (Mazel *et al.*, 1998; Fluit and Schmitz, 2004). Most of the known gene cassettes are identified as resistance cassettes carrying genes for antibiotic resistance determinant, notwithstanding the fact that they can also transfer a diverse suite of genes unrelated to antibiotic resistance (Holmes *et al.*, 2003; Nemergut *et al.*, 2004; Moura *et al.*, 2007; Gillings *et al.*, 2008b).

Class 1 integrons have been reported from bacteria isolated from diverse niches like human and animal clinical settings, agricultural environments, urban and pristine areas, food animals, pets, wild animals and also in aquatic environments (Rosser and Young, 1999; Nandi *et al.*, 2004; Agerso and Sandvang, 2005;Gaze *et al.*, 2005; Hardwick *et al.*, 2008; Leverstein-van Hall *et al.*, 2001; Sunde, 2005; Roe *et al.*, 2003; Moura *et al.*, 2007; Wright *et al.*, 2008; Binh *et al.*, 2009; Moura *et al.*, 2010; Byrne-Bailey *et al.*, 2010; Betteridge *et al.*, 2011; Nardelli *et al.*, 2012). However, reports on the incidence of integrons from bacteria isolated from fresh-water ecosystems like creeks or rivers are very few (Petersen *et al.*, 2000; Mukherjee and Chakraborty, 2006; Ozgumus *et al.*, 2007; Ozgumus *et al.*, 2009). There are few studies that are reported from India, particularly outside the clinical settings (Mukherjee and Chakraborty, 2006; Pathak and Gopak, 2008, Deekshit *et al.*, 2012).

Surveillance for the presence of integrons in bacteria has become a tool for clinical infection control and the study of antibiotic resistant mechanism (Jones *et al.*, 2003). Hence, such studies extended to environments outside the clinical settings would enable to assess the antibiotic-resistance gene pool in the environment as such. Bacteria from diverse ecological niches (environmental, animal and human) are able to mix in aquatic system and consequently resistance in sensitive bacteria may develop as a result of illegal exchanges and shuffling of genes using different genetic platforms and vectors (Baquero *et al.*, 2008).Water is the principal carrier of pollutants in the environment and therefore has been attracted by the environmental biologists. Furthermore, water bodies have been identified as ideal carriers for the dissemination of antibiotic-resistance genes (Lupo *et al.*, 2012). When compared to the waters less affected by human habitation (i.e pristine water bodies), the incidence and occurrences of class 1 integrons bearing strains is higher in polluted water sources (Stalder *et al.*, 2012). Hence, detection and determination of integron constituents at the molecular level is an important scientific issue.

The present study was out lined in the following steps: (i) enumeration of oligotrophic bacteria on nutrient poor medium (discussed in previous chapter); (ii) screening the isolates for obligate and facultative bacteria (discussed in previous chapter); (ii) random selection of facultative oligotrophs for determination of antibiotic susceptibility/resistance (described in previous chapter); (iv) molecular analyses of class 1 integron borne determinants (in the present chapter); and (v) the provisional identification of bacteria bearing class 1 integron (to be detailed in chapter 3).

Mostly the integron studies have been conducted on gram negative bacteria but it is also documented that gram positive bacteria do also bear resistance gene associated with integrons (Nesvera *et al.*, 1998; Nandi *et al.*, 2004; Shi *et al.*, 2006; Yan *et al.*, 2010; Xu *et al.*, 2011).

In this study, the test strains were selected by the use of research randomizer (a tool to generate unique random numbers) to prevent any human biasness, hence the collection of randomly selected bacteria (for screening of class 1 integrons) is an overall representation of culturable facultatively oligotrophic bacteria of the River Mahananda at Siliguri, irrespective of gram-reaction and sensitivity towards any of the antibiotics used. Most of the earlier studies on integron were knowingly or unknowingly restricted to the copiotrophs (bacteria that are cultivated on nutrient-rich media). As per existing literature, we do not find any reference of similar work motivated to explore class 1 integrons in oligotrophic bacteria from the environmental samples. Only few references are available regarding antibiotic-resistance in oligotrophic bacteria (Niktin *et al.*, 1988; Zlatkin *et al.*, 1991; Kimura *et al.*, 1995; Oh *et al.*, 1995; Kumar *et al.*, 2011; Bhullar *et al.*, 2012). The current study showed that 76.2% (1667/2188) of the oligotrophic bacterial population recovered was resistant to at least one of the antibiotic (12 antibiotics) tested. These results were similar to those published by Kimura (1995), a study on oligotrophic bacteria of soil in their case, where it was found that 83.7% oligotrophic bacteria exhibited antibiotic resistance. In this study, it was observed that MAR (multiple antibiotic resistant) bacteria were dominant over SAR (single antibiotic resistance) by 6% (Fig. 1.12, chapter 1) and the distribution of MAR population were as follows: 19.86% (331) were resistant to two, 11.64% (194) to three, 6.54% (109) to four, 4.67% (78) to five, 2.46% (41) to six, 3.78% (63) to seven, 1.62% (27) to eight, 1.02% (17) to nine, 0.78% (13) to ten, 0.48% (8) to eleven antibiotics respectively, and 0.24%(4) to all the 12 antibiotics tested (Fig. 1.13, chapter 1). Closer examination of data showed that facultatively oligotrophic MAR bacteria (in total) were dominant at sampling station II in comparison to SS I and SS III (Fig. 1.15, chapter 1). The SAR bacteria were more frequent amongst isolates of SS I and SS III.

A hypothesis, in light of previous existing data on antibiotic resistances in oligotrophic bacteria, was proposed that "oligotrophic bacteria may be potential reservoir of antibiotic-resistance genes in the aquatic (riverine) environment". The results of this study reflect that 3/4th of the oligotrophic bacterial population were resistant to at least one of the 12 different antibiotics tested. Studies done on river water copiotrophs revealed that MAR bacteria were dominant (Ash *et al.*, 2002; Mukherjee and chakraborty, 2006, Pathak and Gopak, 2008; Ozgumus *et al.*, 2009 Li *et al.*, 2009; Dong *et al.*, 2010) in the copiotrophic population. In this study, the MAR isolates were predominant within the enumerated oligotrophic bacterial population. In addition to above data, isolates screened for the presence of class 1 integrons were found to possess antibiotic-resistance genes; however other unknown/and unrelated function encoded by gene cassettes were also traced. Hence, this study has its own merit in terms of antibiotic resistance and data generated on antibiotic-resistance-gene-cassettes which will allow filling the gap and opening a new dimension in the study of oligotrophic bacteria.

A total of 2188 randomly selected facultatively oligotrophic bacteria were used for detection of class 1 integron; of them 90 (4.1%) produced amplicons, corresponding to the variable region of a class 1 integron, with the primer set 5'CS (specifically bind at 5' conserved segment of class 1 integron) and 3'CS (specifically bind at 3'conserved segment of class 1 integron) (Levesque *et al.*, 1995) and remaining 2098 bacterial strains failed to produce any amplicon. This result was comparable to an earlier study where non-selected gram-negative bacteria from an estuarine environment revealed the presence of class 1 integron in 3.6% of the total bacteria tested (Rosser and Young 1999). A study conducted on Rio Grande River showed that 10% of the *E. coli* isolates were MAR and 13% of them were carried class 1 integron sequences (Roe *et al.*, 2003). Class 1 integrons was observed in 3.8% of the isolates recovered from environments polluted by quaternary ammonia compounds (Gaze *et al.*, 2005). It was observed that within the facultatively oligotrophic

MAR bacteria of Mahananda River, the incidence of class 1 integrons remained quite high in the resistance index group which ranged between 0.5- 0.9, meaning that this category of resistance index is best for fishing integrons amongst oligotrophic bacteria (Fig 3.11).

The length of variable region amplified by CS-PCR ranged between 0.15 to ~3.5 kb (Fig.3.2 A and B). Most of the amplicons of length >0.7 kb were found to carry resistance gene cassette. In case of absence of any gene-cassette within the integron platform, the expected size was found 153 bp (Gebreyes and Thakur, 2005,) and these were termed as empty class 1 integron as noticed in MB62 and MB63 isolates (Ac. No. FM998811 and FM958478 respectively). It was seen that amplicons of size ~1.0 kb predominated and was detected in 24.4% (22/90) of the total integron positive isolates (Table 3.3 A, B and C). Maximum carriages of class 1 integron were documented in MAR oligotrophic bacteria; Moura *et al.* (2007) found that 47.6% bacteria resistant to five or more antibiotics (isolated from slaughter house waste water treatment plant) possessed class 1 integron. In clinical settings, 43-75% antibiotic resistant bacteria were found to carry class 1 integrons (Levesque *et al.*, 1995; Jones *et al.*, 1997; Martinez-Freijo *et al.*, 1998; Chang *et al.*, 2000; Jones *et al.*, 2003). On examining the antibiotic-resistance-profiles of the class 1integron positive isolates, it was found that 22% (18/90) isolates were sensitive to all the twelve antibiotics, 7.8% (07/90) were SAR (resistant to only one of the 12 antibiotics), and 72.2% (65/90) were MAR (resistant to two or more antibiotics) (Fig 3.1A, Table 3.11). Within 65 multiple-antibiotic-resistant isolates, 10.77% (7/65) were resistant to two antibiotics, 12.3% (8/65) were resistant to three, 7.7% (5/65) were resistant to four, 13.84% (9/65) were resistant to five, 18.46% (12/65) were resistant to six, 13.84% (9/65) were resistant to seven, 15.4% (10/65) were resistant to eight, 1.53% (1/65) was resistant to nine antibiotics, 4.6% (3/65) were resistant to ten, 1.53% (1/65) isolate was resistant to eleven antibiotics tested (Fig 3.1B). The facultative oligotrophic bacteria resistant to all twelve antibiotics used in this study did not yield any amplicon with primers for class 1 integron. On the basis of resistance index (RI), the isolates could be categorized into 11 groups as shown in Table 3.1 and 3.2. Observations led from this study revealed that incidence of integrons in antibiotic-resistant bacteria were lowest at 0.08 RI and maximum at 0.66RI (Figure3.11).

The sequence analyses revealed that function encoded by gene cassettes were strongly associated to the antibiotic-resistance-phenotype of the respective integron positive isolates. Similar to earlier studies (Bissonnette *et al.*, 1992) majority (71%) of the integron-borne cassettes corresponded to antibiotic- resistance-genes while 29% of the gene cassettes were found to carry genes unrelated to antibiotic resistance. The common carriage of antibiotic resistance genes were observed for aminoglycoside-modifying enzymes and trimethoprim-resistant dihydrofolate reductases. Only a few antibiotic-resistance-gene-cassettes were found to carry genes for other function like resistance for β-lactams (e.g ampicillin, cephalothin, and oxacillin etc), kanamycin and gentamycin. The gene cassettes coding for trimethoprim-resistant dihydrofolate reductase were found to carry two types of dihydrofolate reductases, type A (*dfrA1, dfrA5, dfrA7, dfrA12, dfrA16, dfrA17,* two novel *dfrA* genes, *dfrA28* and *dfrA30*) and type B (*dfr-IIe*), conferring resistance to trimethoprim, were found. Type A and typeB DFRs are also called as type I and type II. Two distinct types of R-plasmid dihydrofolate reductases are known. Plasmid-borne R483 enzyme (Type I enzymes) (Sköld and Widh, 1974), are produced in amounts several fold higher than the chromosomal enzyme. The calculated I_{50} (50% inhibitory concentrations) values of trimethoprim, methotrexate, and aminopterin were found to be increased by over several thousand folds from the corresponding values obtained with the chromosomal enzyme. On the other hand, plasmid-encoded Type II dihydrofolate reductases are synthesized in about the same amount, or less, than the chromosomal counterpart. Both types of plasmid encoded dihydrofolate reductase in terms of binding of dihydrofolate, NADPH, folic acid, and 2,4-diaminopyrimidine showed little difference from that exhibited by the chromosomal enzyme (Pattishall *et al.*, 1977).

A single gene cassette (antibiotic-resistance or unrelated to antibiotic-resistance) was recognized in 65.55% (59/90) and an array of two gene-cassettes were noticed in 31% (28/90) isolates (Table 3.3 A, B and C) while only 3.3% (3/90) isolates were found to carry an array of three gene cassette on amplified amplicons. A single isolate, MB35 was the only representative which carried an array of four gene-cassettes in a single amplicon generated by CS-PCR technique. Sequence analysis of gene cassettes revealed that 7.8% isolates (MB57A, MB67, MB40A, MB74, MB75, MB61, and MB38) of the total integron positive isolate carried a combination of two or more than two gene cassettes where one was coding for unknown protein and other were coding to known antibiotic resistance. It was noted that the gene cassette coding for uncharacterized protein was either located in between the two antibiotic-resistance-gene-cassettes or at second position to the antibiotic-resistance-gene-cassette. The presence of gene-cassette coding for unknown protein, at second position, to the antibiotic-resistance-gene-cassette was probably due to their earlier recruitment or due to later reshuffling which transferred them at second position or due to acquisition of new antibiotic resistance gene-cassette for selective advantage. These types of combination also have been reported by other workers (Nemergut *et al.*, 2001; Kadlec and Schwarz, 2008) but their presence in facultatively oligotrophic bacteria hint another possibility of function related to oligotrophy and may be active at low nutrient condition to provide sustainability in poor nutrient medium. So, one can speculate that these bacteria may have the necessary genetic tool to engineer protein in the face of emergency or threat. An array of three gene cassette (*dfrA12-orf40A-aadA2*) was detected in three integron positive isolates (MB40A, MB61, and MB67) (Table 3.3 B). One bacterium MB79A was having a gene cassette array of type B (also called as typeII) *dfr-IIe (dfrB)* dihydrofolate reductase, and *arr2* gene, responsible for resistance to rifampicin (a semi-synthetic antibiotic agent) (Table 3.3 C). Maximum percentage (10%, of the total antibiotic-resistance-gene-cassettes) of two-gene-cassette array was observed for *dfrA-aadA1* type followed by *dfrA17-aadA5* (~8%). The gene encoding beta-lactamases were rarely observed in this study. Only MB82 and MB35 isolates were found to carry *oxa1* and *oxa10* sequence respectively. The *oxa1* and *oxa10* genes codes for class D enzymes which possess a distinct catalytic mechanism for the beta-lactam hydrolysis than the other β- lactamases. The *aadA* gene cassette derived from MB 18 was found 99% identical at nucleotide level to the class 1 integron borne *aad*A5 gene (Ac. No. EF105289) of *Riemerella anatipestifer*, and 98% identical at amino acid level to the aminoglycoside adenyltransferase of *Escherichia coli, Klebsiella pneumoniae,* and *Salmonella enteritidis* (Ac. No. YP190214; AF180469; and BAD02332 respectively). It depicts that the *aadA5* gene in strain MB18 (gram positive bacterium which was classified as *Brevibacterium siliguriense*) probably transferred via horizontal gene transfer from one of these source bacteria. The unoccupied integration site of class 1 integron (empty class 1 integron) which was detected in two oligotrophic bacterium, MB62 and MB63, was assumed as ancestor of resistance/nonresistance integron. The noticeable point was that the MB62 was MAR having no resistance-gene cassettes in integron structure (resistance gene functions were rendered by determinants present elsewhere in its genome) while MB63 corresponding to the same genus having class 1 integron devoid of any cassette was sensitive to all antibiotics. It is speculated that these integron structures are the future recipients of gene cassette(s) of special function to get selective advantage in response to upcoming environmental stresses. The present study also describes two new dihydrofolate reductase genes, *dfrA28* and *dfrA30,* harboured in class 1 integron from the isolate MB52 and MB45 respectively (for detail please see chapter 4 and 5). Sequence analyses of Dfr proteins exhibited that most of the proteins carried an intact "VIGngpdIPWsakg.EqllFkaiT" dihydrofolate reductase signature sequence in their sequences. The homology modelling of DfrA and AadA1 proteins derived from MB47 isolate was found similar to the dihydrofolate reductase (PDB ID: 3e0bB) and nucleotide binding domain of nucleotidyl transferase protein derived from *Haemophilus influenzae* (PDB ID: 1no5B) (Fig 2.10H to 2.10Hz and 2.10N to 2.10Nz).

Besides antibiotic resistance gene cassettes, a number of gene cassettes (~29% of the total) bearing ORFs for unrelated function than the antibiotic resistance were observed (Fig. 2.7). Reports on gene cassettes unrelated to antibiotics are very rare from culturable bacteria (Nemergut *et al.*, 2004; Stokes *et al.*, 2006; Gillings *et al.*, 2008a). Most of the uncharacterized gene cassettes have been identified from gene-cassette-metagenome sequences (Holmes *et al.*, 2003; Gillings *et al.*, 2008b). A class 1 integron borne gene cassette harbouring an ORF of a hypothetical protein similar to hypothetical protein of *Acidovorax* strain MUL2G8 was detected from bacteria *Hydrogenophaga, Imtechium,* and *Aquabacterium* (Stokes *et al.*, 2006). This ORF was also homologus to the hypothetical protein VP1784 of *Vibrio parahaemolyticus* (Gillings *et al.*, 2008a). The said authors also detected a second gene cassette containing an ORF with no homology to any known gene, ORF186 (coding for hypothetical protein) and six cassettes of which first encoded putative IS*91*-like transposition function and rest for hypothetical or unknown function from the bacteria *Hydrogenophaga,* bacterium B4 and bacterium E7 respectively. Diverse gene cassettes coding for protein function other than antibiotic resistance were noticed in the present study. The CS-PCR product sequencing of OB05 and MB83 showed that the derived sequences was 75% identical (nucleotide-nucleotide) to the part of the genome of *Acidovorax avenae* subsp. *citrulli* (Ac. No. CP000512). The feature present in that part of *Acidovorax* genome included a portion of ribosomal large subunit pseudouridine synthase B. However the predicted polypeptide of the ORF generated from OB05 DNA sequence did not produce any significant similarity with existing sequences of the protein database. Sequence analyses of the amplicons of MB05 and MB70 revealed the presence of a truncated ORF of 64 amino acids that showed 73% identity with the methyl transferase protein from *Ralstonia eutropha* (Ac no. AAZ62060). Partial sequencing of the CS-PCR product obtained from MB12 showed the presence of a single ORF (*livM1*) of length 154 amino acids (Fig 2.8A). BlastP analysis (Table 2.4) of this putative polypeptide product exhibited best scores with ABC type branched chain amino acid transport system with 72% identity at amino acid level to the ABC transporter permease of bacterium *Aromatoleum aromaticum* EbN1 (Ac. No. NC_006513). The multiple sequence alignment of LivM1 derived from amplicon sequence of bacterium MB12 with its neighbouring ABC transporter proteins is shown in Fig. 2.8B. The transmemebrane (TM) regions predicted within the ABC transporter protein of MB12 are shown in Fig. 2.8C and 2.8D. Analysis revealed the presence of four transmembrane regions in the predicted LiVM1 protein of MB12. The predicted transmembrane (TM) regions (AA position 2-20, 50-74, 87-110, and 119-138) in ABC transporter protein of bacterium MB12 (Protein ID: CAQ53856) are presented in table 2.5. The conserved domain search for putative translated product obtained from translated ORF of MB12 integron sequence revealed that the predicted protein was one of the members of two TM subunits which play role in uptake of branched chain amino acids (Fig 2.8E). Mapping of signature motifs (Fig 2.8F) showed that 19 of 19 [TM_PBP1_LivM_like, a type of transporters which consist a PBP (periplasmic binding protein), two TMs (trans-membrane subunits) and two cytoplasmic ABCs (ATP-binding cassette)] residues which were recognized on the TM-ABC transporter protein sequences of other bacterial species were also found conserved on ABC protein derived of MB12. Hence, it was assumed that these types of transporters probably play a vital role in the import of solutes from environment. Finding of these transporters in class 1 integrons has a greater significance since oligotrophic bacteria thriving in low nutrient milieu may require improved transporter molecules to concentrate and channelize nutrients from outside to the interior of the cells. The theoretical pI and instability index of the putative ABC transporter of MB12 was computed 9.3 and 19.13. The protein was predicted as stable protein with an estimated half life of 10 h (*in vivo* in respect to *E coli*) with a high aliphatic index of 127.21. MB16 was found to carry gene cassette carrying *appA* gene encoding bacterial extracellular solute binding protein. ProtParam computing showed that the extracellular binding protein derived from bacterium MB16 was stable and its instability index was computed 18.94. The GRAVY (grand average hydropathicity), aliphatic index

and theoretical pI was computed -0.087, 78.5, and 9.26 respectively. Psortb, a tool for subcellular localization prediction showed that the translated product of *appA* gene was periplasmic with localization score of 9.44.

The Complete sequence of 704 bp amplicon of MB 40B (Table 2.3B) yielded no significant homology with any nucleotide sequence available in the database. The same sequence yielded a complete ORF of 102 amino acids and BlastP analysis of this protein sequence revealed 58% identity with helicase domain protein of *Verminephrobacter eiseniae* EF01 (Ac. No. ABM585806). The sequences derived from amplicons of OB 12, MB 08, MB41, MB 44, MB 48, MB 51, MB 54, MB55, MB80 and MB81 did not produce any significant homology with any protein sequences available in the database. All of them were characterized as hypothetical proteins. All these polypeptides shared very low level of identity (2.03 to 25.19%) among either with each other or to pre-deposited hypothetical protein sequences. Some of the predicted proteins were found homologous to certain protein sequences present in database, for example, translated product of bacterium MB54 was carrying a conserved domain belonged to the UPF0153 super family(Fig. 2.9A and B). The proteins of this family contain 8 conserved cysteines, hence it was expected that they may constitute a metal binding site and therefore might play a role in metal sequestering like Fe-S cluster as part of oxido-reductase complex. Molecular iron is an important cofactor for growth, because it is used by bacteria during aerobic respiration, DNA replication, and other biological processes. Due to requirement of iron as a cofactor, bacteria must have to develop mechanisms for the acquisition of iron from their environment, for example some bacteria secrete siderophores molecules to bind free iron and then the iron molecules are captured by receptors present on bacterial surface (surface receptors that transport iron for transport across the cytoplasmic membrane). However iron also plays a crucial role in establishment of host pathogen relation that has been proved by many researchers. Such proteins in oligotrophic bacteria might play an important role in concentrating essential elements from the nutrient limited environment.

Since all the earlier studies on integrons were focused on copiotrophic bacteria (grow in rich nutrient media) while no such study was found concerning oligotrophic bacteria, a focused study was required to fill this gap. The present study uncovers the diversity of gene cassettes associated with class 1 integrons in oligotrophic bacteria of riverine origin.

2.5. Conclusion

The results have revealed that Class 1 integrons are prevalent in facultatively oligotrophic bacteria irrespective of being resistant or sensitive to antibiotics. Oligotrophic bacteria isolated from River Mahananda are good source of novel genes as well as potential reservoir of antibiotic gene cassettes. The high percentage of occurrence of MAR bacteria (and high incidence of class 1 integron in them) at sampling station II (where Mahananda flows within the Siliguri city) is possibly due to selection induced by pollution generated from anthropogenic activities. The presence of class 1 integrons in oligotrophic bacteria is indicative of the spread of stress-combating-genes including antibiotic resistance and other putative genes coding for known or unknown function in the aquatic environment. The predominance of aminoglycoside and trimethoprim resistance gene cassettes are probably sign of abuse of these drugs or the high frequency of horizontal gene transfer in that environment. This study has also demonstrated that integron typing can be a useful tool for studying the dissemination of resistance genes among oligotrophic bacteria. Integron-driven gene capture is likely to be an important factor in the more general process of horizontal gene transfer in the evolution of bacterial genomes as shown by earlier studies (Rowe-Magnus and Mazel, 2001; Holmes *et al.*, 2003; Mazel, 2006). The study of integrons and their associated gene cassettes could provide information about antibiotics which should be used most carefully to prevent further dissemination of resistance. Hence, now it has become more relevant to design a tighter antibiotic policy or other measures that can lower the amount of horizontal transfer in different environmental settings.

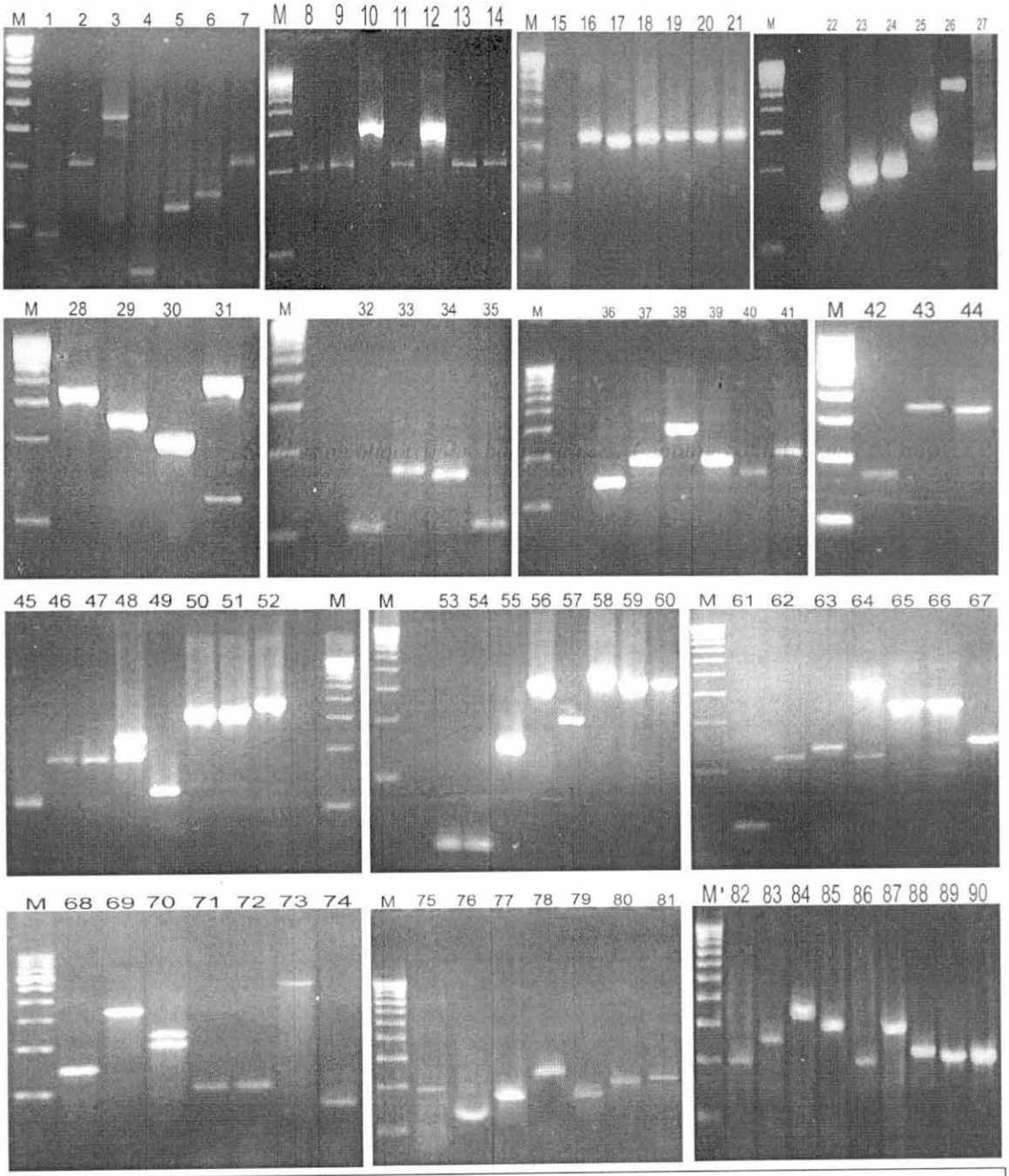

Fig. 2.5: Variable regions of class 1 integrons amplified using 5' CS and 3' CS primers. The PCR products were electrophoresed on 1% agarose gel.

Lane: M, 500 bp DNA ladder; 1, OB05; 2, OB12; 3, MB03; 4, MB05; 5, MB08; 6, MB09; 7, MB12; 8, MB16; 9, MB18; 10, MB19; 11, MB20; 12, MB21; 13, MB22; 14, MB23; 15, MB24; 16, MB25; 17, MB26; 18, MB27; 19, MB28; 20, MB29; 21, MB30; 22, MB31; 23, MB32; 24, MB33; 25, MB34; 26, MB35; 27, MB36; 28, MB37; 29, MB38; 30, MB39; 31, MB40; 32, MB41; 33, MB42; 34, MB43; 35, MB44; 36, MB45; 37, MB46; 38, MB47; 39, MB48; 40, MB49; 41, MB50; 42, MB51; 43, MB52; 44, MB53; 45, MB54; 46, MB55; 47, MB56; 48, MB57; 49, MB58; 50, MB59; 51, MB60; 52, MB61; 53, MB62; 54, MB63; 55, MB64; 56, MB65; 57, MB66; 58, MB67; 59, MB68; 60, MB69; 61, MB70; 62, MB71; 63, MB72; 64, MB73; 65, MB74; 66, MB75; 67, MB76; 68, MB77; 69, MB78; 70, MB79; 71, MB80; 72, MB81; 73, MB82; 74, MB83; 75, MR 01; 76, MR 02; 77, MR 03, 78, MR 04; 79, SR19; 80, NV66; 81, OD 05; 82, OD 08; 83, OD 10; 84, OD 16; 85, OD 24; 86, OC 74; 87, OC 75; 88, OC78; 89, OD 21; 90, OD 24

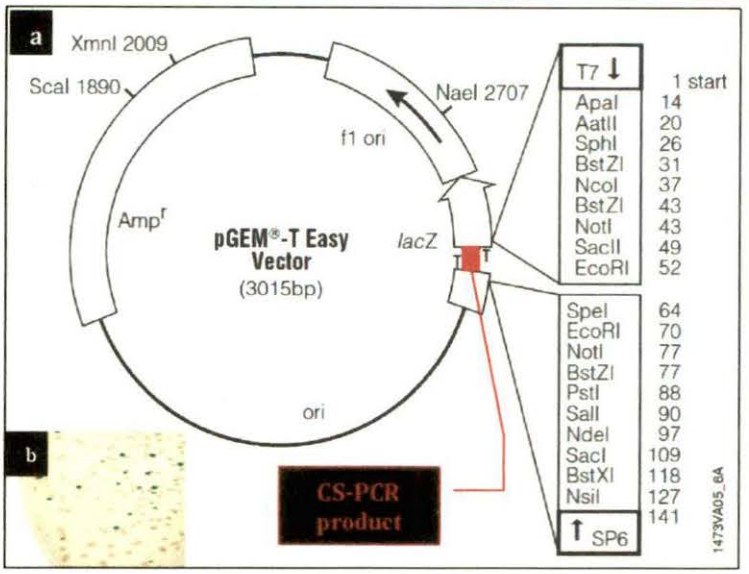

Fig. 2.6: a, Cloning of CS-PCR product in pGEMT-Easy vector and b, screening (Blue-white) of recombinant clone on Luria agar plate amended with ampicillin (100mg/L). Gene of interest ligated in the multiple cloning site of T-vector is shown in red colour.
Amp^r, gene for ampicillin resistance; *ori*, orgin of replication; *lacZ*, gene for beta-galactosidase synthesis.

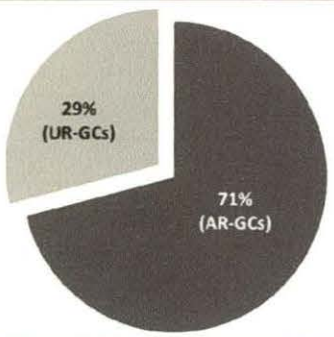

Fig. 2.7: Distribution of gene cassettes identified within the amplified variable regions of class 1 integron.

UR-GCs, cassette unrelated to antibiotics resistance;
AR-GCs, cassette related to antibiotic resistance.

```
TAAGCAGACTTGACCTGACCGACAACACCCACatgtacctgttcgtactggtcatctttctggcgggctt
                          livM1->  M  Y  L  F  V  L  V  I  F  L  A  G  F
cctgctgctgtggcgcgtggttcattcacccttggccagatcctcaaggcgattcgtgaacatcaggac
  L  L  L  W  R  V  V  H  S  P  F  G  Q  I  L  K  A  I  R  E  H  Q  D
cgcgccacgtccccgggctatgacaccgacaccttcaagctgctggcctttgtcatctcgggcgcactgg
R  A  T  S  P  G  Y  D  T  D  T  F  K  L  L  A  F  V  I  S  G  A  L  A
ccgggacggccggagcgaccaaggcgctggtgtttcagctggcctcgctcaccgatgtgcactggagcat
  G  T  A  G  A  T  K  A  L  V  F  Q  L  A  S  L  T  D  V  H  W  S  M
gtcgggcgaggtggtgctgatgaccctggtgggtggcatgggcacgctgtttggtccggtggctggtgcg
  S  G  E  V  V  L  M  T  L  V  G  G  M  G  T  L  F  G  P  V  A  G  A
gccgtgatcgtgtccatgcagaactacctggcacagctgggggcatgggtgacggtggtgcagggtgtga
A  V  I  V  S  M  Q  N  Y  L  A  Q  L  G  A  W  V  T  V  V  Q  G  V  I
tcttcgtggtctgcgtgctcgccttccgccgcggcatcattggcgagatcgccaacctcatcaagaaacc
  F  V  V  C  V  L  A  F  R  R  G  I  I  G  E  I  A  N  L  I  K  K  P
cctctgaCCGGTTGGTGCCATGGGCCCGGCTAGACTGCAGGTTTTCCTGTAGTCGGCGCCTCGCATGTCT
 L  #(stop)
TCTTCCCGCGTTTCCGGTTTGTGGCTGATCAGCCTGGCCGCTTTCAGCAGCATGGCTTCCATGCGCGTGT
GCGACCCGATGCTGGTGGCGCTCGCGACGGAGTTCCAGGTCAGCGTGGGGGAGGCCTCTCGTGTGATTTC
GGCGT
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Fig. 2.8A: Partially sequenced variable region (Ac. No. AM997273) of class 1 integron derived from MB12 showing ORF and the putative translated product.

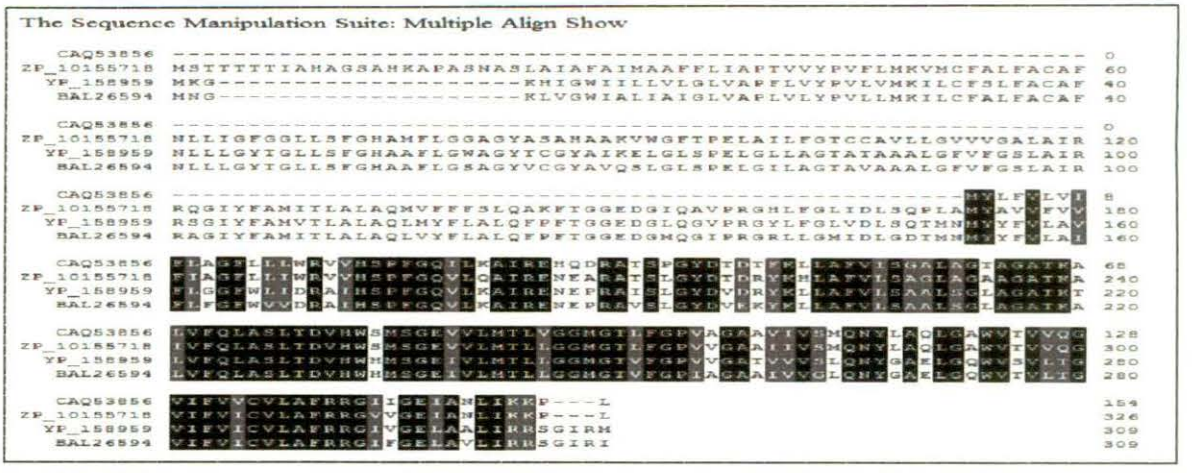


Fig. 2.8B: Multiple sequence alignment between ABC transporter protein obtained from MB12 and the nearest neighboring sequences. Black, identical amino acids and gray, similar amino acids present in all the protein sequences.

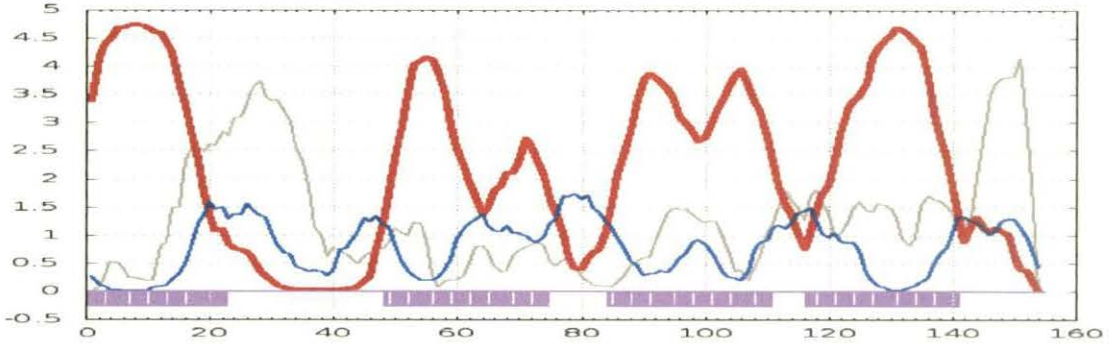


Fig. 2.8C: Graphical presentation of tans-membrane (tm) region within LivM1 protein of MB12.
Colour coding: Red line: Transmembrane helix preference; Blue line: Beta preference; Gray line: Modified hydrophobic moment index; Violet boxes (below abscisa): Predicted trans-membrane helix position.

Predicted amino acid locations of Tm regions: 1-22, 49-74, 85-110, and 117-140 (Protein ID: CAQ53856)

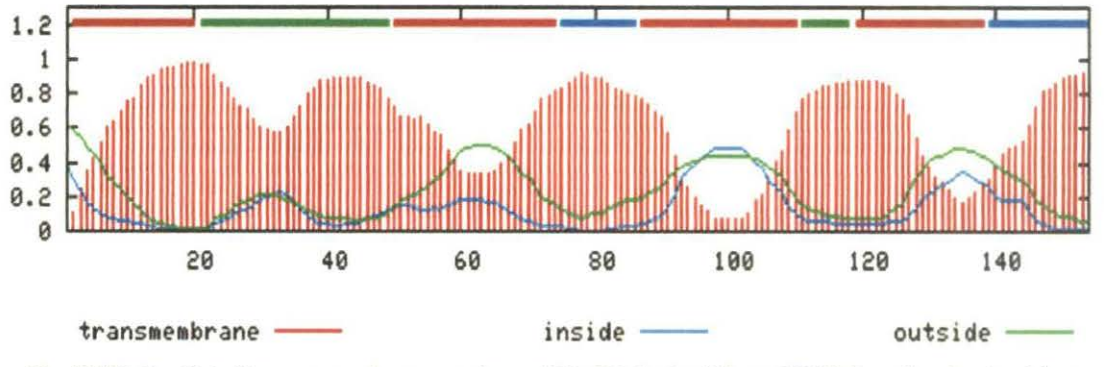


Fig. 2.8D: Predicted trans-membrane regions of LivM1 derived from MB12 (results obtained from Protein Topology Prediction Hidden Markov Model TMMOD tool)

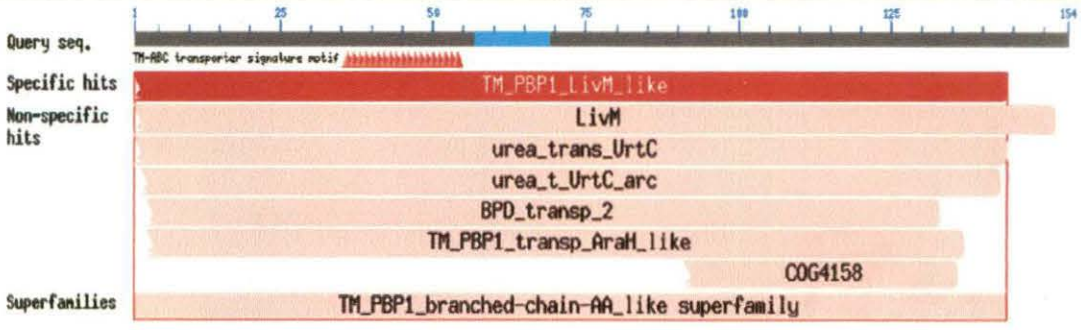


Fig. 2.8E: Result of conserved domain search for the putative translated product obtained from ORF of MB12

Description of CDD search: Result and feature of Specific hit of query sequence with the existing database (cd06581): Transmembrane subunit (TM) of *Escherichia coli* LivM and related proteins. LivM is one of two TMs of the *E. coli* LIV-1/LS transporter, a Periplasmic Binding Protein (PBP)-dependent ATP-Binding Cassette (ABC) transporter involved in the uptake of branched-chain amino acids (AAs). These types of transporters generally bind type 1 PBPs. PBP-dependent ABC transporters consist of a PBP, two TMs, and two cytoplasmic ABCs, and are mainly involved in importing solutes from the environment. The solute is captured by the PBP, which delivers it to a gated translocation pathway formed by the two TMs. The two ABCs bind and hydrolyze ATP and drive the transport reaction. *E. coli* LivM forms a heterodimer with another TM, LivH, to generate the transmembrane pore. LivH is not included in this subgroup. The LIV-1/LS transporter is comprised of two TMs (LivM and LivH), two ABCs (LivG and LivF), and one of two alternative PBPs, LivJ (LIV-BP) or LivK (LS-BP). In addition to transporting branched-chain AAs including leucine, isoleucine and valine, the *E. coli* LIV-1/LS transporter is involved in the uptake of the aromatic AA, phenylalanine

Signature motif on conserved domain

```
Feature 1                                                  ********************
gi 585411     118 .[150].ALLLVVLSLFVINPLLRMPLGRAWEALRED.[2].IACRSLGLSPRRIKLTAFTISAAFAGFAGTLFAARQGF 336
query           1 .[  4].VLVIFLAGFLLLWFVVHSPFGQILKAIREH.[2].DFATSPGYDTDTFKLLAFVISGALAGTAGATKALVFQL  73
gi 27378466    32 .[145].VLVVALIAYLLIARLVHSHFGRAMRGLMEN.[1].TLAVSVGIDVTKTLTLAAVISAAIAGAAGSLYAHYIRI 245
gi 91786264    20 .[156].CLAVAIGSFAAFHLLVSSPFGRALQAIKLD.[2].DAARASGINVTLYKSQAFVTAAVFTGWAGALFTYHNQY 214
gi 55380262   361 .[141].ALLIAVLSFLFARPMMNAPFGSVLQSIRES.[1].ERAEFIGYDVNRYKRRAFVISGGMAGLAGGLLAVSPST 570
gi 51893498   350 .[127].TLTLLLGGIWVAEPLSGSYLGRALRAVRED.[1].VAARAYGVGLERYKSIAFGLSAFIAGLAGVLLAHAYTY 545
gi 15792343    38 .[136].TGGIAIVAVILILNIVYSKYGRAMKAIRDD.[1].DAASAMGINTFWIRTLAFSTSAFLEGVGGLLACLLTT 242
gi 154245652   54 .[133].ILAMLALAILATMVMERSKLGYCLKAIRGD.[1].DAAEALGISPTRYTQAAFAISAFMTALGGSFYAQFFRY 255
gi 92113544    17 .[134].VALFVAGVYFLVERAYASPWGPVLRAIREN.[1].PATAAAGKSIAGFFLQAFVLGSAIMGLGGGLYAHFFGF 219
gi 91974534    45 .[127].TLAIFMVALWLIWRITNSPFGSLLTAIKSN.[1].VRTQYLGYDTALYKLAAIVISGSFSGLAGGLYAWFQYA 240

Feature 1
gi 585411     337 VSP.[1].SFTFAESAFVLAIVVLGGM.[1].SQFAVILAAILLVVSRELMRDF.[ 4].MLMLGGLMVLMMIWRPQGLL 406
query          74 ASL.[1].DVHWSMSGEVVLMTLVGGM.[1].TLFGPVAGAAVIVSMQNYLAQL.[ 4].TVVQGVIFVVCVLAFRRGII 143
gi 27378466   246 IDP.[1].VEAFINTVTMVIMVISGGK.[1].SLAGPVVGGLIFGLLPVALRPI.[ 6].WIAYGGVLIAILFVLPRGIV 317
gi 91786264   215 LNP.[1].DFTFWKSIEIVIMVAVGGI.[1].SLAGAVFGAFAVTLLPEVLGDL.[ 4].MIIFGSLVVLCMGLGHSGLA 284
gi 55380262   571 VIV.[4].TLNWIHSGEVIVIALFGGM.[1].TLYGPMIGSGVFFGAEEFLSSY.[ 4].RLIIGTMFILFVLFVPRGIV 643
gi 51893498   546 IAP.[1].TFTHAMSLQVLTMVVLGGL.[1].NTIGAVAGALALIVIPESARFL.[ 4].FLVYGLLLLVMLRFRPRGLL 615
gi 15792343   243 VSP.[1].QFDFLLTFQLLIIIVLGGL.[1].STTGAIIGAILVIGGSEWLRFL.[11].MVVFSIVLILVMLFARKGIM 329
gi 154245652  256 INP.[2].NMGLELSIDMALMSIIGGQ.[1].TPFGPLVGALLLMPLGEISRGY.[ 5].LVIYGVVLMITVLYFPKGLI 331
gi 92113544   220 LSP.[2].FMPLYGTFLVWVMLIAGGS.[1].NNRGAILGAVLVWGVWSGTELL.[16].VLLIGVLLQVILVTRPEGVL 302
gi 91974534   241 AYP.[1].NLFWIESGNIVILTLLGGG.[2].SFFGPILGAAVFVGAQDLISGY.[ 4].MFFFGLIFIVVVTTFPNGLP 311

Feature 1
gi 585411     407 P 407
query         144 G 144
gi 27378466   318 P 318
gi 91786264   285 G 285
gi 55380262   644 S 644
gi 51893498   616 S 616
gi 15792343   330 G 330
gi 154245652  332 A 332
gi 92113544   303 P 303
gi 91974534   312 E 312
```

Fig. 2.8F: TM-ABC transporter signature motif on conserved domain TM_PBP1_LivM_like. 19 of 19 of the residues that compose this conserved feature have been mapped in the query sequence.

```
GGCATCCAAGCAGCAAGTACCCGACTGTTTACAACTAACACCGGAAACTTTAAAAACCATACACTGG
                              *(start codon)
TTGCCATCGAGTTGTGCCTATAAACGTttgccatcgagttgtgcctataaacgtttgaatgaaggca
            1R                L  P  S  S  C  A  Y  K  R  L  N  E  G  K
aaaatttaccttcttggcactatttaaatacgggttccagacaaagcgtagtgaaagcgagaaagtc
  N  L  P  S  W  H  Y  L  N  T  G  S  R  Q  S  V  V  K  A  R  K  S
ggtagcagggcgttgtattcctgaaactgatgtatatgaagatgatatcgaagattatgtggtgcgt
 V  A  G  R  C  I  P  E  T  D  V  Y  E  D  D  I  E  D  Y  V  V  R
tgggtgcgttaaTACGCTCCCGATGAGCATTTTTAATTACAGCGTATAATAAAGTAACATTTTTATC
W  V  R  #(stop codon, end of ORF)                          1L
GTAAATTTTTTAATCATCTCCGTCATATAAAAATAAAAGCAAAAGGAGATAAAAATGATGAGACGAT
TAGCAGCCCCCTTATTATGTAGTAGTTTCTTCTTG//TTAATGGCCTGTGGCTCTAATAATACTAAT
TCAAAAAGTCCTGAACAAAATACAACTACAAAAACAGAGCAGAAGAGTGCTGCCAAACAAATTTATC
AGGTCAAGTCTGCTT
```

Fig. 2.9A: Nucleotide sequence (Ac. No. FM955254) and translated product of variable region obtained from MB54. The ORFs, putative translated product and other features of class 1 integron borne gene cassette have been shown in the sequence. Symbols: *, start codon; #, stop codon; 1R, core site; //, beginning of 3' Conserved segment

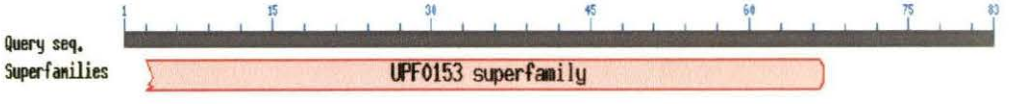


Fig. 2.9B: Result of conserved domain search for the putative translated product obtained from ORF of MB54.

Description of CDD search: This family of proteins contains 8 conserved cysteines that may form a metal binding site. The function of these proteins is unknown but presumably Fe-S cluster as part of an oxidoreductase complex.

```
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACG
                              *(start)
CAGCAGGGCAGTCGCCCTAAAACAAAGatgaaactatcactaatggtagctatatcgaagaatggagtta
                  dfrA1->   M  K  L  S  L  M  V  A  I  S  K  N  G  V  I
tcgggaatggccctgatattccatggagtgccaaaggtgaacagctcctgtttaaagctattacctataa
  G  N  G  P  D  I  P  W  S  A  K  G  E  Q  L  L  F  K  A  I  T  Y  N
ccaatggctgttggttggacgcaagacttttgaatcaatgggagcattacccaaccgaaagtatgcggtc
 Q  W  L  L  V  G  R  K  T  F  E  S  M  G  A  L  P  N  R  K  Y  A  V
gtaacacgttcaagttttacatctgacaatgagaacgtagtgatctttccatcaattaaagatgctttaa
V  T  R  S  S  F  T  S  D  N  E  N  V  V  I  F  P  S  I  K  D  A  L  T
ccaacctaaagaaaataacggatcatgtcattgtttcaggtggtggggagatatacaaaagcctgatcga
  N  L  K  K  I  T  D  H  V  I  V  S  G  G  G  E  I  Y  K  S  L  I  D
tcaagtagatacactacatatatctacaatagacatcgagccggaaggtgatgtttactttcctgaaatc
 Q  V  D  T  L  H  I  S  T  I  D  I  E  P  E  G  D  V  Y  F  P  E  I
cccagcaattttaggccagtttttacccaagacttcgcctctaacataaattatagttaccaaatctggc
P  S  N  F  R  P  V  F  T  Q  D  F  A  S  N  I  N  Y  S  Y  Q  I  W  Q
aaaaggttaaCAAGTGGCAGCAACGGATTCGCAAACCTGTCACGCCTTTTGTACCAAAAGCCGCGCCAG
  K  G  #(stop)          1R      *
GTTTGCGATCCGCTGTGCCAGGCGTTAAACATCatgagggaagcggtgatcgccgaagtatcgactcaac
                          aadA1->M  R  E  A  V  I  A  E  V  S  T  Q  L
tatcagaggtagttggcgtcatcgagcgccatctcgaaccgacgttgctggccgtacatttgtacggctc
  S  E  V  V  G  V  I  E  R  H  L  E  P  T  L  L  A  V  H  L  Y  G  S
cgcagtggatggcggcctgaagccacacagtgatattgatttgctggttacggtgaccgtaaggcttgat
 A  V  D  G  G  L  K  P  H  S  D  I  D  L  L  V  T  V  T  V  R  L  D
gaaacaacgcggcgagctttgatcaacgacctttggaaacttcggcttcccctggagagagcgagattc
E  T  T  R  R  A  L  I  N  D  L  L  E  T  S  A  S  P  G  E  S  E  I  L
tccgcgctgtagaagtcaccattgttgtgcacgacgacatcattccgtggcgttatccagctaagcgcga
  R  A  V  E  V  T  I  V  V  H  D  D  I  I  P  W  R  Y  P  A  K  R  E
actgcaatttggagaatggcagcgcaatgacattcttgcaggtatcttcgagccagccacgatcgacatt
 L  Q  F  G  E  W  Q  R  N  D  I  L  A  G  I  F  E  P  A  T  I  D  I
gatctggctatcttgctgacaaaagcaagagaacatagcgttgccttggtaggtccagcggcggaggaac
D  L  A  I  L  L  T  K  A  R  E  H  S  V  A  L  V  G  P  A  A  E  E  L
tctttgatccggttcctgaacaggatctatttgaggcgctaaatgaaaccttaacgctatggaactcgcc
  F  D  P  V  P  E  Q  D  L  F  E  A  L  N  E  T  L  T  L  W  N  S  P
gcccgactgggctggcgatgagcgaaatgtagtgcttacgttgtcccgcatttggtacagcgcagtaacc
 P  D  W  A  G  D  E  R  N  V  V  L  T  L  S  R  I  W  Y  S  A  V  T
ggcagaatcgcgccgaaggatgtcgctgccgactgggcaatggagcgcctgccggcccagtatcagcccg
G  R  I  A  P  K  D  V  A  A  D  W  A  M  E  R  L  P  A  Q  Y  Q  P  V
tcatacttgaagctagacaggcttatcttggacaagaagaagatcgcttggcctcgcgcgcagatcagtt
  I  L  E  A  R  Q  A  Y  L  G  Q  E  E  D  R  L  A  S  R  A  D  Q  L
                                                        1L
ggaagaatttgttcactacgtgaaaggcgagatcaccaaggtagtcggcaaataTGTCTAACAATTCGT
 E  E  F  V  H  Y  V  K  G  E  I  T  K  V  V  G  K  #(stop)
TCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAAGCG//TTAGATGCACTAAGCACATAATTGCTCA
CAGCCAAACTATCAGGTCAAGTCTGCTT
```

Fig. 2.10A: Nucleotide sequence (Ac. No FM179327) of variable region obtained from MB47 isolate showing ORFs, putative translated product and other features of class 1 integron borne gene cassette.
Symbols: *, start codon; #, stop codon; 1R, core site; 1L, inverse core site; //, beginning of 3' Conserved segment

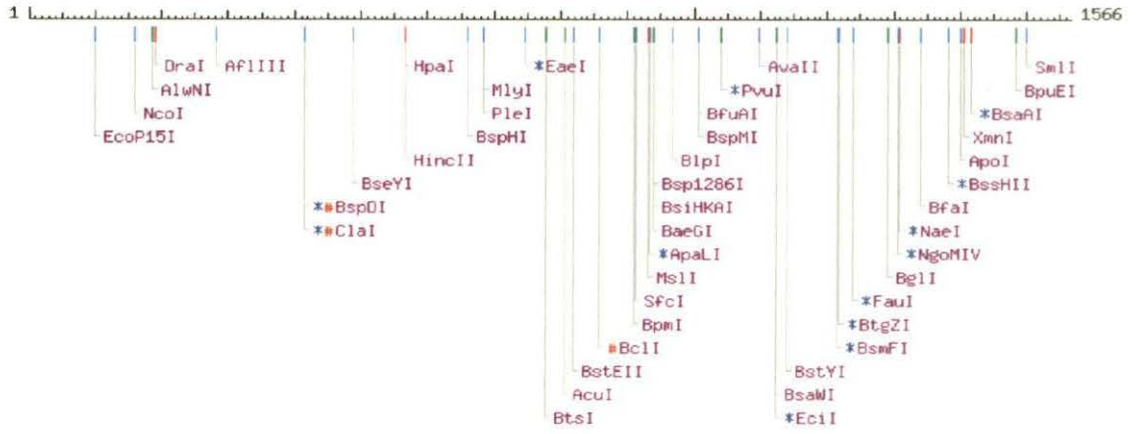


Fig. 2.10B: Restriction map of 1566 bp nucleotide sequence CS-PCR product of MB47.
Symbols: *, cleavage affected by CpG methylation; and #, cleavage affected by other methylations

Fig. 2.10C: Multiple sequence alignment of DfrA1 protein derived from MB47 and it's nearest neighboring sequences. Black, identical amino acids and gray, similar amino acids present in all the Dfr proteins.

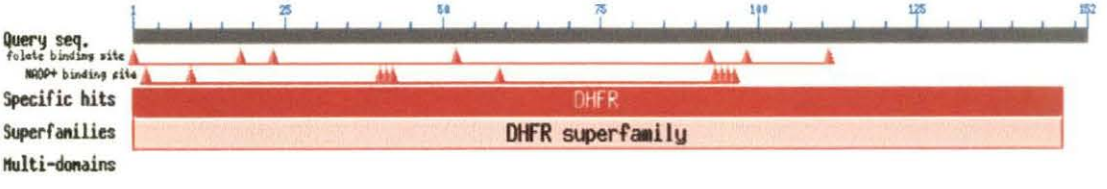
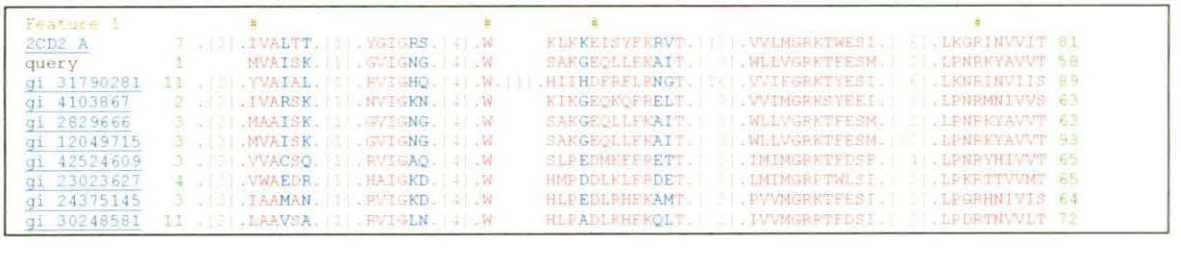
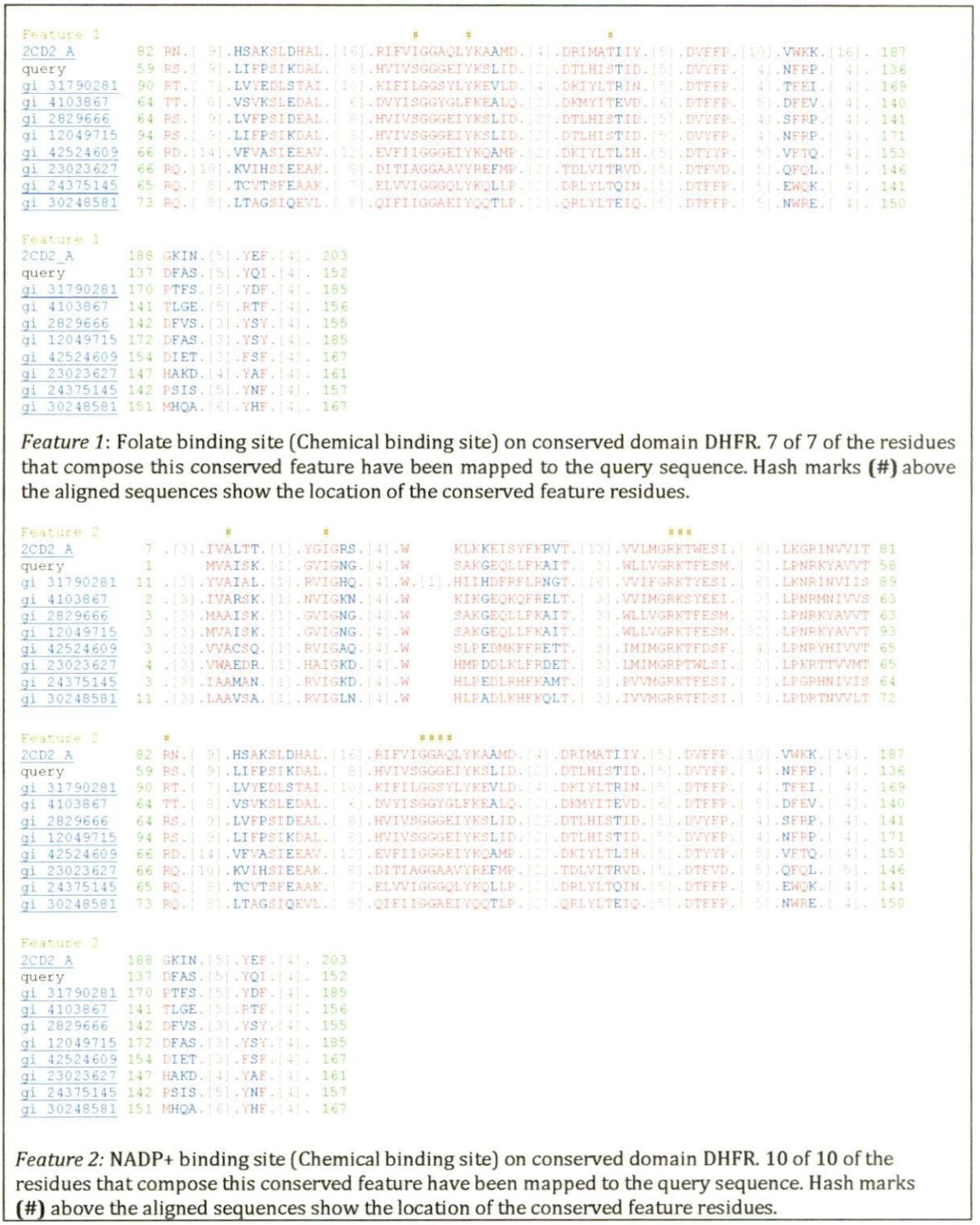


Fig. 2.10D: Detected putative conserved domain in translated product of *orf1* derived MB47 isolate.

Description of CDD search: [Specific hit] cd00209, Dihydrofolate reductase (DHFR). Reduces 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate with NADPH as a cofactor. This is an essential step in the biosynthesis of deoxythymidine phosphate since 5,6,7,8-tetrahydrofolate is required to regenerate 5,10-methylenetetrahydrofolate which is then utilized by thymidylate synthase. Inhibition of DHFR interrupts thymidilate synthesis and DNA replication, inhibitors of DHFR (such as Methotrexate) are used in cancer chemotherapy. 5,6,7,8-tetrahydrofolate also is involved in glycine, serine, and threonine metabolism and aminoacyl-tRNA biosynthesis.





Feature 1: Folate binding site (Chemical binding site) on conserved domain DHFR. 7 of 7 of the residues that compose this conserved feature have been mapped to the query sequence. Hash marks **(#)** above the aligned sequences show the location of the conserved feature residues.

Feature 2: NADP+ binding site (Chemical binding site) on conserved domain DHFR. 10 of 10 of the residues that compose this conserved feature have been mapped to the query sequence. Hash marks **(#)** above the aligned sequences show the location of the conserved feature residues.

Fig. 2.10E: Features that mapped on query sequence in CDD search

Computation determination (using ProtParam tool available at, http://web.expasy.org/protparam/) of various physical and chemical parameters of DfrA1 protein of isolate, MB47 coding for trimethoprim resistance

Computed parameters of DfrA1 are following:

Number of amino acids: *152*

Molecular weight: *16974.2*

Theoretical pI: *5.64*

Total number of negatively charged residues (Asp + Glu): 16

Total number of positively charged residues (Arg + Lys): 14

Ext. coefficient 25440 M^{-1} cm^{-1}, at 280 nm measured in water.
Abs 0.1% (=1 g/l) 1.499
Estimated half-life:
The N-terminal of the sequence considered is M (Met).
The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).
Instability index:
The instability index (II) is computed to be 29.84
This classifies the protein as stable.

Aliphatic index: 90.99
Grand average of hydropathicity (GRAVY): -0.163

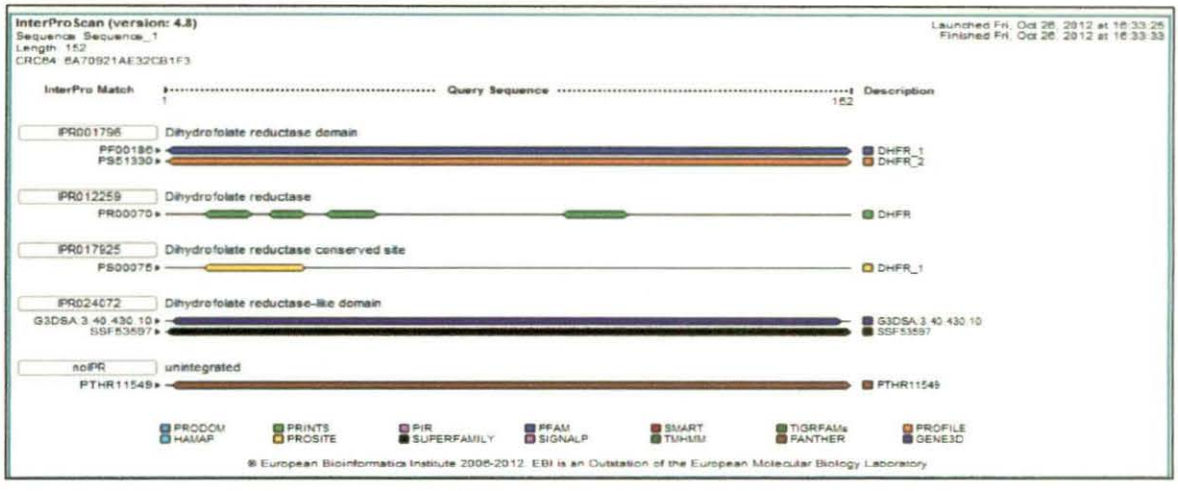


Fig. 2.10F. InterPro's signatures of DfrA1 protein derived from MB47 isolate. The map generated through InterProScan software package.

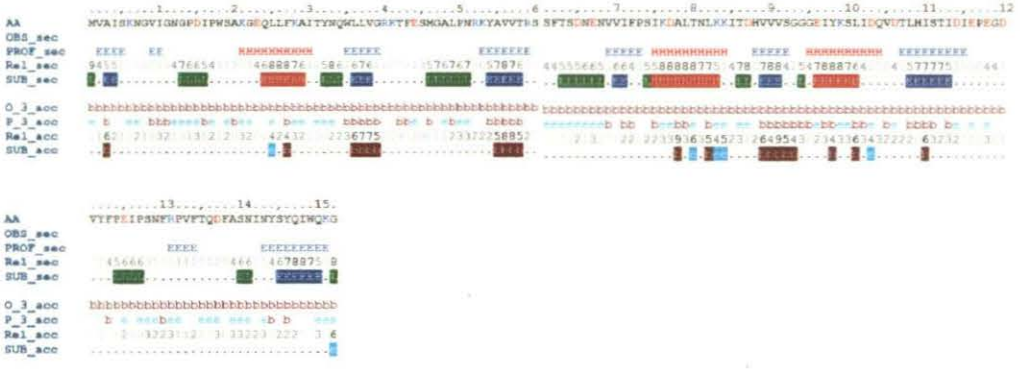


Fig 2.10G: Predicted secondary structures in DfrA1 protein coding for trimethoprim resistant dihydrofolate reductase from bacterium, MB47.

Abbreviations:

AA:	amino acid sequence
OBS_sec:	observed secondary structure [H=helix, E=extended (sheet), blank=other (loop)]
PROF_sec:	predicted secondary structure [[H=helix, E=extended (sheet), blank=other (loop)] PROF: profile network prediction HeiDelberg
Rel_sec:	reliability index for PROF secondary prediction [0=low to 9=high]
SUB_sec:	subset of PROF secondary prediction for all residues with an expected average accuracy >82% (please see table) [L=loop]
O_3_sec:	observed relative solvent accessibility (acc) in 3 states, b=0-9%; i=9-36%; e=36-100%
P_3_sec:	PROF predicted relative solvent accessibility (acc) in 3 states, b=0-9%; i=9-36%; e=36-100%

Homology modeling (automated mode software, http://swissmodel.expasy.org/) of DfrA1 protein derived from class 1 integron borne gene cassette from a facultatively oligotrophic bacterium, MB47.

Fig. 2.10H: graphical presentation of pairwise alignment between target protein dihydrofolate reductase derived from isolate, MB47 (green bar) and *Bacillus anthracis (blue bar)*.

Model information: Modeled residue range: 1 to 151 Based on template: [3e0bB] (2.25 Å) **Quaternary structure information:** Template (3e0b): Monomer Model built: Single chain **Ligand information:** Ligands in the template: N22: 1, NAP: 1. Ligands in the model: none.	

Fig. 2.10Hx: 3D model (ribbon model) of the template: 3e0bB (crystal structure at resolution2.25 Å of dihydrofolate reductase derived from *Haemophilus influenzae*)

Model information: Template: [3e0bB] (2.25 Å) Modeled residue range: 1 to 151 Sequence Identity [%]:30.57 Evalue: 0.00e-1 Quality information: QMEAN score: 0.7 Z-Score: -1.01 Predicted LGscore : 3.971 Predicted MaxSub : 0.455	

Fig 2.10Hy: 3D model (ribbon model) of the DfrA1 protein of bacterium MB47

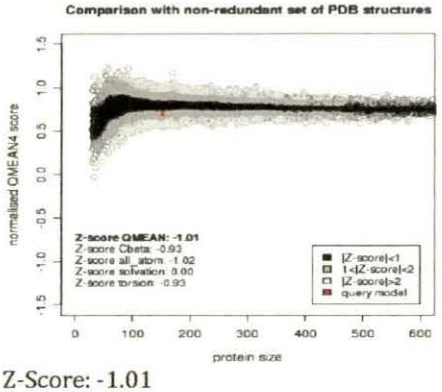
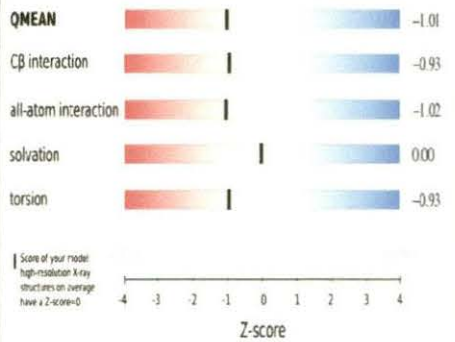
QMEAN4 score	Estimated absolute model quality	Score components
0.7 (Range for reliable model: 0-1)	 Z-Score: -1.01	

Fig 2.10Hz: Global model quality estimation of DfrA1 protein of bacterium MB47.

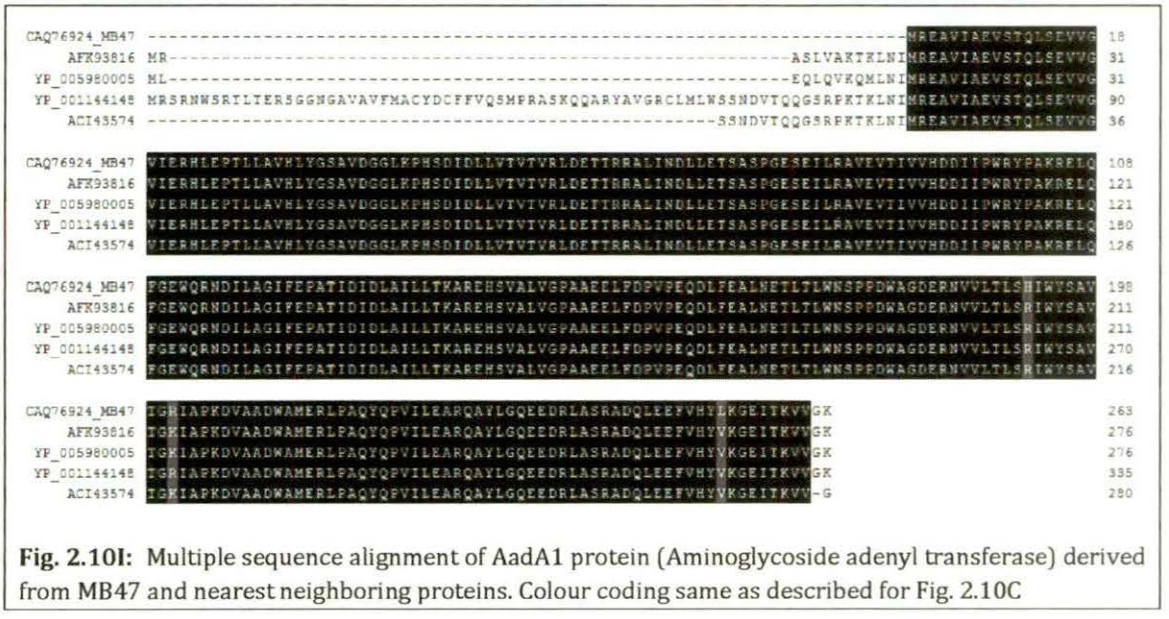


Fig. 2.10I: Multiple sequence alignment of AadA1 protein (Aminoglycoside adenyl transferase) derived from MB47 and nearest neighboring proteins. Colour coding same as described for Fig. 2.10C

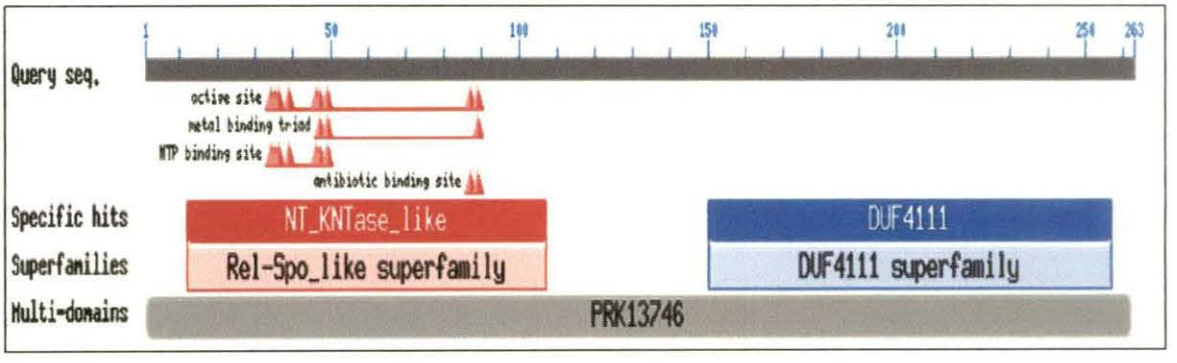
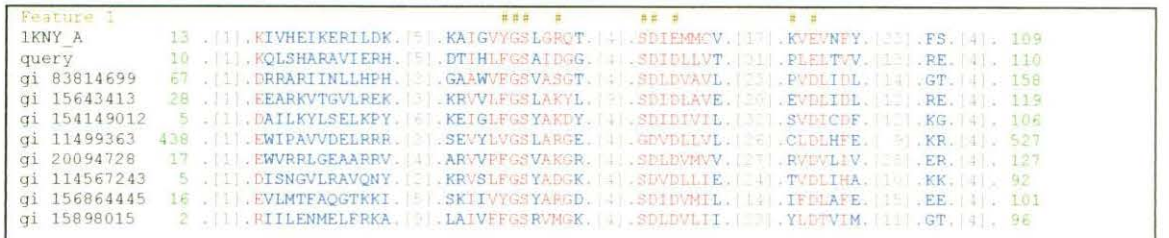
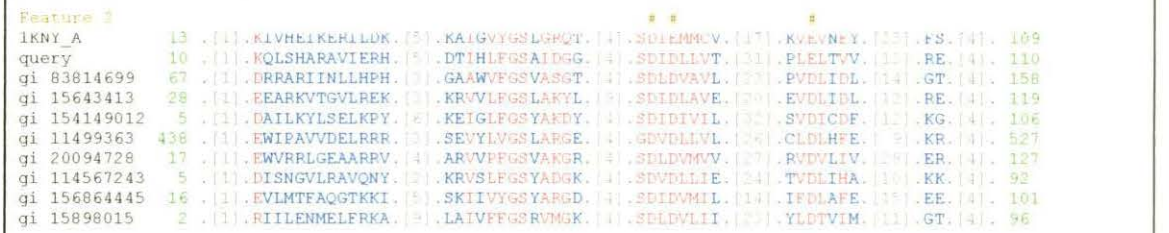


Fig. 2.10J: Detected putative conserved domain in translated nucleotide sequence derived from MB47 bacterium.

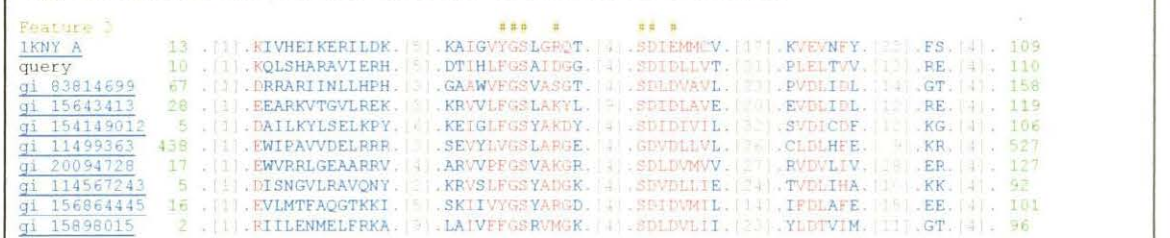
Description of CDD search: Specific hit result cd05403, Nucleotidyltransferase (NT) domain of *Staphylococcus aureus* kanamycin nucleotidyltransferase, and similar proteins. *S. aureus* KNTase is a plasmid encoded enzyme which confers resistance to a wide range of aminoglycoside antibiotics which have a 4'- or 4''-hydroxyl group in the equatorial position, such as kanamycin A. This enzyme transfers a nucleoside monophosphate group from a nucleotide (ATP, GTP, or UTP) to the 4'-hydroxyl group of kanamycin A. This enzyme is a homodimer, having two NT active sites. The nucleotide and antibiotic binding sites of each active site include residues from each monomer. Included in this subgroup is *Escherichia coli* AadA5 which confers resistance to the antibiotic spectinomycin and is a putative aminoglycoside-3'-adenylyltransferase. It is part of the aadA5 cassette of a class 1 integron. This subgroup also includes *Haemophilus influenzae* HI0073 which forms a 2:2 heterotetramer with an unrelated protein HI0074. Structurally HI0074 is related to the substrate-binding domain of S. aureus KNTase. The genes encoding HI0073 and HI0074 form an operon. Little is known about the substrate specificity or function of two-component NTs. The characterized members of this subgroup may not be representative of the function of this subgroup. This subgroup belongs to the Pol beta-like NT superfamily. In the majority of enzymes in this superfamily, two carboxylates, Dx [D/E], together with a third more distal carboxylate, co-ordinate two divalent metal cations involved in a two-metal ion mechanism of nucleotide addition. These carboxylate residues are conserved in this subgroup.



Feature1: Active site on conserved domain. 9 of 9 of the residues that compose this conserved feature have been mapped to the query sequence. Hash marks **(#)** above the aligned sequences show the location of the conserved feature residues.



Feature 2: metal binding triad [ion binding site] on conserved domain. 3 of 3 of the residues that compose this conserved feature have been mapped to the query sequence. Hash marks **(#)** above the aligned sequences show the location of the conserved feature residues.



Feature 3: NTP binding site [chemical binding site] on conserved domain. 7 of 7 of the residues that compose this conserved feature have been mapped to the query sequence. Hash marks **(#)** above the aligned sequences show the location of the conserved feature residues.

Fig. 2.10K: Features showing conserved domain (NT_KNTase_like) in query sequence

Computational determination using ProtParam tool (http://web.expasy.org/protparam/) of various physical and chemical parameters of AadA1 protein (responsible for streptomycin /spectinomycin resistance) of isolate, MB47

Computed parameters of AadA1 are following:

Number of amino acids: 263; Molecular weight: 29355.3; Theoretical pI: 4.60

Total number of negatively charged residues (Asp + Glu): 45
Total number of positively charged residues (Arg + Lys): 23

Ext. coefficient 41940 M^{-1} cm^{-1}, at 280 nm measured in water.
Abs 0.1% (=1 g/l) 1.429

Estimated half-life:
The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:
The instability index (II) is computed to be 41.28
This classifies the protein as unstable.
Aliphatic index: 110.49; Grand average of hydropathicity (GRAVY): -0.075

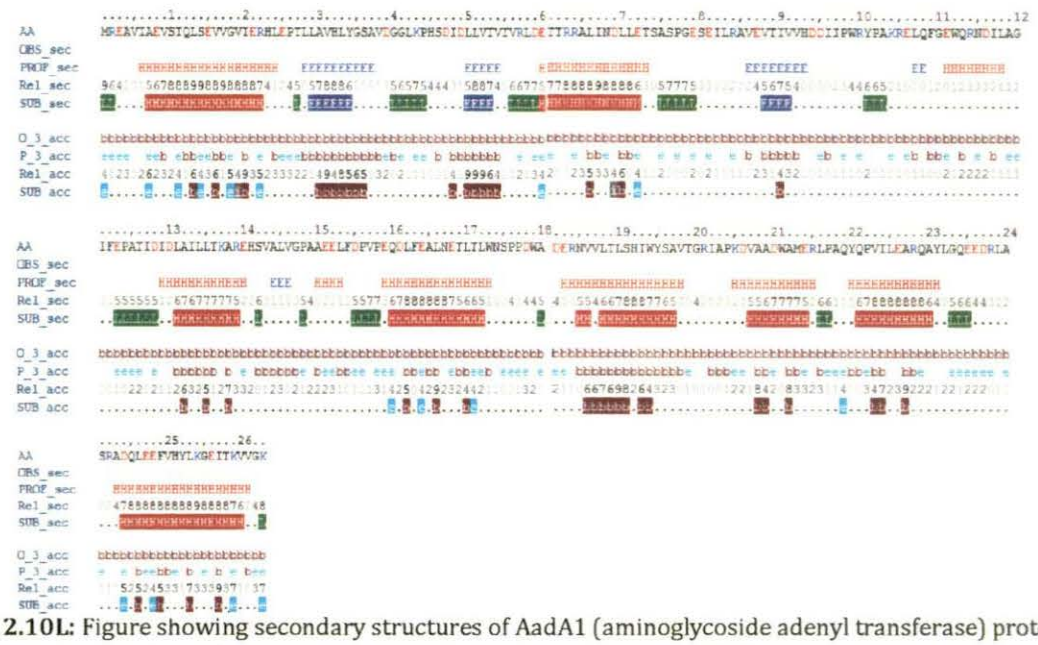


Fig 2.10L: Figure showing secondary structures of AadA1 (aminoglycoside adenyl transferase) protein coding for streptomycin/spectinomycin resistance derived from bacterium MB47.

Abbreviations: same as stated for Fig. 2.9F.

Homology modeling (automated mode, website *http://swissmodel.expasy.org/*) of AadA1 protein derived from class 1 integron borne gene cassette from a facultatively oligotrophic bacterium, MB47.

Fig 2.10M: graphical presentation of pairwise alignment between target nucleotide binding domain of the protein nucleotidyl transferase of AadA1 (green) derived from MB47 and *Haemophilus influenzae (blue)*. The blue bar with red outer line showed the modeled residues range and identity of amino acids present in-between two protein sequences.

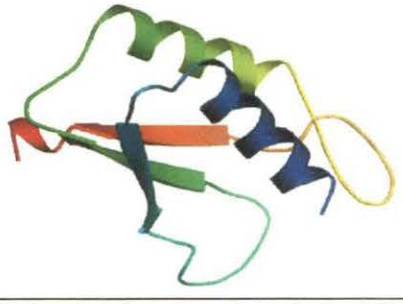
Model information: Modeled residue range: 10 to 96 Template: [1no5B] (1.80 Å) **Quaternary structure information:** Template (1no5): MONOMER Model built: SINGLE CHAIN **Ligand information:** Ligands in the template: SO4:1, ZN: 4. Ligands in the model: none.	

Fig. 2.10Mx: 3D model (ribbon model) of the template: 1no5B (crystal structure at resolution1.80 Å of HI0073, the nucleotide binding domain of the Hi0073/Hi0074 two protein nucleotidyl transferase from *Haemophilus influenzae*)

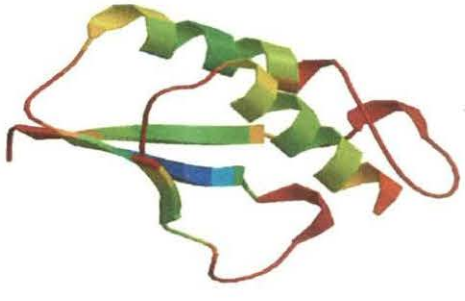
Model information: Template: [1no5B] (1.80 Å) Modeled residue range: 10 to 96 Sequence Identity [%]:21.84 Evalue: 2.00e-9 QMEAN score: 0.51 Z-Score: -2.21 Predicted LGscore : 2.112 Predicted MaxSub : 0.312	

Fig. 2.10My: 3D model (ribbon model) of the AadA1 protein of bacterium MB47

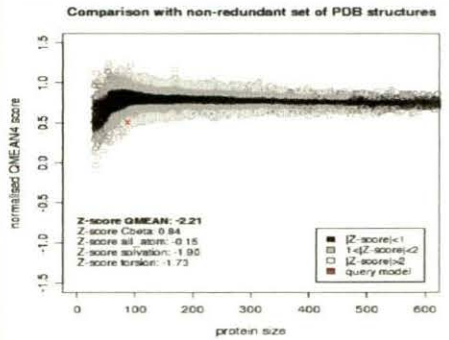
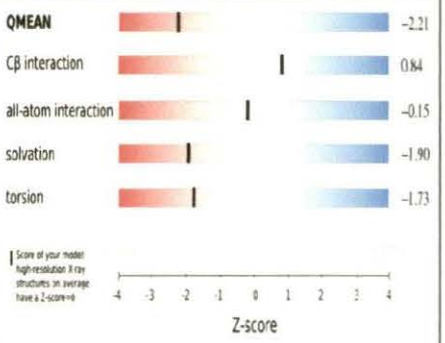
QMEAN4 score	Estimated absolute model quality	Score components
0.51 (Range for reliable model:0-1)	 Z-Score: -2.21	

Fig. 2.10Mz: Global model quality estimation of AadA1 protein of bacterium MB47

```
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACG
                                1R                          *(start)
CAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATTAAGGGAGTTAAAatgaaaatatcattgatttctg
                                          dfrA17-- >      M  K  I  S  L  I  S  A
cagtgtcagaaaatggcgtaatcggtagtggtcctgatatcccgtggtcagtaaaaggtgagcaactact
  V  S  E  N  G  V  I  G  S  G  P  D  I  P  W  S  V  K  G  E  Q  L  L
ctttaaagcgctcacatataatcaatggctccttgtcggaagaaaaacatttgactctatgggtgttctt
 F  K  A  L  T  Y  N  Q  W  L  L  V  G  R  K  T  F  D  S  M  G  V  L
ccaaatcgcaaatatgcagtagtgtcaaagaacggaatttcaagctcaaatgaaaacgtcctagtttttc
P  N  R  K  Y  A  V  V  S  K  N  G  I  S  S  S  N  E  N  V  L  V  F  P
cttcaatagaaaatgctttgaaagagctatcaaaagttacagatcatgtatatgtctctggcggggtca
  S  I  E  N  A  L  K  E  L  S  K  V  T  D  H  V  Y  V  S  G  G  G  Q
aatctataatagccttattgaaaaagcagatataattcatttgtctactgttcacgttgaagtcgaaggt
 I  Y  N  S  L  I  E  K  A  D  I  I  H  L  S  T  V  H  V  E  V  E  G
gatatcaaattccctataatgcctgagaatttcaatttggtttttgaacagtttttttatgtctaatataa
D  I  K  F  P  I  M  P  E  N  F  N  L  V  F  E  Q  F  F  M  S  N  I  N
                                1L
attatacataccagatttggaaaaaaggctaaCAATGCGTTGCAGCACCAGTCGCTTCGCTCCTTGGACA
  Y  T  Y  Q  I  W  K  K  G  #(stop
GCTTTTAAGTCGCGTCTTTGTGGTTTTGCTGCGCAAAAGTATTCCACAAAGCCGCAACTTAAAAGCTGCC
               1R       *(start)
GCTGAACTTAACGTTAGGCATCatggtgaatttttccctgcacaagttttcaagcagctgtcccacgct
            aadA5-->   M  G  E  F  F  P  A  Q  V  F  K  Q  L  S  H  A
cgcgcggtgatcgagcgccatctggctgcgacactggacacaatccacctgttcggatctgcgatcgatg
R  A  V  I  E  R  H  L  A  A  T  L  D  T  I  H  L  F  G  S  A  I  D  G
gagggctgaagccggacagcgacatagacttgctcgtgaccgtcagcgccgcacctaacgattcgctccg
  G  L  K  P  D  S  D  I  D  L  L  V  T  V  S  A  A  P  N  D  S  L  R
gcaggcgctaatgctcgatttgctgaaagtctcatcaccgccaggcgatggcggaacatggcgaccgctg
 Q  A  L  M  L  D  L  L  K  V  S  S  P  P  G  D  G  G  T  W  R  P  L
gagctaactgttgtcgctcgaagcgaagtagtgccttggcgctatccggcgcggcgtgagcttcagttcg
E  L  T  V  V  A  R  S  E  V  V  P  W  R  Y  P  A  R  R  E  L  Q  F  G
gtgagtggctccgccacgacatcctttccggaacgttcgagcctgccgttctggatcacgatcttgcgat
  E  W  L  R  H  D  I  L  S  G  T  F  E  P  A  V  L  D  H  D  L  A  I
tttgctgaccaaggcgaggcaacacagccttgcgcttctaggcccatccgcagccacgtttttcgagccg
 L  L  T  K  A  R  Q  H  S  L  A  L  L  G  P  S  A  A  T  F  F  E  P
gtgccgaaggagcatttctccaaggcgcttttcgacactattgcccagtggaatgcagagtcggattgga
V  P  K  E  H  F  S  K  A  L  F  D  T  I  A  Q  W  N  A  E  S  D  W  K
agggtgacgagcggaacgtcgttcttgctcttgctcgcatttggtacagcgcttcaactggtctcattgc
  G  D  E  R  N  V  V  L  A  L  A  R  I  W  Y  S  A  S  T  G  L  I  A
tcctaaggacgttgctgccgcatgggtatcggagcgtttgcctgccgagcatcggcccctcatctgcaag
 P  K  D  V  A  A  A  W  V  S  E  R  L  P  A  E  H  R  P  L  I  C  K
gcacgcgcggcgtacctgggtagcgaggacgacgacctagcaatgcgcgtcgaagagacggccgcgttcg
A  R  A  A  Y  L  G  S  E  D  D  D  L  A  M  R  V  E  E  T  A  A  F  V
ttcgatatgccaaagcaacgattgagagaatcttgcgttgaGCGGCATGTGCGAAAAGTGCATCGACCCG
  R  Y  A  K  A  T  I  E  R  I  L  R  #  (stop)
                     1R
CGCCGAGGGCATCTGATGCCTAACTCGGCGTTCAAGCGGACGGGCTGCGCCCGCCGCTCAACTATGCG//T
(59 base element)
TAGATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTT
```

Fig. 2.11: Nucleotide sequence (Ac. No. FM179325) of variable region obtained from MB53 showing ORFs, putative translated product and other features of class 1 integron borne gene cassette (symbols: *, start codon; #, stop codon; 1R, core site; 1L, inverse core site; //, beginning of 3' Conserved segment).

```
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGCTATGGAGCAGCAACGATGTTA
                                  1R                    * (start)
CGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATTAAGGGAGTTAAAttgaaaatatcattgatt
                                                  dfrA7-> L  K  I  S  L  I
tctgcagtgtcagaaaatggcgtaatcggtagtggtcctgatatcccgtggtcagtaaaaggtgagca
S  A  V  S  E  N  G  V  I  G  S  G  P  D  I  P  W  S  V  K  G  E  Q
actactctttaaagcgctcacatataatcaatggctccttgtcggaagaaaaacatttgactctatgg
 L  L  F  K  A  L  T  Y  N  Q  W  L  L  V  G  R  K  T  F  D  S  M  G
gtgttcttccaaatcgcaaatatgcagtagtgtcaaagaacggaatttcaagctcaaatgaaaacgtc
  V  L  P  N  R  K  Y  A  V  V  S  K  N  G  I  S  S  S  N  E  N  V
ctagtttttccttcaatagaaaatgctttgaaagagctatcaaaagttacagatcatgtatatgtctc
L  V  F  P  S  I  E  N  A  L  K  E  L  S  K  V  T  D  H  V  Y  V  S
tggcgggggtcaaatctataatagccttattgaaaaagcagatataattcatttgtctactgttcacg
 G  G  G  Q  I  Y  N  S  L  I  E  K  A  D  I  I  H  L  S  T  V  H  V
ttgaagtcgaaggtgatatcaaattccctataatgcctgagaatttcaatttggtttttgaacagttt
  E  V  E  G  D  I  K  F  P  I  M  P  E  N  F  N  L  V  F  E  Q  F
                                                  1L
tttatgtctaatataaattatacataccagatttggaaaaaaggctaaCAATGCGTTGCAGCACCAGT
F  M  S  N  I  N  Y  T  Y  Q  I  W  K  K  G  #(stop)
CGCTTCGCTCCTTGGACAGCTTTTAAGTCGCGTCTTTGTGGTTTTGCTGCGCAAAAGTATTCCACAAA
                                  1R          *(start)
GCCGCAACTTAAAAGCTGCCGCTGAACTTAACGTTAGGCATCatggtgaatttttccctgcacaagt
                                                  aadA5-> M  G  E  F  F  P  A  Q  V
tttcaagcagctgtcccacgctcgcgcggtgatcgagcgccatctggctgcgacactggacacaatcc
 F  K  Q  L  S  H  A  R  A  V  I  E  R  H  L  A  A  T  L  D  T  I  H
acctgttcggatctgcgatcgatggagggctgaagccggacagcgacatagacttgctcgtgaccgtc
  L  F  G  S  A  I  D  G  G  L  K  P  D  S  D  I  D  L  L  V  T  V
agcgccgcacctaacgattcgctccggcaggcgctaatgctcgatttgctgaaagtctcatcaccgcc
S  A  A  P  N  D  S  L  R  Q  A  L  M  L  D  L  L  K  V  S  S  P  P
aggcgatggcggaacatggcgaccgctggagctaactgttgtcgctcgaagcgaagtagtgccttggc
 G  D  G  G  T  W  R  P  L  E  L  T  V  V  A  R  S  E  V  V  P  W  R
gctatccggcgcggcgtgagcttcagttcggtgagtggctcgcacgacatctttcggaacgttcgagc
  Y  P  A  R  R  E  L  Q  F  G  E  W  L  A  R  H  L  S  E  R  S  S
ctgccgtctggatcacgatctgcgattttgctgaccaaggcgaggcaacacagccttgcgcttctagg
L  P  S  G  S  R  S  A  I  L  L  T  K  A  R  Q  H  S  L  A  L  L  G
cccatccgcagccacgtttttcgagccggtgccgaaggagcatttctccaaggcgcttttcgacacta
 P  S  A  A  T  F  F  E  P  V  P  K  E  H  F  S  K  A  L  F  D  T  I
ttgcccagtggaatgcagagtcggattggaagggtgacgagcggaacgtcgttcttgctcttgctcgc
  A  Q  W  N  A  E  S  D  W  K  G  D  E  R  N  V  V  L  A  L  A  R
atttggtacagcgcttcaactggtctcattgctcctaaggacgttgctgccgcatgggtatcggagcg
I  W  Y  S  A  S  T  G  L  I  A  P  K  D  V  A  A  A  W  V  S  E  R
tttgcctgccaagcatcggcccctcatctgcaaggcacgcgcggcgtacctgggtagcgaggacgacg
 L  P  A  K  H  R  P  L  I  C  K  A  R  A  A  Y  L  G  S  E  D  D  D
acctgcaatgcgcgtcgaagagacggccgcgttcgttcgatatgccaaagcaacgattgagagaatct
  L  Q  C  A  S  K  R  R  P  R  S  F  D  M  P  K  Q  R  L  R  E  S
                                                  1L
tgcgttgagcggcatgtgcgaaaagtgcatcgacccgcgccgagggcatctgatgcctaaCTCGGCGT
C  V  E  R  H  V  R  K  V  H  R  P  A  P  R  A  S  D  A  #(stop)
TCAAGCGGACGGGCTGCGCCCGCCGCTCAACTATGCG//TTAGATGCACTAAGCACATAATTGCTCAC
AGCCAAACTATCAGGTCAAGTCTGCTT
```

Fig. 2.12A: Nucleotide sequence (Ac. No. HE650979) of variable region obtained from MB29 showing ORFs, putative translated product and other features of class 1 integron borne gene cassette.
Symbols: *, start codon; #, stop codon; *1R*, core site; *1L*, inverse core site; //, beginning of 3' conserved segment.

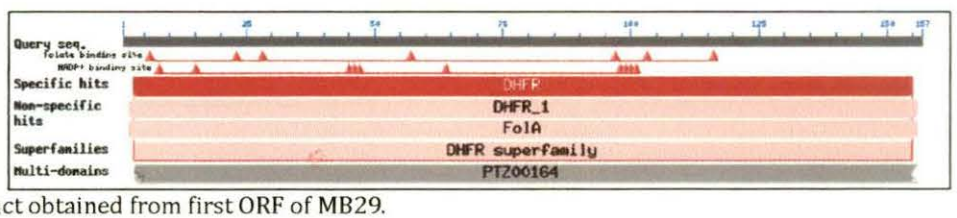


Fig. 2.12B: Result of conserved domain search for the putative translated product obtained from first ORF of MB29.

Description of CDD search: same as described in Fig. 2.9C.

BlastP: Homology of predicted orf1 of MB29 with the existing protein sequences database

gb|EGB89678.1| dihydrofolate reductase [*Escherichia coli* MS 117-3]
Length=210
Score = 320 bits (820), Expect = 3e-109, Method: Compositional matrix adjust.
Identities = 157/157 (100%), Positives = 157/157 (100%), Gaps = 0/157 (0%)

```
Query  1    LKISLISAVSENGVIGSGPDIPWSVKGEQLLFKALTYNQWLLVGRKTFDSMGVLPNRKYA  60
            LKISLISAVSENGVIGSGPDIPWSVKGEQLLFKALTYNQWLLVGRKTFDSMGVLPNRKYA
Sbjct  54   LKISLISAVSENGVIGSGPDIPWSVKGEQLLFKALTYNQWLLVGRKTFDSMGVLPNRKYA  113

Query  61   VVSKNGISSSNENVLVFPSIENALKELSKVTDHVYVSGGGQIYNSLIEKADIIHLSTVHV  120
            VVSKNGISSSNENVLVFPSIENALKELSKVTDHVYVSGGGQIYNSLIEKADIIHLSTVHV
Sbjct  114  VVSKNGISSSNENVLVFPSIENALKELSKVTDHVYVSGGGQIYNSLIEKADIIHLSTVHV  173

Query  121  EVEGDIKFPIMPENFNLVFEQFFMSNINYTYQIWKKG  157
            EVEGDIKFPIMPENFNLVFEQFFMSNINYTYQIWKKG
Sbjct  174  EVEGDIKFPIMPENFNLVFEQFFMSNINYTYQIWKKG  210
```

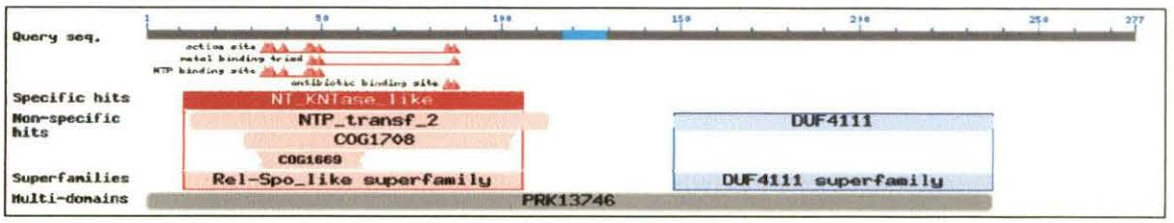


Fig. 2.12C: Result of conserved domain search for the putative translated product obtained from second ORF derived from MB29 bacterium.

Description of CDD search: same as described earlier in fig. 2.9l.

BlastP result of ORF2 of MB29

gb|AEQ26263.1| aminoglycoside adenyltransferase [Riemerella anatipestifer]
Length=262

Score = 439 bits (1129), Expect = 3e-153, Method: Compositional matrix adjust.
Identities = 221/239 (92%), Positives = 225/239 (94%), Gaps = 2/239 (1%)

```
Query  1    MGEFFPAQVFKQLSHARAVIERHLAATLDTIHLFGSAIDGGLKPDSDIDLLVTVSAAPND  60
            MGEFFPAQVFKQLSHARAVIERHLAATLDTIHLFGSAIDGGLKPDSDIDLLVTVSAAPND
Sbjct  1    MGEFFPAQVFKQLSHARAVIERHLAATLDTIHLFGSAIDGGLKPDSDIDLLVTVSAAPND  60

Query  61   SLRQALMLDLLKVSSPPGDGGTWRPLELTVVARSEVVPWRYPARRELQFGEWLARHLSER  120
            SLRQALMLDLLKVSSPPGDGGTWRPLELTVVARSEVVPWRYPARRELQFGEWL   +
Sbjct  61   SLRQALMLDLLKVSSPPGDGGTWRPLELTVVARSEVVPWRYPARRELQFGEWLRHDILSG  120

Query  121  SSLPS--GSRSAILLTKARQHSLALLGPSAATFFEPVPKEHFSKALFDTIAQWNAESDWK  178
            +  P+       AILLTKARQHSLALLGPSAATFFEPVPKEHFSKALFDTIAQWNAESDWK
Sbjct  121  TFEPAVLDHDLAILLTKARQHSLALLGPSAATFFEPVPKEHFSKALFDTIAQWNAESDWK  180

Query  179  GDERNVVLALARIWYSASTGLIAPKDVAAAWVSERLPAKHRPLICKARAAYLGSEDDDL  237
            GDERNVVLALARIWYSASTGLIAPKDVAAAWVSERLPA+HRPLICKARAAYLGSEDDDL
Sbjct  181  GDERNVVLALARIWYSASTGLIAPKDVAAAWVSERLPAEHRPLICKARAAYLGSEDDDL  239
```

```
gi 123545237    3 ASELFDPVPKESFVKALSDTAAQW.[2].ESDWQGDERNVVLALARIWFSVSTGEIAPKDAAASWLLERLPHEHRAVLMV 79
query         148 AATFFEPVPKEHFSKALFDTIAQW.[2].ESDWKGDERNVVLALARIWYSASTGLIAPKDVAAAWVSERLPAKHRPLICK 224
gi 75401522    11 AATFFEPVPKEHFSKALFDTIAQW.[2].ESDWKGDERNVVLALARIWYSASTGLIAPKDVAAAWVSERLPAEHRPLICK 87
gi 123565762  150 AAALFEQVPARDFVAALLATVAQW.[2].EPDWRGDACNVVLALARIWYSAATGRIAPKDVAATWVLERLPETYRPVVAA 226
gi 123074532  150 ATDLFDPVPRTDLARAFYDTALQW.[2].PADWRGDERTVVLALARIWFSLSTGGIAPKDVAAQWVIERLPDEHQSIMRS 226
gi 123450561  150 AQKVFMPVPEHDFLQVLSDTLKLW.[2].HEDWENEERNIVLTLARIWYSTETGGIVPKDVAAEWVLERLPAEHKPILVE 226
gi 75449966   149 AEEFFDPVPEQDLFEALRETLKLW.[2].QPDWAGDERNVVLTLSRIWYSAITGKIAPKDVAADWAIKRLPAQYQPVLLE 225
gi 239820329  150 ADELFDPVPRADFSRSLLDTVAQW.[2].APDWQGDERNVVLALARIWFSACTGQIAPKDTAAAWALERLPSEHRPVLAS 226
gi 146307205  178 AAELFEPVPEADMIHALHDTAAQW.[2].PDDWLGDECNVVLALARIWLTLSSGEIAAKDVAADWLLERLPPEHRPLLET 254
gi 146282523  145 AAELFDPVPEEDMRQALRDTLAQW.[2].PADWQGDECNVVLAVARIWLTLTTDAIAPKDVAAIWLLERLPDEHRAVLEK 221

gi 123545237   80 ALAAYLGTARDDL.[18]. 110
query         225 APAAYLGSEDDDL.[18]. 255
gi 75401522    88 APAAYLGSEDDDL.[18]. 118
gi 123565762  227 APAAYLGGEEEAA.[18]. 257
gi 123074532  227 APASYLGETQDDL.[16]. 257
gi 123450561  227 APQAYLGLCKDSL.[18]. 257
gi 75449966   226 APQAYLGQKEDHL.[18]. 256
gi 239820329  227 ALAAYLGRAPDNL.[18]. 257
gi 146307205  255 APDVYLGQARDDL.[18]. 285
gi 146282523  222 ASQAYRGDAIEKP.[18]. 252
```

Fig. 2.12D: Sequence alignment of part of protein sequence of orf2 (coding for Aad) derived from MB29 bacterium showing domain similarity with DUF4111 family.

Domain of unknown function (DUF4111): the protein-protein homology of downstream sequence of *orf 2* which was coding for AadA5 protein showed similarity with an unknown function domain. Although the exact function of this domain is not known however it frequently appears downstream of sequences of the family, Nucleotidyl transferase, pfam01909. It is also found in species associated with methicillin-resistant bacteria.

```
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTAC
                                                                      *
GCAGCAGGGCAGTCGCCCTAAAACAAAGTTAACCTCTGAGGAAGAATTGTGAAACTATCACTAatggta
                             1R                                DfrA1->  M  V
gctatatcgaagaatggagttatcgggaatggccctgatattccatggagtgccaaaggtgaacagctc
A  I  S  K  N  G  V  I  G  N  G  P  D  I  P  W  S  A  K  G  E  Q  L
ctgtttaaagctattacctataaccaatggctgttggttggacgcaagacttttgaatcaatgggagca
L  F  K  A  I  T  Y  N  Q  W  L  L  V  G  R  K  T  F  E  S  M  G  A
ttacccaaccgaaaatatgcggtcgtaacacgttcaagttttacatctgacaatgagaacgtattgatc
L  P  N  R  K  Y  A  V  V  T  R  S  S  F  T  S  D  N  E  N  V  L  I
tttccatcaattaaagatgctttaaccaacctaaagaaaataacggatcatgtcattgtttcaggtggt
F  P  S  I  K  D  A  L  T  N  L  K  K  I  T  D  H  V  I  V  S  G  G
ggggagatatacaaaagcctgatcgatcaagtagatacgctacatatatctacaatagacatcgagccg
G  E  I  Y  K  S  L  I  D  Q  V  D  T  L  H  I  S  T  I  D  I  E  P
gaaggtgatgtttactttcctgaaatccccagcaattttaggccagtttttacccaagacttcgcctct
E  G  D  V  Y  F  P  E  I  P  S  N  F  R  P  V  F  T  Q  D  F  A  S
aacataaattatagttaccaaatctggcaaaaggttaaCAAGTGGCAGCAACGGATTCGCAAACCTGT
N  I  N  Y  S  Y  Q  I  W  Q  K  G  #(end of dfrA1)
                                                  1R                *
CACGCCTTTTGTACAAAAGCCGCGCCAGGTTTGCGATCCGCTGTGCCAGGCGTTAAGGCTACatgaaaa
                                                  ORF38->  M  K  I
tcgtacattacgaagcgaatgcaccatggataggaagaatgaaatgcccaaacccaaagtgtgggaagg
  V  H  Y  E  A  N  A  P  W  I  G  R  M  K  C  P  N  P  K  C  G  K  E
aaactcctgcctggcaatcgagcggcatgagcgacagttgcccgcatttttctgtgatacttgctcga
  T  P  A  W  Q  S  S  G  M  S  D  S  C  P  H  F  F  C  D  T  C  S  N
atgtaatccatagagagcaggaccatgcattactgtacgaaaatgaaatcaatcaagagctcttggatc
  V  I  H  R  E  Q  D  H  A  L  L  Y  E  N  E  I  N  Q  E  L  L  D  R
gaatagcagcaactcttccagattgcccttgcggggataggtttgttcctggtgcaaacccaaagtgtc
  I  A  A  T  L  P  D  C  P  C  G  D  R  F  V  P  G  A  N  P  K  C  P
cgagttgcaagaccgagtacgtgcaccaatgggatgcagtgaaaaggttgaatgtacctttatgccaa
  S  C  K  T  E  Y  V  H  Q  W  D  A  V  K  R  L  N  V  P  F  M  P  I
tcttgtatggttcctgcttgattcgagataggctgtattcgtatgaagtatgcattggttctaaaccaa
  L  Y  G  S  C  L  I  R  D  R  L  Y  S  Y  E  V  C  I  G  S  K  P  K
                          1L
aatactggtggcgtttgttcacaaatgccttaacaagtttaggcaagggacgctcctgaCGTCGCGCCC
  Y  W  W  R  L  F  T  N  A  L  T  S  L  G  K  G  R  S  #
CTGCTAAAAGCG//TTAGATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCT
T
```

Fig. 2.13A: Nucleotide sequence (Ac. No. AM997276) of variable region amplified through CS-PCR from MB38 isolate, showing putative translated product and other features of class 1 integron borne gene cassette. Symbols: *, start codon; #, stop codon; 1R, core site; 1L, inverse core site; //, beginning of 3' Conserved segment.

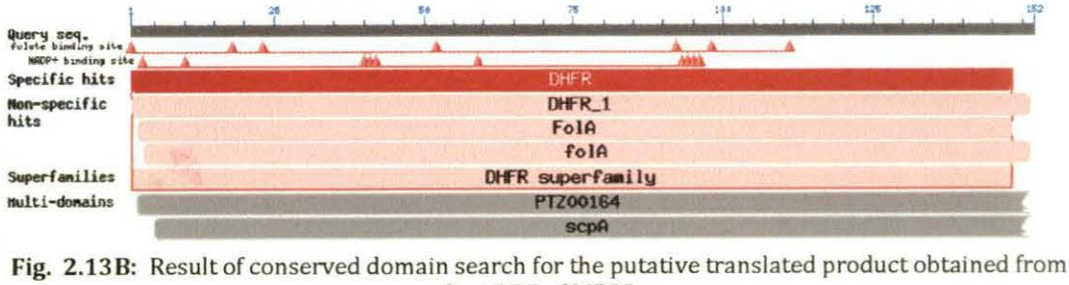


Fig. 2.13B: Result of conserved domain search for the putative translated product obtained from first ORF of MB38

BlastP result of second ORF38 of MB38,

gb|AAM33365.1|AF455254_2 unknown [*Vibrio cholerae*]
dbj|BAD02391.1| unknown [*Vibrio cholerae* non-O1/non-O139]
dbj|BAD08521.1| hypothetical protein [*Escherichia coli*]
36 more sequence titles
Length=159

```
Score =  330 bits (846),  Expect = 1e-115, Method: Compositional matrix adjust.
Identities = 157/159 (99%), Positives = 157/159 (99%), Gaps = 0/159 (0%)

Query  1    MKIVHYEANAPWIGRMKCPNPKCGKETPAWQSSGMSDSCPHFFCDTCSNVIHREQDHALL  60
            MKIVHYEANAPWIGRMKCPNPKCGKETPAWQSSGMSDSCPHFFCDTCSNVIHREQDHALL
Sbjct  1    MKIVHYEANAPWIGRMKCPNPKCGKETPAWQSSGMSDSCPHFFCDTCSNVIHREQDHALL  60

Query  61   YENEINQELLDRIAATLPDCPCGDRFVPGANPKCPSCKTEYVHQWDAVKRLNVPFMPILY  120
            YENEINQELLDRIAATLPDCPCG RFVPGANPKCPSCKTEYVHQWDAVKRLNVPFMPIL
Sbjct  61   YENEINQELLDRIAATLPDCPCGGRFVPGANPKCPSCKTEYVHQWDAVKRLNVPFMPILD  120

Query  121  GSCLIRDRLYSYEVCIGSKPKYWWRLFTNALTSLGKGRS  159
            GSCLIRDRLYSYEVCIGSKPKYWWRLFTNALTSLGKGRS
Sbjct  121  GSCLIRDRLYSYEVCIGSKPKYWWRLFTNALTSLGKGRS  159
```

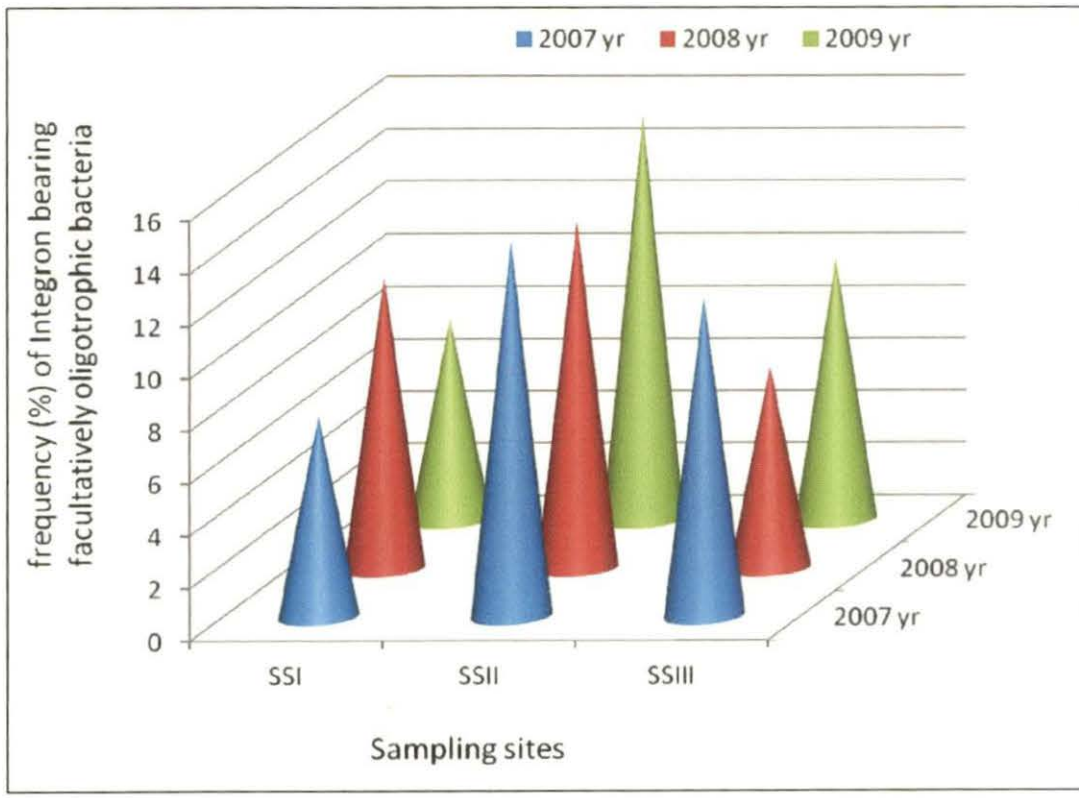


Fig. 2.14: Integron carriage in facultative oligotrophic bacteria at three different sampling sites in three consecutive years

Table 2.1: Susceptible/resistance profile of ninety class-1 integron positive oligotrophic bacterial isolates (symbols: please see table 2.2 footnote)

Group	RI	No. of strains[a]	Phenotypes[b]											
			Amp	Azi	Cef	Cft	Chl	Cip	Cot	Kan	Lev	Net	Str	Tet
0	0.00	18	-	-	-	-	-	-	-	-	-	-	-	-
		1	+	-	-	-	-	-	-	-	-	-	-	-
		1	-	+	-	-	-	-	-	-	-	-	-	-
1	0.08	1	-	-	-	-	-	-	+	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	+	-	-
		1	-	-	-	-	-	-	-	-	-	-	-	+
		2	+	-	-	-	-	-	-	-	-	+	-	-
		1	+	-	-	-	-	-	-	-	-	-	+	-
2	0.16	1	-	-	+	+	-	-	-	-	-	-	-	-
		1	-	-	-	-	-	-	+	-	-	-	+	-
		1	-	-	-	-	-	-	+	-	-	-	-	+
		1	-	-	-	-	-	-	-	-	-	-	+	+
		1	+	-	-	-	-	-	+	-	-	-	+	-
		1	+	-	-	-	+	-	+	-	-	-	-	-
3	0.25	1	+	-	-	-	-	-	+	-	-	-	-	+
		2	-	-	-	-	-	-	-	-	-	+	+	+
		1	+	-	-	-	-	-	-	+	-	-	-	+
		2	+	-	-	-	-	-	+	-	-	-	-	+
		1	-	-	-	-	-	-	+	-	+	+	-	+
		1	+	-	-	-	-	-	+	-	-	+	-	+
4	0.33	1	+	-	-	+	+	-	+	-	-	-	-	-
		1	+	-	-	+	+	-	-	-	-	-	+	-
		1	-	+	-	-	-	-	-	-	+	+	-	+
		2	+	-	+	-	+	-	+	-	-	+	-	-
		2	+	-	-	-	+	-	+	-	-	-	+	+
5	0.41	1	+	-	-	+	-	-	+	+	-	+	-	-
		1	+	-	+	-	-	+		+	-	+	-	-
		2	+	-	-	-	-	-	+	+	+	-	-	+
		1	-	-	-	+	+	-	+	-	-	-	+	+
		1	+	-	+	+	-	+	+	-	-	-	-	+
		1	+	+	-		-	+	+	-	+	-	-	+
		1	+	-	-	+	+	-	-	-	-	+	+	+
		1	+	-	-	-	-	+	+	+	-	+	-	+
6	0.50	1	+	+	-	+		+	+	-	-	+	-	-
		1	+	-	-	+	+	-	+	-	-	-	+	+
		1	+	-	+	+	+	-	+	-	+	-	-	-
		2	+	-	+	+	-	-	+	-	-	-	+	+
		1	+	-	-	+	-	-	+	-	-	+	+	+
		1	-	-	-	-	+	-	+	-	+	+	+	+
		1	-	+	+	-	+	+	-	+	+	-	-	-
		1	+	-	+	+	-	+	+	-	+	-	-	+
		1	+	-	+	+	-	+	+	+	-	+	-	+
		1	+	-	+	-	-	+	+	+	-	+	-	+
7	0.58	1	+	+	-	-	-	+	+	-	+	+	-	+
		1	+	-	+	+	-	+	+	-	-	+	-	+
		1	+	-	+	+	+	+	+	-	-	+	-	-
		1	+	-	+	+	+	+	+	+	-	-	+	-
		1	+	-	+	+	+	+	-	-	-	+	-	+
		1	+	-	+	+	-	-	+	-	+	+	-	+
		2	+	-	+	+	-	+	+	-	+	+	-	+
		1	+	-	+	+	-	+	+	+	-	+	-	+
		1	+	-	+	+	-	+	+	-	-	+	+	+
8	0.66	1	+	-	-	+	+	+	+	-	+	-	+	+
		1	+	+	+	+	-	-	+	-	-	+	+	+
		1	+	+	+	+	+	+	+	-	+	-	-	-
		1	+	+	+	+	-	+	+	-	+	-	-	+
		1	+	+	+	+	-	+	+	+	-	+	-	-
		1	+	+	+	-	-	+	+	-	+	-	+	+
9	0.75	1	+	-	+	+	+	+	+	-	+	+	+	-
10	0.83	1	+	-	+	+	+	+	+	+	+	+	-	+
		2	+	+	+	+	+	+	+	-	+	-	+	+
11	0.91	1	+	+	+	+	+	+	+	-	+	+	+	+

Table 2.2: Resistance phenotype of class 1integron positive oligotrophic bacterial isolates

Group	Resistance index (RI)	Isolate	Phenotype[a]
		OB 05	Amp
		MB 09	Tet
1		MR 01	Azi
	0.08	MB 20	Net
		MB 24	Net
		MB 50	Net
		MB 57	Cot
		MR 02	Cef, Cft
2	0.16	MB 23	Amp, Str
		MB 36	Str, Tet
		MB 49	Amp, Net
		MB 52	Cot, Str
		MB 51	Amp, Net
		MB 64	Cot, Tet
		MB 03	Amp, Cot, Str
3	0.25	MB 21	Amp. Chl, Cot
		MB 26	Amp, Cot, Tet
		MB 32	Net, Str, Tet
		MB 33	Net, Str, Tet
		MB 42	Amp, Kan, Tet
		MB 67	Amp, Cot, Tet
		MB 77	Amp, Cot, Tet
		MR 03	Cot, Liv, Net, Tet
4	0.33	MB 47	Amp, Cot, Net, Tet
		MB 62	Amp, Cft, Chl, Cot
		MB 81	Amp, Cft, Chl, Str
		MR 04	Azi, Lev, Net, Tet
		MB 18	Amp, Cef, Chl, Cot, Net
		MB 31	Amp, Chl, Cot, Str, Tet
5		MB 40	Amp, Cft, Cot, Kan, Net
	0.41	MB 59	Amp, Cef, Chl, Cot, Net,
		MB 66	Amp. Chl, Cot, Str, Tet
		MB 72	Amp, Cef, Cip, Kan, Net
		MB 74	Amp, Cot, Kan, Liv, Tet
		MB 75	Amp, Cot, Kan, Liv, Tet
		MB 80	Cft, Chl, Cot, Str, Tet
		MB 27	Amp, Cef, Cft, Cip, Cot, Tet
		MB 30	Amp, Azi, Cip, Cot, Liv, Tet
6		MB 41	Amp, Cft, Chl, Net, Str, Tet
	0.50	MB 60	Amp, Cip, Cot, Kan, Net, Tet
		MB 61	Amp, Azi, Cft, Cip, Cot, Net
		MB 69	Amp, Cft, Chl, Cot, Str, Tet
		MB 78	Amp, Cef, Cft, Chl, Cot, Liv
		SR 19	Amp, Cef, Cft, Cot, Str, Tet
		OD 05	Amp, Cft, Cot, Net, Str, Tet
		OD 08	Amp, Cef, Cft, Cot, Str, Tet
		OC 16	Chl, Cot, Liv, Net, Str, Tet
		MB 82	Azi, Cef, Chl, Cip, Kan, Liv
		MB 19	Amp, Cef, Cft, Cip, Cot, Liv, Tet
		MB 28	Amp, Cef, Cft, Cip, Cot, Kan, Net,
7	0.58	MB 29	Amp, Cef, Cip, Cot, Kan,Net, Tet
		MB34B	Amp, Azi, Cip, Cot, Liv, Net, Tet
		MB37A	Amp, Cef, Cft, Cip, Cot, Net, Tet
		MB 53	Amp, Cef, Cft, Chl, Cip, Cot, Net
		MB 70	Amp, Cef, Cft, Chl, Cot, Kan, Str
		MB 73	Amp, Cef, Cft, Chl, Cip, Net, Tet
		OC 78	Amp, Cef, Cft, Cot, Liv, Net, Tet
		MB 25	Amp, Cef, Cft, Cip, Cot, Liv, Net, Tet
		OC 24	Amp, Cef, Cft, Cip, Cot, Liv, Net, Tet
8	0.66	MB 35	Amp, Cef, Cft, Cip, Cot, Kan, Net, Tet,
		MB 45	Amp, Cef, Cft, Cip, Cot, Net, Str, Tet
		MB 79	Amp, Cft, Chl, Cip, Cot, Liv, Str, Tet
		OD 10	Amp, Azi, Cef, Cft, Cot, Net, Str, Tet
		OD 21	Amp, Azi, Cef, Cft, Chl, Cip, Cot, Lev
		OD 24	Amp, Azi, Cef, Cft, Cip, Cot, Lev, Tet
		MB 65	Amp, Azi, Cef, Cft, Cip Cot, Kan, Net
		MB 76	Amp, Azi, Cef, Cip, Cot, Liv, Str, Tet

Table 2.2: continue.....

Group	Resistance index (RI)	Isolate	Phenotype[a]
9	0.75	OC 74	Amp, Cef, Cft, Chl, Cip, Cot, Liv, Net, Str
		MB 38	Amp, Cef, Cft, Chl, Cip, Cot, Kan, Liv, Net, Tet
10	0.83	MB 68	Amp, Azi, Cef, Cft, Chl, Cip, Cot, Liv, Str, Tet
		OC 75	Amp, Azi, Cef, Cft, Chl, Cip, Cot, Liv, Str, Tet
11	0.91	NV 66	Amp, Azi, Cef, Cft, Chl, Cip, Cot, Lev, Net, Str, Tet

[a] number of strains sensitive/or resistant to a particular antibiotics or set of antibiotics.
[b]Amp: ampicillin; Azi: azithromycin; Cef: cefipime; Cft: cefotaxime; Chl: chloramphenicol; Cip: ciprofloxacin; Cot: co-trimoxazole; Kan: kanamycin; Lev: levofloxacin; Net: netilmicin; Str: streptomycin; Tet: oxytetracycline

Table 2.3A: Isolates bearing gene cassettes unrelated to antibiotic resistance.

RI[a]	Isolate	Approx length (bp)	Nature of the gene cassette	Cassette encoded Function	Accession Number
0.0	OB 12	1000	*orf1*	Hypothetical protein	AM997272
	MB 05	223	*mts*	Ribosomal methyl transferase	AM991331
	MB 08	608	*orf1*	Hypothetical protein	FN178516
	MB 12	1000	*livM1*	Branched chain ABC transporter	AM997273
	MB 16	1000	*appA*	Oligopeptide ABC transporter	AM991327
	MB 22	1010	*fgam*	Phosphoribosyl formyl glycinamide synthase	AM991334
	MB 44	522	*orf1*	Hypothetical protein	MB41[b]
	MB 48	1095	*orf1*	Hypothetical protein	FN178520
	MB 54	527	*orf1*	Hypothetical protein	FM955254
	MB 55	959	*orf55, fgam*	Hypothetical protein, Phosphoribosyl formyl glycinamide synthase	HE653232
	MB 56	900	*fhaC*	Putative hemolysin activator protein	FM955483
	MB 58	624	*tnp*	Transposase, IS4 family protein	FM955255
	MB 71	627	*tnp*	Transposase, IS4 family protein	MB58[b]
	MB 83	435	*rsu*	Ribosomal large subunit pseudouridine synthase B	OB05[b]
0.08	OB 05	489	*rsu*	Ribosomal large subunit pseudouridine synthase B	AM997271
	MB 09	682	*hsdR*	DNA degradation	AM991332
	MR 01	951	*fgam*	Phosphoribosyl formyl glycinamide synthase	FN561626
0.16	MB 51	794	*orf1*	Hypothetical protein	AM997281
	MR 02	513	GGDEF	Signal transduction	FN561627
0.33	MB 81	522	*orf1*	Hypothetical protein	MB 41[b]
	MR 04	1300	SNF2 family	ATP-dependent helicase	FN561629
0.41	MB 80	522	*orf1*	Hypothetical protein	MB41[b]
0.50	MB 41	522	*orf1*	Hypothetical protein	HE653229
0.58	MB 28	1620	*ydcR*	Bifunctional putative Transcriptional regulator	FM179326
	MB 19	1500	*yfkc*	Putative reverse transcriptase maturase	AM997282
	MB 70	223	*mts*	Ribosomal methyl transferase	MB05[b]

[a]Resistance index; [b] gene sequence identical to that isolate

Table 2.3B: Isolates bearing gene cassettes related to antibiotic resistance, and bearing no cassettes (empty class 1 integron).

RI[a]	Isolate	Approx length (bp)	Nature of the gene cassette	Cassette encoded Function	Accession Number
0.0	MB 39	1009	*aadA2*	STR	AM997277
	MB 43	792	*aacA4*	KAN	HE653231
	MB 46	1009	*aadA2*	STR	AM997280
	MB 63	153	*In*0	Empty class 1 integron	FM958478
0.08	MB 20	1009	*aadA1*	STR	MB 32[b]
	MB 24	1003	*aadA1*	STR	AM991326
	MB 50	1009	aadA1	STR	MB 32[b]
	MB 57A MB 57B	1242 (i) 973 (ii)	*dfrA1-orfB* (i) *aadA1* (ii)	TMP-Hypothetical (i) STR (ii)	HE653233 (i) HE653234 (ii)
0.16	MB 23	1009	*aadA1*	STR/SPEC	MB 32[b]
	MB 36	1009	*aadA1*	STR/SPEC	AM937245
	MB 49	792	*aacA4*	KAN	MB43[b]
	MB 52	1694	*dfrA28- aadA1*	TMP-STR	FN263373
	MB 64	769	*dfrA7*	TMP	MB31[b]
0.25	MB 03	1647	*dfrA28-aadA1*	TMP-STR	AM937241
	MB 21	1569	*dfrA1- aadA*	TMP-STR	AM937243
	MB 26	1571	*dfrA1-aadA1*	TMP-STR	AM991328
	MB 32	1009	*aadA1*	STR/SPEC	AM991330
	MB 33	1009	*aadA1*	STR/SPEC	AM991333
	MB 42	862	*aacA4*	KAN	HE653230
	MB 67	1913	*dfrA12-orf40A- aadA2*	TMP-Hypothetical-STR	MB40A[b]
	MB 77	730	*dfrA30*	TMP	MB72[b]
0.33	MR 03	737	*dfrA16*	TMP	FN561628
	MB 47	1569	*dfrA1- aadA1*	TMP-STR	FM179327
	MB 62	153	*In*0	Empty class 1 integron	FM998811
0.41	MB 18	1048	*aadA5*	STR/SPE	AM937242
	MB 31	769	*dfrA7*	TMP	HE650981
	MB 40A MB 40B	1913 (i) 704 (ii)	*dfrA12- orf40A-aadA2* (i) *yrf1* (ii)	TMP-Hypothetical-STR(i) Helicase (ii)	FM179328 (i) AM997278 (ii)
	MB 59	1543	*dfrA1-aadA1*	TMP-STR	HE653235
	MB 66	1009	*aadA1*	STR/SPE	HE650982
	MB 72	730	*dfrA30*	TMP	HE650983
	MB 74	1242	*dfrA1-orf38*	TMP-Hypothetical	MB38[b]
	MB 75	1242	*dfrA1-orf38*	TMP-Hypothetical	MB38[b]
0.5	MB 27	1614	*dfrA17-aadA5*	TMP-STR	AM937244
	MB 30	1664	*dfrA7-aadA5*	TMP-STR	HE650980
	MB 60	1556	*dfrA1-aadA1*	TMP-STR	HE653236
	MB 61	1913	*dfrA12-orf40A-aadA2*	TMP-Hypothetical-STR	MB40A[b]
	MB 69	1543	*dfrA1-aadA1*	TMP-STR	MB59[b]
	MB 78	1694	*dfrA28-aadA1*	TMP-STR	MB52[b]
	MB 82	2013	*Oxa1-aadA1*	Bla-STR	HE650986
	SR 19	729	*dfrA5*	TMP	FN396373
	OD 05	1009	*aadA*	STR	FN396375
	OD 08	1009	*aadA*	STR	FN396376
	OC 16	1664	*dfrA17-aadA5*	TMP-STR	FN396368
0.58	MB 29	1657	*dfrA7-aadA5*	TMP-STR	HE650979
	MB 34B	1663	*dfrA17-aadA4*	TMP-STR	AM997275
	MB 37A	1661	*dfrA17-aadA5*	TMP-STR	AM991329
	MB 53	1664	*dfrA17-aadA5*	TMP-STR	FM179325
	MB 73	1664	*dfrA7-aadA5*	TMP-STR	[b]MB30
	OC 78	1170	*aac-6'-Ib*	STR	FN396372

Table 2.3B: continue.....

RI[a]	Isolate	Approx length (bp)	Nature of the gene cassette	Cassette encoded Function	Accession Number
0.66	MB 25	1606	*dfrA17-aadA4*	TMP-STR	AM997274
	MB 35	3454	*blaIMP-9-aacA4-oxa-10-aadA2*	Kan-Bla-STR	FN178517
	MB 45	667	*dfrA30*	TMP	AM997279
	MB 65	1556	*dfrA1-aadA1*	TMP-STR	MB60[b]
	MB 76	769	*dfrA7*	TMP	MB31[b]
	MB 79A MB 79B	1167 (i) 1009 (ii)	*dfrIIe-arr2* (i) *aadA1* (ii)	TMP-RMP (i) STR (ii)	HE650984 (i) HE650985 (ii)
	OD 10	1350	*aadA*	STR	FN396374
	OC 24	1521	*dfrA17-aadA5*	TMP-STR	FN396369
	OD 21	1009	*aadA2*	STR	FN396378
	OD 24	1000	*dfrA12*	TMP	FN396377
0.75	OC 74	1000	*aac-6'-Ib*	STR	FN396370
0.83	MB 38	1241	*dfrA1-orf38*	TMP-Hypothetical	AM997276
	MB 68	1543	*dfrA1-aadA1*	TMP-STR	MB59[b]
	OC 75	1400	*dfrA17*	TMP	FN396371
0.91	NV 66	1009	*aadA2*	STR	FN396367

[a]Resistance index (RI)
[b] gene sequence identical to that isolate
TMP, trimethoprim; STR, streptomycin; KAN, kanamycin; SPEC, spectinomycin
Gene name, described in main text of the manuscript

Table 2.4: The nearest protein sequence to the ABC transporter protein derived from MB12 obtained through protein-protein homology searching (BlastP).

Protein id	Description and source of origin
CAQ53856	ABC type branched chain amino acid transport system [bacterium AK-**MB12**]
ZP_10155718	branched-chain amino acid ABC transporter, permease protein [*Hydrogenophaga* sp. PBC
	ABC transporter permease [*Aromatoleum aromaticum* EbN1]
YP_158959	branched-chain amino acid ABC transporter system, permease protein [*Azoarcus* sp. KH32C]
BAL26594	

Table 2.5: Predicted trans-membrane regions of LivM1 protein (154AA) derived from bacterium, MB12.

Annotation	TM PROTEIN	
Number of predicted TMHs	4	
inside	1	1
TMhelix	2	20
outside	21	49
TMhelix	50	74
inside	75	86
TMhelix	87	110
outside	111	118
TMhelix	119	138
inside	139	154

TMH, trans-membrane helices;
AA, amino acid

Table 2.6: *Protein sequences with protein IDs that produces significant alignment score with the putative protein sequence derived from first orf1 (dfrA1) of class 1integron sequence of facultative oligotrophic bacterium, MB47.*

Protein id	Description and source of origin
CAQ76923	dihydrofolate reductase [bacterium AK-**MB47**]
YP_002527542	dihydrofolate reductase type I DhfrA1 [*Escherichia coli*]
YP_133853	dihydrofolate reductase type 1 [uncultured bacterium]
BAD99259	dihydrofolate reductase [*Escherichia coli*]
ACJ22953	dihydrofolate reductase type 1 [*Salmonella* sp. D76]

Table 2.7: Data summary of DfrA1_MB47 protein analyses [predicted secondary structure composition and solvent accessibility composition (core/surface ratio)] obtained through Protparam computing.

Sec. structure type	H	E	L	Accessibility type	b	e
% in protein	19.74	32.89	47.37	% in protein	35.53	64.47

H: helix, E=extended, L=loop

Classes used in Predicted solvent accessibility composition (core/surface ratio)

- e: residues exposed with more than 16% of their surface
- b: all other residues

Table 2.8: Protein sequences with protein IDs that produces significant alignment score with the putative protein sequences derived from *orf2* (*aadA1*) of class 1integron sequence of facultative oligotrophic bacterium, MB47.

Protein id	Description and source of origin
CAQ76924	Aminoglycoside adenyl transferase [bacterium AK-**MB47**]
AFK93816	aac(3'')-Ia [Proteus mirabilis]
YP_005980005	Streptomycin adenyltransferase [*Pseudomonas aeruginosa* NCGM2.S1]
YP_001144148	Aminoglycoside resistance protein [*Aeromonas salmonicida* subsp. *salmonicida* A449]
ACI43574.1	AadA1 [*Escherichia coli*]

Table 2.9: Data summary of AadA1_MB47 protein analyses [predicted secondary structure composition and solvent accessibility composition (core/surface ratio)] obtained through Protparam computing.

Sec. structure type	H	E	L	accessibility type	b	e
% in protein	49.43	10.65	39.92	% in protein	42.97	57.03

H: helix, E=extended, L=loop

Classes used in Predicted solvent accessibility composition (core/surface ratio)

- e: residues exposed with more than 16% of their surface
- b: all other residues

Table 2.10: Fraction (in %) of facultative oligotrophic bacteria of river Mahananda carrying integron [summarized data of 90 samples].

Year	Integron frequencies at three sampling sites (%)		
	SSI	**SSII**	**SSIII**
2007 yr	7.7	14.4	12.2
2008 yr	11.2	13.3	7.7
2009 yr	7.7	15.5	10

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Identification of Oligotrophic Bacteria bearing Class 1 integron

[A]: Identification of isolates using basic approaches

3A.1. Background

Scientific naming of organisms dates back to 18th century. A famous Swedish botanist, Carolus Linnaeus, also called as "Father of taxonomy", proposed binomial nomenclature, the system that is still used today to name all living things. In addition to binomial system of nomenclature, Linnaeus also propounded a hierarchy of taxonomic ranks: species, genus, family, order, class, phylum or division, and kingdom. An order of taxonomic hierarchy from larger to smaller taxon is shown in Fig. 3.1. The overall philosophy of Linnaeus is also adapted in classifying bacteria as well. The Bacterial taxonomy or systematics is generally defined as "the branch of bacteriology that deals characterization and naming of organism and organizing them into groups". It can be separated into three branches-

(a) nomenclature: naming of bacteria
(b) classification: grouping of bacteria sharing common properties
(c) identification: to determine bacterial genus followed by species

"Bergey's Manual of Determinative Bacteriology", coming to existence in late 1957, published reports relating to the identification of bacteria largely based on Artificial classification and Numerical taxonomy: Artificial classification is based on the characteristics expressed by bacteria that we observe, and results generated increases the accuracy with which we can detect similarities among them and the organizing of these data in a special manner to conclude how that species are closer to each other in terms of characteristics leads to numerical taxonomy. In numerical taxonomy, each characteristic is allocated a value of '1' if present and '0' if not present, means all characteristics are given equal weight. Characteristics may be genetical, morphological, physiological or biochemical response of bacteria like cell shape, cell margin, colour, reaction to Gram staining, aerobic, anaerobic, capsule present or absent, catalyse production, citrate utilization, properties of nucleic acids and proteins, and the presence or absence of particular enzymes and chemical reactions can be evaluated. Bacteria are then compared, and patterns of similarities and differences are computed and analysed. In this system, each strain is compared with everyone and if two organisms shared a 90% or more of the characteristics studied, they are supposed to be the same species. Though the idea of numerical taxonomy was developed much before the introduction of computers applications in biology, but after the development of user-friendly computer algorithms, a large number of data now can be compared in a fraction of second. Hence computerized numerical taxonomy offers great promise for improving our understanding of relationships among all organisms. A dendorgram can be generated from computed similarity values. Separating isolates according to colour, cell shape, size, flagella,

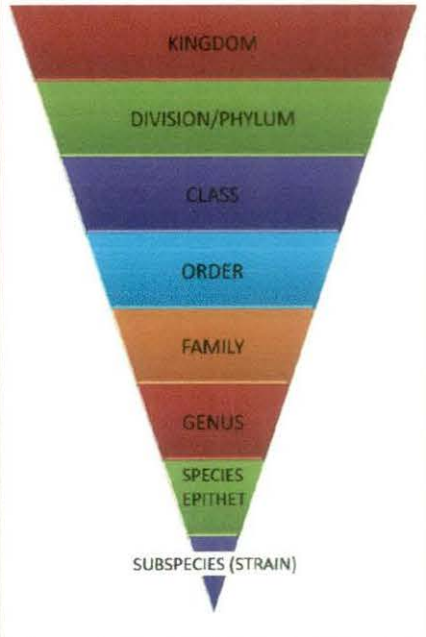


Fig. 3.1: inverted pyramid showing hierarchical relation of different taxa.

endospores, capsules and other physiological characteristics neither produce a very significant data to classify bacteria nor allow identification of particular species. For that reason, other criteria must be considered. However, Gram test is still the first requirement of all bacteriologists among all the known characteristics used to classify bacteria. However, with time and advancement of modern biology, some molecular techniques were perceived by some as useful in standard bacterial classification. Several efforts, though in lesser scale were mounted to improve classification by various molecular approaches. Genetic characterization, deoxyribonucleic acid (DNA) base ratios, nucleic acid hybridization studies, cell wall analyses, and protein sequencing began to reveal phylogenetically valid groupings. "These early molecular approaches, though useful, were not powerful enough to reveal the higher bacterial taxa, and in any case conventional wisdom did not perceive doing so as important" (Woese, 1987, Woese *et al.*, 1990). The sequencing of proteins and nucleic acids further strengthened the methodology to measure evolutionary relationships and a novel way of looking at them, in terms of the "evolutionary clock" (Wilson *et al.*, 1977).

In 1987, Carl Woese's seminal work has added a new dimension to this field. An important observation was that by examining structural feature in the small subunit rRNA, the hairpin loop (a side lump made-up of six nucleotide in all eubacteria protruding from stalk of the structure) found between positions 500 and 545 (*E. coli* numbering), could distinguish eubacteria from archaebacteria and eukaryotes (Woese *et al.*, 1990). In subsequent years, ease of sequencing of rRNA gene sequences revolutionized the molecular phylogeny. Genetic information, i.e., sequence information, was found as superior tool over phenotypic data in two main ways: (a) more readily, reliably, and precisely interpreted and (b) inherently more informative of evolutionary relationships than phenotypic information is. "Unlike three-dimensional phenotypic patterns, a sequence pattern is one dimensional and One-dimensional pattern can be measured in simple ways, in terms of simple relationships" (Woese, 1987). Five important points summarized by Woese *et al.* (1990), as stated below have provided the strongest logic in support of using ribosomal RNA for evolutionary phylogeny.

"

I. They possess a high degree of functionally fidelity, which assures relatively good clock like behaviour over other.
II. They found in all organisms, and different positions in their sequences change at very different rates, allowing most phylogenetic relationships (including the most distant) to be measured, which makes their range all-encompassing.
III. Their sizes are large and they consist of many domains.
IV. There are about 50 helical stalks in the 16S rRNA secondary structure and roughly twice that number in the 23S rRNA which makes them accurate chronometers on two counts.
V. The most compelling reason for using rRNAs as chronometers is that they can be sequenced directly."

As multiple copies of 16S rRNA gene(s) possessing highly conserved regions at both upstream and downstream and hypervariable regions providing conserved species-specific signature sequences has advantage of easy PCR based amplification, it has become more relevant for phylogenetic studies.

Later on, bacteriologist realized that there should be a uniform rule for naming. In 1992, bacteriologists at international level agreed to set a rule for naming Bacteria and Archea. These rules are termed as "International Code for the Nomenclature of Bacteria" (ICNB). ICNB stated that each bacterium will be named in the same manner as of plant and animals i.e Linnaeus's binomial system. *International Journal of Systematic and Evolutionary Microbiology* (IJSEM) is the journal devoted to the taxonomy for the publication of newly discovered bacterial species. Hence it has been accepted universally that any claim for a new species requires validation by the authorities of IJSEM. The

recent edition of *"Bergey's Manual of Systematic Bacteriology (2001-2008)"*, published by Springer contains a complete list of prokaryotic species and their classification.

There were many rules formulated for bacterial classification. For example, rule 9 (Lapage *et al.*, 1992) of the *Bacteriological code* (1990 Revision), the name of taxon between genus and class will be figured by the addition of a suitable suffix to the stem of the name of the type genus. Stackebrandt *et al.* (1997) proposed a list of suffixes to denote class and subclass. However, there is no such hard-and-fast official document in prokaryotic taxonomy (Sneath and Brenner, 1992) and still it is a matter of debate. The latest "Taxonomic Outline", now known as "Taxonomic Outline of the Bacteria and Archaea" (TOBA) release 7.7 (Garrity *et al.*, 2007). At present, to define a bacterial species accurately, a set of tests like phenotypic features, 16S rRNA gene based phylogenetic data; results of DNA-DNA hybridization assay, typing of housekeeping genes, DNA base composition, chemical composition of cell, fatty acid methyl ester analysis and some genus or species specific characteristics are required. This collective species identification approach called as polyphasic characterization.

In the first phase, phenotypic characterization along with total cellular protein profiling of the isolates were performed. The results of the first phase enabled to create the dendrogram based on similarity coefficient. In the second phase, representative isolate(s) from each cluster were picked up for determining phylogeny using 16S rRNA gene sequences for identification at least up to the genus level. A polyphasic approach was also utilized to characterize and classify one novel Gram-positive bacterium, *Brevibacterium siliguriense* discovered during the study. This novel species have been validated and published in IJSEM (Kumar *et al.*, 2012).

3A.2. Materials and methods

3A.2.1. Bacterial isolates

All ninety facultatively oligotrophic bacteria which yielded amplicon(s) of the variable region of class 1 integron were characterized up to genus level.

3A.2.2. Morphological and physiological characterization of isolates

Conventional tests for catalase, oxidase, IMViC, casein hydrolysis, citrate utilization, gelatin hydrolysis, amylase production, H2S production, acid production from carbohydrate were performed following methodology described earlier (Cappuccino and Sherman, 1996; Aneja, 2001). Ready to use test kits for biochemical characterization were also used as per manufacturer's instructions (HiMedia, India Ltd). The data generated from these tests were converted into binary numbers and used for numerical taxonomy for categorizing the bacteria.

3A.2.3. Numerical taxonomy followed by total protein profiling

The tentative genus of class 1 integron positive oligotrophic bacteria was ascertained by the application of numerical taxonomy. The phenotypic data generated from physiological and biochemical tests were converted into binary characters (1, for positive character and 0, for negative character) and similarity matrix was generated by using the Sneath and Sokal methodology (Sneath and Sokal, 1973). The Jaccard (Tanimoto) coefficient was computed from the set of variable (similarity: a /a+b, where a, is the homolog character present in two bacterial isolate and b is the number of non-homolog character present in two bacterial isolate). The similarity dendogram was generated using unweighted pair group method with arithmetic means (UPGMA) tree building method with the help of DendroUPGMA tool available at http://genomes.urv.cat/UPGMA/. The obtained output also can be visualized in tree view tool. The isolates clustered together in groups were subjected for total protein profiling. The total protein of individual isolates of each cluster was extracted followed by one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE) in running buffer of pH 8.3 [composition (g/L): Glycine, 14.4; Tris-buffer, 3; and SDS, 1) at 100v 30 amps for 3 h

(Sambrook and Russell, 2001). For extracting total proteins, 3-4 colonies were suspended in 200 µL 2X SDS-gel loading buffer [composition: 100mM Tris-cl, pH 6.8; 4% (w/v) SDS; 0.2% (w/v) bromophenol blue; 20% (v/v) glycerol and 200mM DTT or β-mercaptoethanol (β-ME) (should be added at time of experiment)]. Total proteins separated on 12% polyacrylamide gel, according to their molecular size (molecular weight), was stained in 0.25% (w/v) coomassie-blue [staining solution: coomassie blue 0.25% (w/v); 90 mL 50% methanol+ 10 mL absolute glacial acetic acid] for 3h at gel dancing apparatus. Gel was visualized under white light. Pattern were compared and considered identical when all the protein bands (migrated bands) were at same distances.

3A.2.4. Amplification, cloning and sequencing of 16S rRNA gene sequence

One representative culture from each group of bacteria, exhibiting similar phenotype and nearly identical proteins band pattern, was used for amplification of 16S rRNA gene sequence using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) primer pair in DNA engine (BioRAD, USA). For PCR assay total DNA was extracted by boiling lysis method as described in chapter 2. A 100 µL of supernatant from the lysed cell suspension (after centrifugation step) was aspirated and transferred to the fresh micro-centrifuge tube and stored at 4 °C until use. The supernatant was used as template in the PCR-reaction mixture for amplification of 16S rRNA gene sequence (or 16S rDNA). PCR cycling conditions used were: initial denaturation at 94 °C for 3 min; followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec and amplification at 72 °C for 1 min and final extension at 72 °C for 7 min. Amplified product was resolved on 0.7% or 1% agarose gel containing 0.3- 0.5 µg/mL ethidium bromide. Amplified product was purified using commercial PCR purification kit [Elute™ PCR Clean-up kit (Sigma-Aldrich, St. Louis, MO)]. If the multiple bands of PCR product appeared on gel then the gel extraction method was followed to get the desire amplicon for cloning and sequencing purpose. Desired band of ~1.5kb was visually identified by comparing with the marker lane. The DNA band was excised with the help of sterile surgical blade and DNA was extracted using gel extraction kit (Genie, India) following manufacturer's instruction. The purified DNA was cloned in to pGEMT easy vector-II (Fig. 3.2) and transformed in to *E. coli* JM109 host cell. The cells bearing recombinant plasmid, were screened by blue-white screening on ampicillin (100 µg/mL; ampicillin is the selective marker of pGEMT easy vector), X-gal (5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside, concentration used: 0.5mM) and IPTG (isopropylthio-β-galactoside) containing Luria agar plate. The white colonies (colony containing recombinant plasmid) along with one blue (for negative control) were picked and purified on ampicllin containing Luria agar plate. The pure white and blue colonies were transferred to the fresh Luria broth containing ampicllin [concentration, 100µg/mL] and incubated at 37°C for overnight. The recombinant and non-recombinant plasmids were isolated from overnight grown culture following alkaline lysis method (Sambrook and Russell, 2001). The extracted plasmids were visualized over UV trans-illuminator (Genie, India) after run on 0.7% agarose gel containing ethidium bromide and compared with control (non-recombinant). An upward shifting in test plasmid in compare to control is the indication of chimeric DNA (clone containing desired insert). Further the cloning was confirmed by restriction digestion of recombinant plasmid DNA using *Eco*RI endonuclease (Genie, India). The clone was sequenced as described in chapter 2.

3A.2.5. Analyses of 16S rRNA gene sequences (derived from the isolates) using tools of Bioinformatics

DNA sequences of cloned 16S rRNA genes were edited and vector sequences were removed from both the ends after examining via vec-Screen tool (www.ncbi.nlm.nih.gov) or manually. Orientation of the gene sequence was examined manually by searching forward primer location on sequenced 16S rRNA gene sequence. Nearest matches were determined in the GenBank database using BlastN (www.ncbi.nlm.nih.gov) program. Best five or more similar 16S rRNA gene sequences were retrieved from databank and compared using Clustal W/or X. A pairwise sequence alignment

(http://www.ebi.ac.uk/Tools/psa/) was conducted to determine the homology percent. The closest known genera with maximum score and percentage homology were considered as nearest probable genus.

3A.2.6. 16S rRNA gene sequence based phylogeny

The 16S rRNA gene sequence (16S rDNA sequence) of representative isolate was used as a query for search of homologous sequence in the nucleotide sequence databases by using BlastN program (Altschul *et al.*, 1997). Sequences showing high similarities with maximum scores were retrieved from the GenBank database and were aligned with 16S rRNA gene sequences of the isolate by using CLUSTAL W software (Thompson, 1994). Distances were calculated according to Jukes & Cantor (1969) one-parameter/ or Kimura two-parameter (1980)/ and or Tamura and Nei (1993) four-parameter methods. Phylogenetic trees were inferred by using the neighbor-joining (Saitou and Nei, 1987)/ or maximum-likelihood (Yang, 1999)/ and or by maximum-parsimony (Eck and Dayhoff, 1966) algorithms. The tree topology was evaluated by the bootstrap analysis based on 1000 re-samplings (Felsenstein, 1985). For tree building, a MEGA 4.0 software package was used (Tamura *et al.*, 2007).

Accession numbers: The 16S rRNA gene sequences of the isolates were deposited in GenBank/EMBL nucleotide database (Table 3.2).

3A.3. Results and Discussions

The biochemical characteristics exhibited by all class-1 integron-positive isolates are shown in Table 3.1. Out of ninety, eighty nine integron positive oligotrophic isolates were Gram-negative and a single isolate was determined to be Gram-positive. Two isolates, one Gram positive, MB18, and one gram negative, MB12, were excluded from the numerical taxonomy analyses because they responded differently and were not amenable for comparison [Isolate, MB18 was excluded because it was the only isolate which was Gram-positive, while MB12 did not respond conclusively to any of the biochemical test performed]. **Extensive characterization of the strain MB18 has been presented separately** (under the subtitle: *Polyphasic taxonomy of Brevibacterium siliguriense, strain MB18^T, a novel Gram positive facultative oligotrophic bacterium isolated from River Mahananda, Silguri, India*). All gram negative facultatively oligotrophic isolates were grouped in two major categories (i) isolate showing positive reaction to oxidase test and (ii) isolates showing negative reaction to oxidase test. All phenotypic characters were converted in to binary numbers and similarity matrix was calculated using DendroUPGMA tool. Similarity matrix of phenotypic data computed with Jaccard (Tanimoto) coefficient is shown in supplementary table (Annexure-I, available in the soft copy of the thesis). An UPGMA dendogram of all the isolates were constructed from binary data generated from phenotypic characters (Fig. 3.3). It was observed that bacteria possessing same phenotype were found to exhibit nearly same protein pattern under the same cultural condition (Fig. 3.3).

The 16S rRNA gene sequencing (molecular approach) and phenotypic data (similarity matrix, a numerical taxonomic approach) of representatives of each cluster revealed that all oxidase positive and oxidase negative isolates fell under two main classes, *Betaproteobacteria* and *Gammaproteobacteria*. Results revealed that *Betaproteobacteria* comprised of only two genera, *Comamonas* and *Acidovorax* of family *Comamonadaceae* while other isolates were identified under super class *Gammaproteobacteria*. The representative genera of class *Gammaproteobacteria* were constituted by families, *Moraxellaceae, Pseudomonadaceae, Aeromonadaceae* and *Enterobacteriaceae*. Similar to previous reports published on presence of integrons in copiotrophic bacteria, majority of the identified integron positive oligotrophic bacteria of super class *Gammaproteobacteria* were detected from the family *Enterobacteriaceae*. (Mukherjee and Chakraborty, 2007; Chen *et al.*, 2011;

Xu *et al.*, 2011; Mokracka *et al.*, 2012). Eight facultative oligotrophic isolates MB25, MB28, MB41, MB44, MB48, MB54, MB81, MB83 and one oligotrophic isolate MB12 could not be assigned a specific genus from the data derived either phenotypically or by 16S rRNA gene sequence (Fig. 3.3 to 3.8). The 16S rRNA gene sequences of these unclassified isolates (the isolates which could not be assigned any genus) shared insignificant homologies with the known genera of different classes. The 16S rRNA phylogeny showed that they were branching with uncultured bacteria. However on comparing the phenotypic data, the group of unknown species was found to cluster with members of family *Enterobacteriaceae* (Fig 3.3). Due to taxonomic uncertainties, these strains were kept under category of unknown spp.

Majority of the integron positive oligotrophic bacteria were detected from the family *Enterobacteriaceae* similar to earlier reports on presence of integrons in copiotrophic bacteria (Mukherjee and Chakraborty, 2006; Chen *et al.*, 2011; Han *et al.*, 2012; Su *et al.*, 2012). The results indicate that the members of this family are more prone to acquire antibiotic-resistance genes to get selective advantages over other. In the present study fifty five integron-positive oligotrophic isolates were identified as the members of the family *Enterobacteriaceae*. Isolate MB05 and MB24 was identified as member of genus *Shigella* (Table 3.2; Fig 3.3 and 3.9). Genus *Kluyvera* was only represented by the single isolate MB66 (Fig. 3.10 and Table 3.2). Eight isolates, MB19, MB26, MB29, MB42, MB45, MB49, MB51 and MB72 were recognized as the member of genus *Klebsiella* (Table 3.2; Fig.3.3, 3.11 and 3.12). Genus *Enterobacter* were represented by six integron positive oligotrophic bacterial isolates named as OD21, SR19, MR01, MB40, MB59, and MB73 (Table 3.2; Fig. 3.13). Four isolates MB30, MB34, MB47 and MB57 were identify as member of genus *Proteus* (Table 3.2; Fig. 3.13). Phenotypic data and 16S rRNA sequence homology revealed that isolates MB20, MB38, MB43, MB64, MB67, MB74, MB75, MB76 and MB82 belongs to the genus *Salmonella* (Table 3.2; Fig. 3.13). Only two isolates MR04 and NV66 represented the genus *Providencia* (Table 3.2; Fig. 3.14). Genus *Serratia* was represented by isolates MB23 and MB53 (Table 3.2; Fig. 3.15). Phenotypic and genotypic study revealed that the isolates MR03, MB35, MB61, OC16, OC24, OD05, OD08, OD10 and OD24 represented the genus *Citrobacter* (Table 3.2; Fig. 3.13 and 3.15). Twelve isolates, OC75, OC78, MB27, MB31, MB32, MB33, MB36, MB37, MB60, MB65, MB68, MB69, and MB79 were tentatively classified under genus *Escherichia* (Table 3.2; Fig. 3.13 and 3.16).

Family *Comamonadaceae* was represented by nine integron positive isolates, MB09, OB05, MR02, MB16, MB50, MB56, MB58, MB70 and MB71 (Fig.3.3 and Table 3.2). Isolate MB09 was identified as the member of genus *Comamonas* while rest eight isolates were tentatively characterized under genus *Acidovorax* (Table 3.2; Fig. 3.17 and 3.18). All known species of *Acidovorax* are aerobic and less is known about them, few of them are found to cause diseases in vegetables. *Acinetobacter* was the only genus identified amongst the isolates that was under the family *Moraxellaceae*. Seven isolates, MB03, MB22, MB46, MB52, MB55, MB63 and MB80 were assigned to the genus *Acinetobacter* (Table 3.2; Fig 3.3 and 3.19). *Acinetobacter* strains are capable of surviving in extremely nutritionally deficient (oligotrophic) abiotic environments of hospitals for a substantial period, for example, up to ten days on dust and dry particles (Webster *et al.*, 2000) or for more than 4 months on both moist and dry surfaces such as polyvinyl chloride, rubber, ceramics, and various types of medical equipments (Wendt *et al.*, 1997) and are an important cause of infection in immunocompromised patients.

Aeromonas was the only genus that was identified for the family *Aeromonadaceae* of super class *Gammaproteobacteria*. On the basis of habitat genus *Aeromonas* divided in two groups-psychrophilic and mesophilic. Psychrophilic are omnipresent aquatic environmental species and are mostly pathogenic to the aquatic (mainly fish) and terrestrial animals while mesophilic *Aeromonas* spp. are grow in temperature ranging 10-42 °C and are important pathogens for humans (Lee *et al.*, 2008). In a study from conducted in Taiwan it was observed that 13.9% of the total were carrying class 1 integron (Lee *et al.*, 2008). A report on *Aeromonas* isolated from aquaculture showed that

~60% members were found to carry gene cassettes in variable region of class 1 integrons (Lukkana *et al.*, 2012; Ol Ndi and Barton, 2011). Four isolates MB21, MB39, MB77 and MB78 were classified under this genus (Table 3.2; Fig 3.3, 3.20, 3.21 and 3.22). Family *Pseudomonadaceae* was covered by a single genus *Pseudomonas*. Four isolates OB12, MB08, MB62, and OC74 were categorized in the genus *Pseudomonas* (Table 3.2; Fig 3.3 and 3.23). Pseudomonads are recognized as opportunistic pathogen of human and other animals (Haenen and Davidse, 2001; Fonseca *et al.*, 2005) and have been recovered from diverse sources even from distilled water (Favero *et al.*, 1971). Reports revealed that member of *Pseudomonas* are potential carrier of antibiotic resistance genes in class 1 integrons (Poirel *et al.*, 2001; Yan *et al.*, 2007).

[B]: Identification of a novel gram positive bacterium using polyphasic approach

3B: Polyphasic taxonomy of *Brevibacterium siliguriense*, strain MB18^T, a novel Gram positive facultative oligotrophic bacterium isolated from River Mahananda, Siliguri, India

The genus *Brevibacterium* was established by Breed (1953) for some non-sporulating, non-branching, Gram-positive rods, which were earlier assigned to genus *'Bacterium'*. While several gram-positive, non-spore-forming, non-branching rods were classified as members of the genus *Brevibacterium*, further chemotaxonomic studies showed that not all of these species are the members of the genus *Brevibacterium*. Eventually, the genus *Brevibacterium* has been described after the type species, *Brevibacterium linens* (Collins *et al.*, 1980). As per available reports, *Brevibacterium* spp. have been isolated from diverse sources such as milk products (Kollöffel-*et al.*, 1999), clinical specimens (Collins *et al.*, 1983; Pascual *et al.*, 1996; Wauters *et al.*, 2001; Wauters *et al.*, 2003; Wauters *et al.*, 2004; Mages *et al.*, 2008; Roux and Raoult, 2009), human body parts (McBride *et al.*, 1993), soil (Gavrish *et al.*, 2004; Tang *et al.*, 2008), sediment (Lee, 2006; Bhadra *et al.*, 2008), brown algae (Ivanova *et al.*, 2004), paintings (Heyrman *et al.*, 2004), poultry (Pascual and Collins, 1999), marine environments (Lee, 2008), fresh water (Manage *et al.*, 2009), insects (Kati *et al.*, 2010), wall colonized by moulds (Kämpfer *et al.*, 2010), salt-lake (Tong-Wei Guan *et al.*, 2010) and recently, *B. daeguense*, isolated from industrial wastewater treatment plant (Cui *et al.*, 2012). So far 46 species were classified in genus *Brevibacterium* (http://www.bacterio.cict.fr/b/brevibacterium.html).

A facultatively oligotrophic *Brevibacterium siliguriense* sp. nov. strain MB18^T, isolated from waters of river Mahananda at Siliguri (Longitude, 88°25'22.89"E; Latitude, 26°44'23.20"N) West Bengal, India, representing a novel member of the genus *Brevibacterium*, has been described in details in this chapter.

Oligotrophic growth of the strain MB18^T was demonstrated. In diluted (10^{-3}) Luria Broth (LB), an increase of approximate 12 times from the initial cell number was noted in span of 4 days incubation, at 28 °C (Fig. 3.24). Oligotrophic growths of the strain were also recorded in other diluted (10^{-3}) media like nutrient broth (NB, HiMedia, India) and tryptone soy broth (TSB, HiMedia, India). However strain MB18 could grow well in the undiluted rich media like LB, NB and TSB. Since the strain was able to grow on both nutrient-rich and nutrient-deficient media, it has been described as a facultative oligotrophic bacterium.

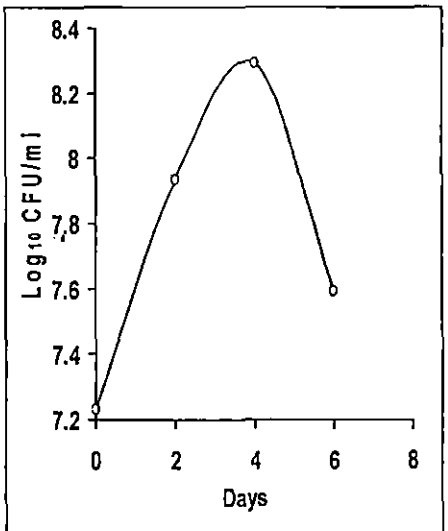


Fig. 3.24: Viability and growth of MB18^T in diluted (10^{-3}) Luria broth

Cell morphology and motility were determined under phase contrast microscope (Olympus, Japan); detail of the cell shape (Fig. 3.25) was ascertained with help of scanning electron microscope (LEO 1430 VP). Result of Gram-staining (Claus, 1992) was confirmed by the KOH lysis method (Murray *et al.*, 1999). Growth of the strain MB18 was tested at 10, 20, 28, 30, 37, 40 and 45 °C (±1). Salt tolerance was tested in peptone-yeast extract (PY) medium (composition g/L; Peptone 10, yeast extract 5) supplemented with following concentrations (%w/v): 0, 2, 4, 6, 8, 10, 15 and 20 of KCl and NaCl. To assess growth at different pH, the pH of the sterile LB medium was adjusted from pH 3.0 to 12.0 by using either 0.1 M HCl or 0.1 M NaOH. Catalase activity was examined by air bubble production after the addition of few drops of 3% (v/v) H_2O_2. Ability to hydrolyze starch was

determined by assessing the development of clear zones around the streaked culture. Lipase production was determined by standard procedure. Hemolytic activity and gelatin hydrolysis were tested according to the method described earlier (Kumar *et al.*, 2010). Pyrazinamidase activity and acid production from 2, 3-butylene glycol were detected as described by Wauters *et al.* (2001). Acid production from ethylene glycol and phenyl acetate; and Alkali production from sodium-formate was detected by methods described earlier (Wauters *et al.*, 1998, 2003). Hydrolysis of casein, tyrosine and xanthine were examined using the method described by Gordon *et al.* (1974). Oxidase, lysine utilization, ornithine utilization, urease activity, deamination of phenyl alanine, reduction of nitrate, H_2S production, citrate utilization, VP test , MR test, malonate utilization and carbon source utilization/fermentation tests were carried out using HiBio-ID/*HiCarbo* system (HiMedia, Mumbai, India) and GP card (VITEK 2 system, BioMérieux) according to manufacturer's instruction. Results were scored after 7 day at 28 °C. The biochemical characteristics of strain MB18^T were also determined using the Biolog GP2 MicroPlate system (BioMérieux). Bacterial suspensions prepared in GP sterile inoculation fluid transferred to GP2 Microplates as described by the manufacturer.The tests were repeated three times. The metabolic fingerprint patterns were noted. Antibiotics susceptibility tests (specific for oligotrophic bacteria) determined according to the method described in chapter 2. Susceptibility to some of the drugs was tested using GP card (VITEK 2 system, BioMérieux) and results were interpreted according to manufacturer's instructions.

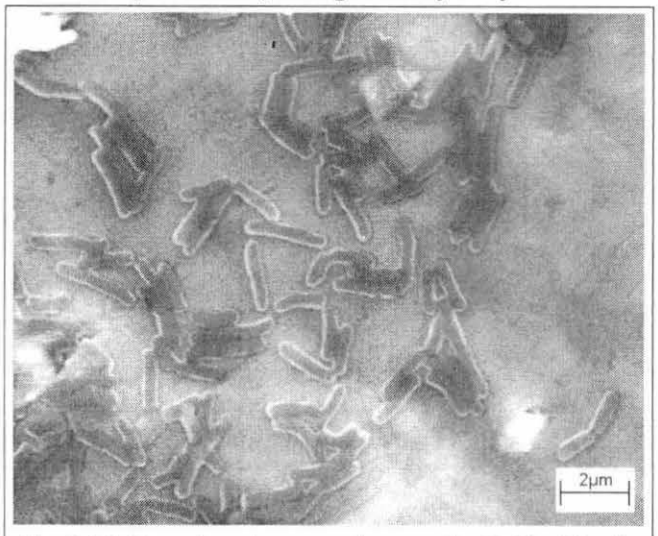


Fig. 3.25: Scanning electron micrograph of 16 h old cells of *Brevibacterium siliguriense* sp. nov. strain MB18^T grown in undiluted Luria broth at 28 °C. Bar, 2 µm.

For amplification of 16S rRNA gene, whole cell DNA was extracted according to the method described in chapter 3. The amplified 16S rRNA gene sequence was purified, cloned and sequenced. Nearly complete 16S rRNA gene sequence comprising 1433 bp was obtained from strain MB18 using 27F and 1492R primers. 16S rRNA gene sequence comparisons with entries in the updated GenBank and EMBL databases were performed with the FASTA and BLAST programs (Pearson, 1990; Altschul *et al.*, 1990, 1997). To determine the phylogenetic affiliation, the 16S,rRNA gene sequence of strain MB18 was aligned with the sequences of species of the genus *Brevibacterium* using Clustal-W (Thomson *et al.*, 1994). Approximately 1360 bp long stretch of 16S rRNA gene sequences present in all strains of *Brevibacterium* between position 13 and 1371 selected from nucleotide database from EMBL European Bioinformatics Institute (http://www.ebi.ac.uk) for further analysis. The rest of the lanking nucleotide sequences were omitted due to alignment ambiguities. Phylogenetic tree(s) [maximum-parsimony (MP) and neighbour-joining (NJ)] were constructed from continuous stretch containing ~1360 bp (Fig. 3.26 and 3.27). Evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). The fidelity of the tree topologies were evaluated by the bootstrap analysis with 1000 replicates (Felsenstein, 1985). The phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

The phylogenetic tree (Fig. 3.27) showed that strain, MB18 belong to the genus *Brevibacterium*. In the phylogenetic tree, strain formed a cluster comprising five *Brevibacterium* species: *B. epidermidis* NCDO 2286^T, *B. iodinum* DSM 2062^T, *B. permense* VKM Ac-2280^T and *B. linens* DSM 20425^T and *B. oceani* LMG 23457^T. Strain MB18 makes a deep branching with *B. epidermidis* NCDO 2286^T (= DSM 20660 = LMG 21455) with 16S rRNA gene sequence similarity value of 96%.

According to previous studies if any strain, showing 3% or more than 3% 16S rRNA gene sequence dissimilarity with their neighbours, it can be assigned as separate species (Stackebrandt and Goebel, 1994; Stackebrandt *et al.*, 2002; Lee, 2006 and Tindall *et al.*, 2010) without the need of DNA-DNA hybridization. As the level of similarity between 16S rRNA gene sequences of strain MB18 and the closest *Brevibacterium* species, *Brevibacterium epidermidis* NCDO 2286^T was 96%, therefore, this strain, MB18 can be endorsed to a separate genospecies without entering into DNA–DNA hybridization. The differences are also evident between the strains in terms of biochemical and chemotaxonomic characters.

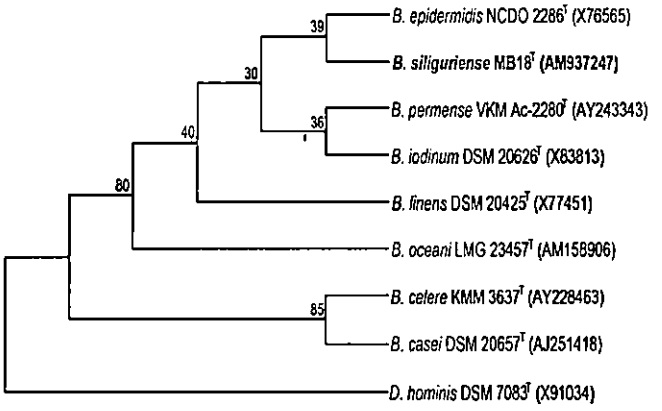


Fig. 3.26: MP tree based on 16S rRNA gene sequences, showing the position of strain MB18^T (Bold face) within the closely related members of the genus *Brevibacterium*. Bootstrap values (expressed as percentages of 1000 replications) are given at the branch nodes. *Dermabacter hominis* DSM 7083^T was used as outgroup.

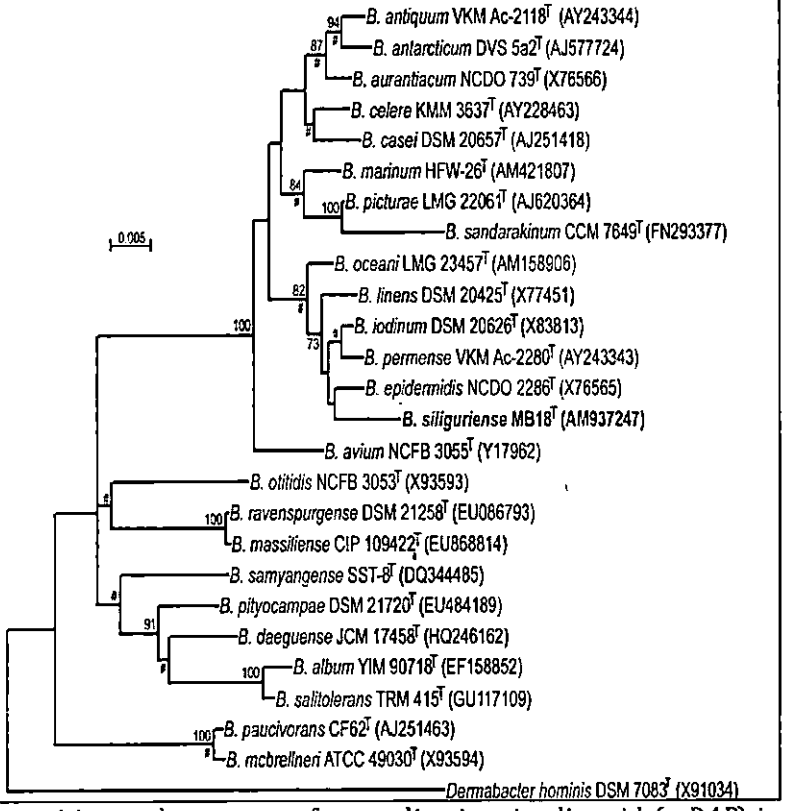


Fig. 3.27: Consensus NJ tree derived from 16S rRNA gene sequences showing the position of strain MB18^T within the members of the genus *Brevibacterium*. The tree was reconstructed using maximum parsimony method and common clusters obtained in both NJ and MP tree are indicated by hash (#). Bootstrap values (>70%) expressed as percentages of 1000 replications are given at each branch point. *Dermabacter hominis* DSM 7083^T was used as out-group. Bar, 5 nucleotide substitutions per 1000 nucleotide.

To determine the sugar composition and presence of *meso*-diaminopimelic acid (*mDAP*) in strain MB18^T, 200 mg (wet weight) cells were scraped from LA plate grown at 28 °C for 3 days. The cell walls were prepared according to the method of Boone and Pine (1968), and sugars and *m*DAP in acid hydrolysates were identified by one-dimensional paper chromatography following the method described earlier (Staneck and Roberts, 1974) with slight modification (Whatman paper No.1 was used instead of cellulose thin- layer chromatographic plate). Using butanolic ninhydrin (0.3% w/v ninhydrin in isobutanol) spray, the appearance of gray-green spot with low Rf value (0.37-0.38) which turned yellow on keeping in dark for more than 24 h, confirmed the characteristic

presence of *m*DAP (Fig. 3.28). *m*DAP standard was kindly provided by Dr. B. Bhadra (CCMB Hyderabad, now at dupont, Hyderabad).

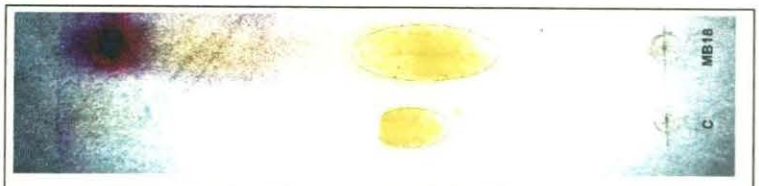


Fig. 3.28: mDAP (yellow spot) of *Brevibacterium siliguriense* sp.nov. strain MB18^T. C, control

Sugars present in cell wall acid hydrolysate were detected by spraying the acid aniline phthalate (0.325% 0-pthalic acid dissolved in water saturated n-butanol containing 0.2 mL aniline). Galactose as the sole sugar was identified in the cell wall of strain MB18 (Fig. 3.29). Polar lipids were isolated from the strain MB18 by the method described earlier (Minnikin *et al.*, 1984). The isolated polar lipids were detected on aluminum backed silica gel 60 F_{254} plate (Merck, Germany) by one and two-dimensional thin-layer chromatography (TLC) according to the method (Counsel and Murray, 1986). Polar lipid profile (Fig 3.30) contained, phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) phospholipids as found in the other members of *Brevibacterium*. For detection of mycolic acid, method described by Minnikin *et al.* (1980) was used. No mycolic acid was detected in cell wall of the strain MB18. Menaquinones were extracted from lyophilized cells and analyzed by HPLC following methods described by Collins *et al.* (1977) and Groth *et al.* (1997) respectively.

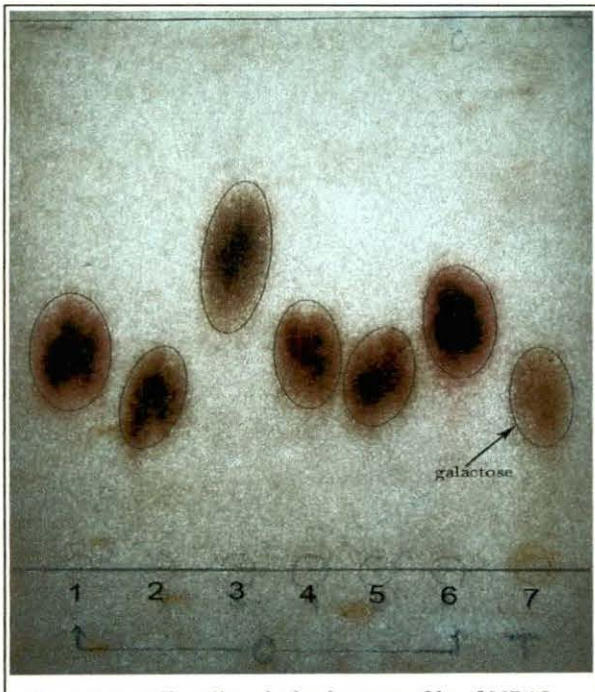


Fig. 3.29: Cell wall carbohydrate profile of MB18. 1, arabinose; 2, galactose; 3, rhamnose; 4, mannose; 5, glucose; 6, xylose; 7, Acid hydrolysate of *Brevibacterium siliguriense* sp. nov. strain MB18^T. Top gray colored line showed solvent front.

Fig. 3.30: The lipid profile of strain MB18

PG, phosphatidylglycerol;
DPG, diphosphatidylglycerol

For analysis of fatty acids, fatty acid methyl esters (FAMEs) were extracted from 36 h old (exponentially growing) cells grown in Tryptone-soy-agar (M290; HiMedia, India) at 28 °C. It was then analyzed by gas chromatography (Hewlett Packard 5890 II plus, Palo alto, CA, USA) and the Sherlock Microbial Identification System using version 4.10 of the TSBA40 library (Microbial ID, Newark, DE, USA). The analyzed FAME peaks are shown in Fig 3.31. Fatty acids of strain MB18^T [anteiso-$C_{15:0}$ (50.82%), anteiso-$C_{17:0}$ (26.73%), iso-$C_{15:0}$ (14.04%), iso$C_{16:0}$ (4.07%); iso-$C_{17:0}$ (3.16%); and trace amount of $C_{16:0}$ (0.48%); iso$C_{14:0}$ (0.47%) and $C_{14:0}$ (0.22%)] were typical of the genus *Brevibacterium*, but the proportion differed from those reported for *B. epidermidis* DSM 20660^T

{anteiso-$C_{15:0}$ (70.0%), anteiso-$C_{17:0}$ (21.5%), iso-$C_{15:0}$ (2.0%), iso$C_{16:0}$ (3.0%); and trace amount of anteiso-A $C_{17:1}$ (2.0%), $C_{18:0}$ (1.5%), and $C_{16:0}$ (1.0%) [Collins *et al.*, 1983]}.

For determination of G+C content, the genomic DNA of the strain was prepared by disrupting cells with French pressure cell and purified on hydroxyapatit following standard procedure (Cashion *et al.*, 1977). The purified DNA was hydrolyzed with p1 nuclease and the nucleotides de-phosphorylized with bovine alkaline phosphates (Mesbah *et al.*, 1989). The resulting deoxyribonucleside were analyzed by HPLC system (Shimdzu Corp. Japan,) having LC-20AD solvent delivery module, DGU-3A online degasser, CTO-10AC column oven, SIL-20A automatic sample injector and a SPD-6A UV spectrophotometric detector. The system was calibrated with non-methylated lambda DNA (Sigma) (49.86 mol% G+C) and three sample DNAs, *Bacillus subtillis* DSM 402 (43.52 mol% G+C), *Xanthomonas campestris* pv. *campestris* DSM 3586^T (65.07 mol% G+C), and *Streptomyces violaceoruber* DSM 40783 (72.12 mol% G+C). GC was calculated from the ratio of deoxyguanosine (dG) and deoxythymidine (dT) according to the method of Mesbah *et al.* (1989). The DNA G+C content of the strain MB18 was found to be 64.6 mol% which was very near to its neighbour, *B. epidermidis* DSM 20660^T (63.5 mol %).

Table 3.3: Differential phenotypic characteristics between *B. siliguriense* sp. nov. strain MB18^T and *B. epidermidis* LMG 21455^T, the nearest phylogenetic neighbor.
Symbols: –, negative result; +, positive result.

Characteristics	MB18^T	*B. epidermidis*
Growth at :.		
37°C	poor	good
40°C	–	+
Hydrolysis of Casein	–	+
Hydrolysis of Xanthine	–	+
Acid from Phenyl acetate	+	–
Carbon utilization:		
Glucose	–	+
Arabinose	–	+
Succinic acid	–	+
Xylose	–	+
Inositol	–	+
Mannose	+	–
Raffinose	–	+
Glycerol	+	–
Galactose	–	+
Gluconate	–	+
Enzymes:		
Alkaline phosphatase	–	+
b-glucoronidase	–	+
Fermentation of D-mannitol	+	–
DNA G+C content (mol%)	64.6	63.5
Origin of isolation	River water	Human skin

In spite of the similarities, strain MB18 can be readily differentiated from *B. epidermidis* with reference to some physiological and biochemical characteristics (Table 3.3). Strain MB18 can also be distinguished from *B. epidermidis* NCDO 2286^T by 16S rRNA gene sequence similarity, DNA G+C content, cellular fatty acids, and other chemical analysis. On the basis of the data obtained with our polyphasic taxonomic approach, strain MB18 merits recognition as a member of a novel species of the genus *Brevibacterium*, for which the name *Brevibacterium siliguriehse* sp. nov. was proposed.

3B.1. Description of *Brevibacterium siliguriense* sp. nov.

Brevibacterium siliguriense: si.li.gu.ri.en'se. N.L. neut. adj. siliguriense of or pertaining to town Siliguri, the location from where the water sample was collected.

Cells are Gram-positive, non-motile, non-spore-forming, rods, 2.0 ±0.23 µm long and 0.4±0.06 µm wide, catalase-positive and oxidase-negative. Colonies are off-white, circular and low convex with entire margin. Growth is observed at 20-37°C (optimum 28 °C; no growth at 40 °C), pH 5–12 (optimum 7.0); and 0-15% (w/v) KCl and NaCl (optimum 2 %). The supplementation of KCl in PY media shows better growth than the NaCl. Urease, gelatinase, arginine dihydrolase 1, β–galactosidase, β-glactopyranosidase, leucine arylamidase, L-proline arylamidase, α-galactosidase, alanine arylamidase and tyrosine arylamidase are positive. Nitrate is reduced to nitrite. Tests for α-glucosidase, ala-phe-pro arylamidase, L-aspartate, arylamidase, α-mannosidase, phosphatase, β-glucoronidase, H_2S production, indole production, L-pyrrolidonyl-arylamidase and fermentation of

D-xylose, D-sorbitol, D-galactose, D-ribose, lactose, N-acetyl D-glucosamine, D-maltose, D-raffinose, sacchorose, D-trehalose, D-amygdalin are negative. Additional detailed phenotypic characteristics and antibiogram are given in Table 3.4A and 3.4B. Strain MB18^T contains *mDAP* in peptidoglycan. Phosphatidylglycerol and diphosphatidylglycerol are the major polar lipids. The major menaquinone is MK-8(H2) and fatty acid profile contains predominantly anteiso-$C_{15:0}$ (50.82%), anteiso-$C_{17:0}$ (26.73%) and iso-$C_{15:0}$ (14.04%).The G+C content of the type strain is 64.6 mol%.

The type strain MB18^T (=DSM **23676**^{T} = LMG **25772**^{T}) was isolated from water sample of river Mahananda, Siliguri, West Bengal, INDIA.

3.4. Conclusion

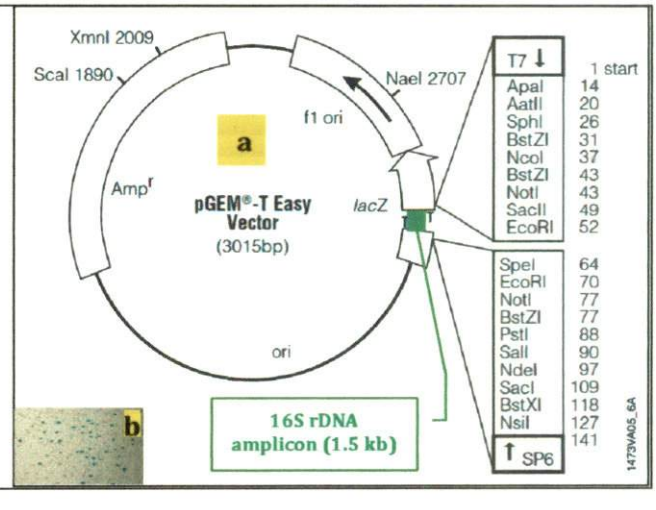
Analyses of phenotypic characteristics and 16S rRNA gene sequence phylogenies of the facultatively oligotrophic isolates with class 1 integron carriages indicated the diversity of oligotrophs in the Mahananda River at Siliguri. They belonged to different families like *Comamonadaceae, Moraxellaceae, Pseudomonadaceae, Aeromonadaceae,* and *Enterobacteriaceae.* These families fell under two super classes: *Betaproteobacteria* and *Gammaproteobacteria.* Despite of the several isolates placed in a specific genera, nine isolates, MB25, MB28, MB41, MB44, MB48, MB54, MB81, MB83 and MB12 could not be assigned to any of the known genera. The 16S rRNA gene sequences of these isolates (the isolates which could not be assigned any genus) poorly shared with the known genera of different classes. Each of these isolates demand further studies for establishment of novel genus. However, on examining their positions in the dendogram (based on numerical taxonomy), they were found to branch separately with isolates clustered under the family *Enterobacteriaceae.* Majority of the isolates were detected from the family *Enterobacteriaceae* comprising of genera: *Shigella, Kluyvera, Klebsiella, Salmonella, Citrobacter, Serratia, Enterobacter, Proteus, Providencia* and *Escherichia. Acinetobacter* was the only genus identified amongst the isolates that was under the family *Moraxellaceae.* The only culturable oligotrophic Gram positive strain isolated in the course of this study, MB18, was identified as a novel species of the genus *Brevibacterium* on the basis of 16S rRNA gene sequence similarity, DNA G+C content, cellular fatty acids, and other chemical analysis. On the basis of the data obtained with our polyphasic taxonomic approach, strain MB18 merits recognition as a member of a novel species of the genus *Brevibacterium,* for which the name *Brevibacterium siliguriense* sp. nov. was proposed.

Thus it may be concluded that exploring river water for culturable oligotrophic bacteria provides not only an opportunity to discover novel bacteria but also strengthens the study of microbial biodiversity at large.

Remaining figures with their legends:

Fig. 3.2: a, Cloning of 16S rDNA-amplicon in pGEMT-easy vector vector-II and b, Blue-white screening of recombinant clone on Luria agar plate containing ampicillin (100 mg/L), X-gal and IPTG. Red color shows the gene of interest, ligated within the multiple cloning site of T-vector.

Amp^r, gene for ampicillin resistance; *ori*, orgin of replication; *lacZ*, gene for beta-galactosidase synthesis.



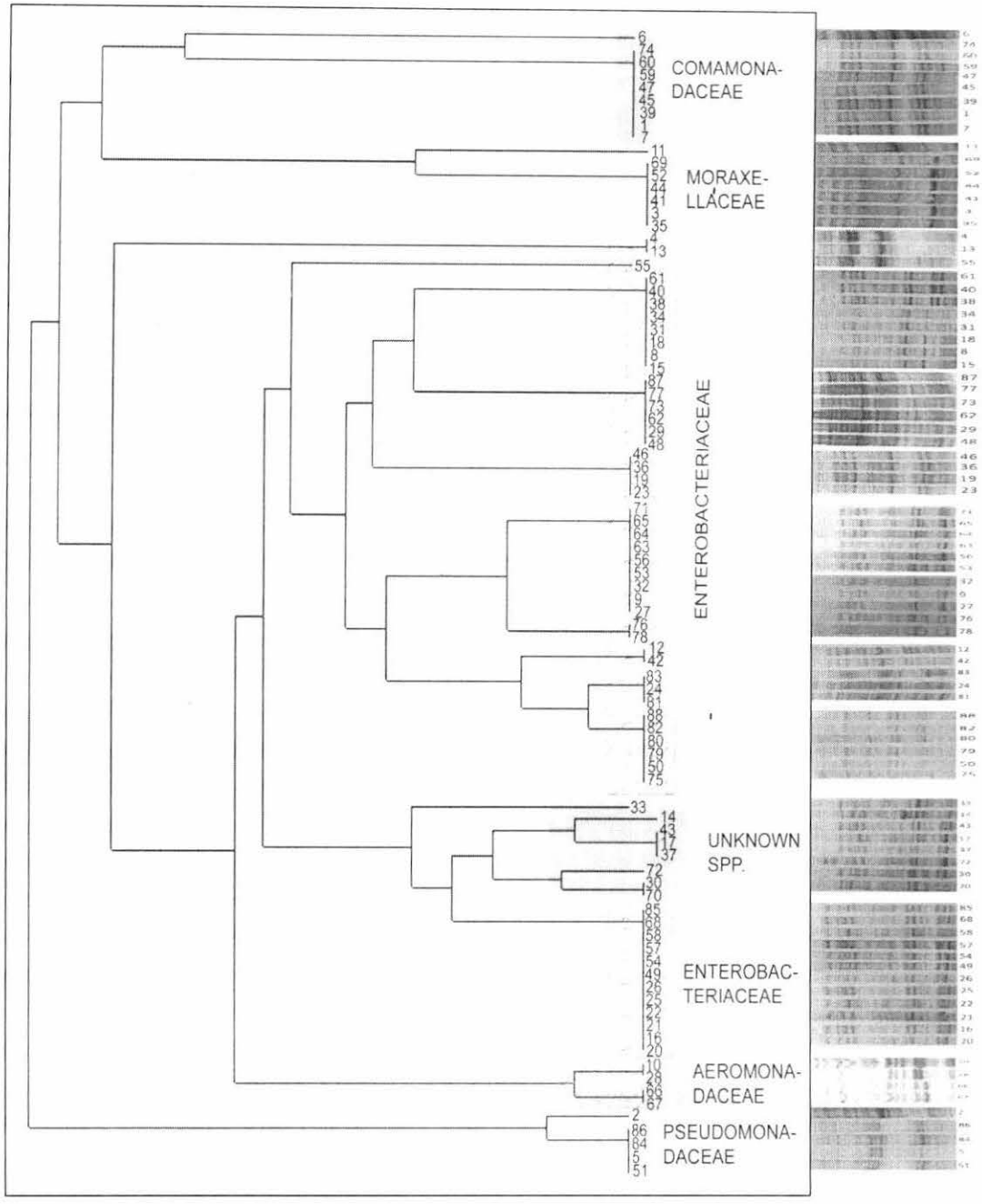


Fig. 3.3: Dendogram based on similarity matrix (numerical analysis) computed from phenotypic characters and One-dimensional SDS-PAGE analysis of total proteins extracted from bacteria possessing class 1 integrons. MB12 and MB18 are excluded from this analysis, for detail please see manuscript. Similarity matrix of phenotypic data is available as supplementary table in the soft copy of the thesis (Annexure-I).

Numbers corresponding to the isolates: 1, OB 05; 2, OB 12; 3, MB 03; 4, MB 05; 5, MB 08; 6, MB 09; 7, MB 16; 8, MB 19; 9, MB 20; 10, MB 21; 11, MB 22; 12, MB 23; 13, MB 24; 14, MB 25; 15, MB 26; 16, MB 27; 17, MB 28; 18, MB 29; 19, MB 30; 20, MB 31; 21, MB 32; 22, MB 33; 23, MB 34B; 24, MB 35; 25, MB 36; 26, MB 37A; 27, MB 38; 28, MB 39; 29, MB 40; 30, MB 41; 31, MB 42; 32, MB 43; 33, MB 44; 34, MB 45; 35, MB 46; 36, MB 47; 37, MB 48; 38, MB 49; 39, MB 50; 40, MB 51; 41, MB 52; 42, MB 53; 43, MB54; 44, MB 55; 45, MB 56; 46, MB 57; 47, MB 58; 48, MB 59; 49, MB 60; 50, MB 61; 51, MB 62; 52, MB 63; 53, MB 64; 54, MB 65; 55, MB 66; 56, MB 67; 57, MB 68; 58, MB 69; 59, MB 70; 60, MB 71; 61, MB 72; 62, MB 73; 63, MB 74 ; 64, MB 75; 65, MB 76; 66, MB 77; 67, MB 78; 68, MB 79; 69, MB 80; 70, MB 81; 71, MB 82; 72, MB 83; 73, MR 01; 74, MR 02; 75, MR 03; 76, MR 04; 77, SR 19; 78, NV 66; 79, OD 05; 80, OD 08; 81, OD 10; 82, OC 16; 83, OC 24; 84, OC 74; 85, OC 75; 86, OC 78; 87, OD 21; 88, OD 24.

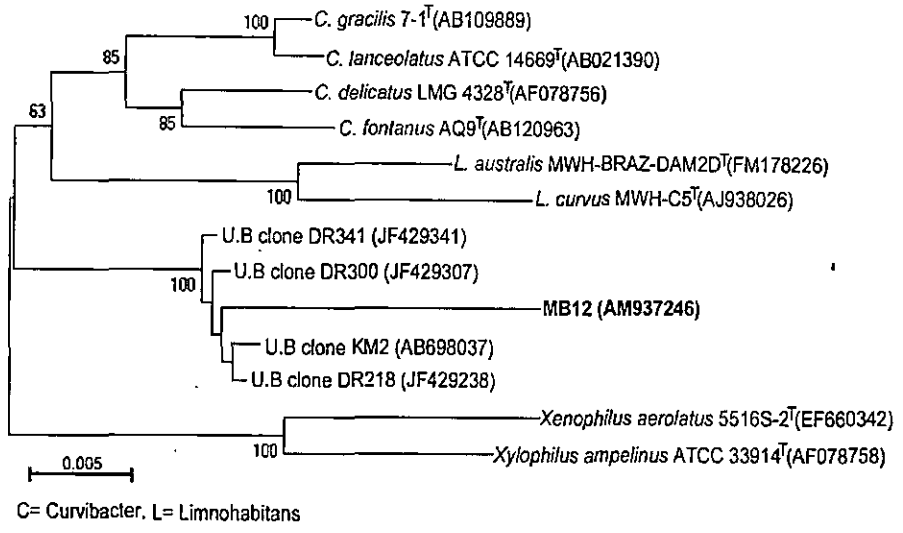
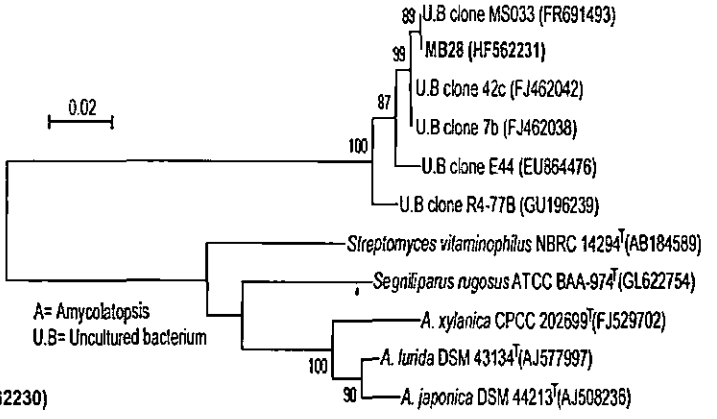


Fig. 3.4: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB-12 (Bold face) within uncultured bacteria and related genera. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are indicated at branching nodes. Accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

Fig. 3.5: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB28 (Bold face) within the uncultured bacterium and members of related genera. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 2 nucleotide substitutions per 100 nucleotides.



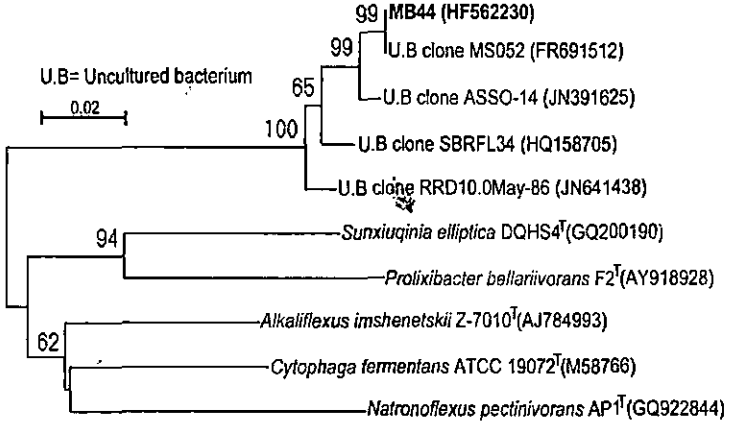
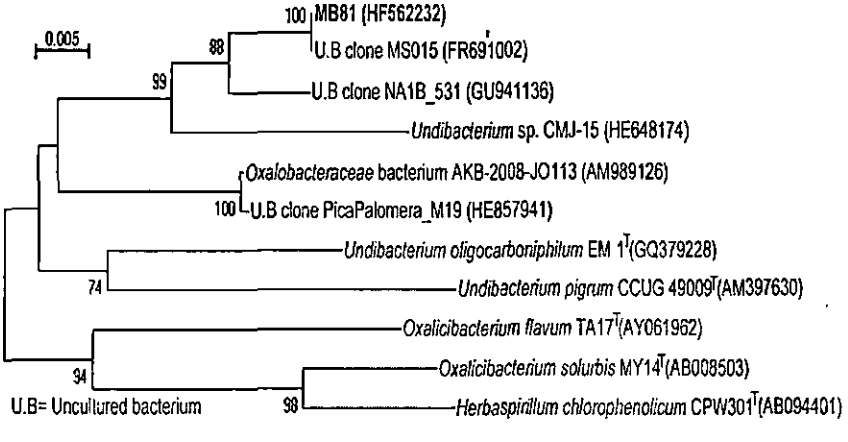


Fig. 3.6: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB28 (Bold face) within the uncultured bacterium and members of related genera. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 2 nucleotide substitutions per 100 nucleotides.

Fig. 3.7: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strains MB-81 (Bold face) within uncultured bacteria and related genera. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes.. Bar, 0.005 substitutions per nucleotide.



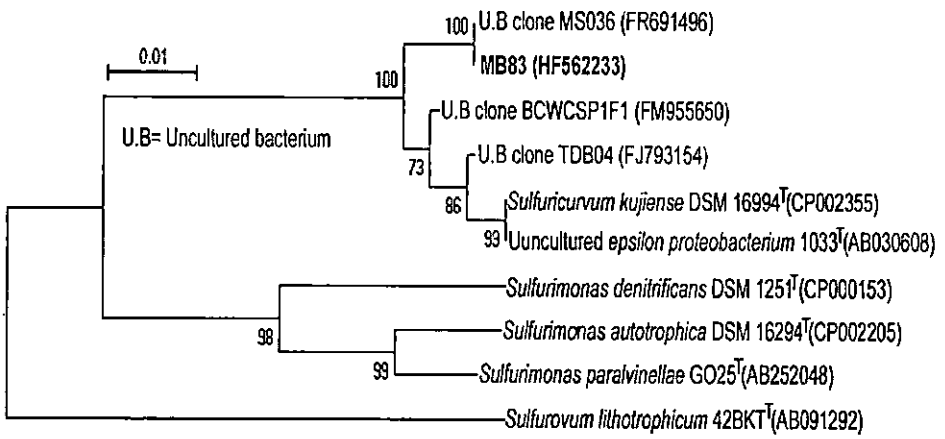


Fig. 3.8: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB-83 (Bold face) within uncultured bacteria and related genera. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide.

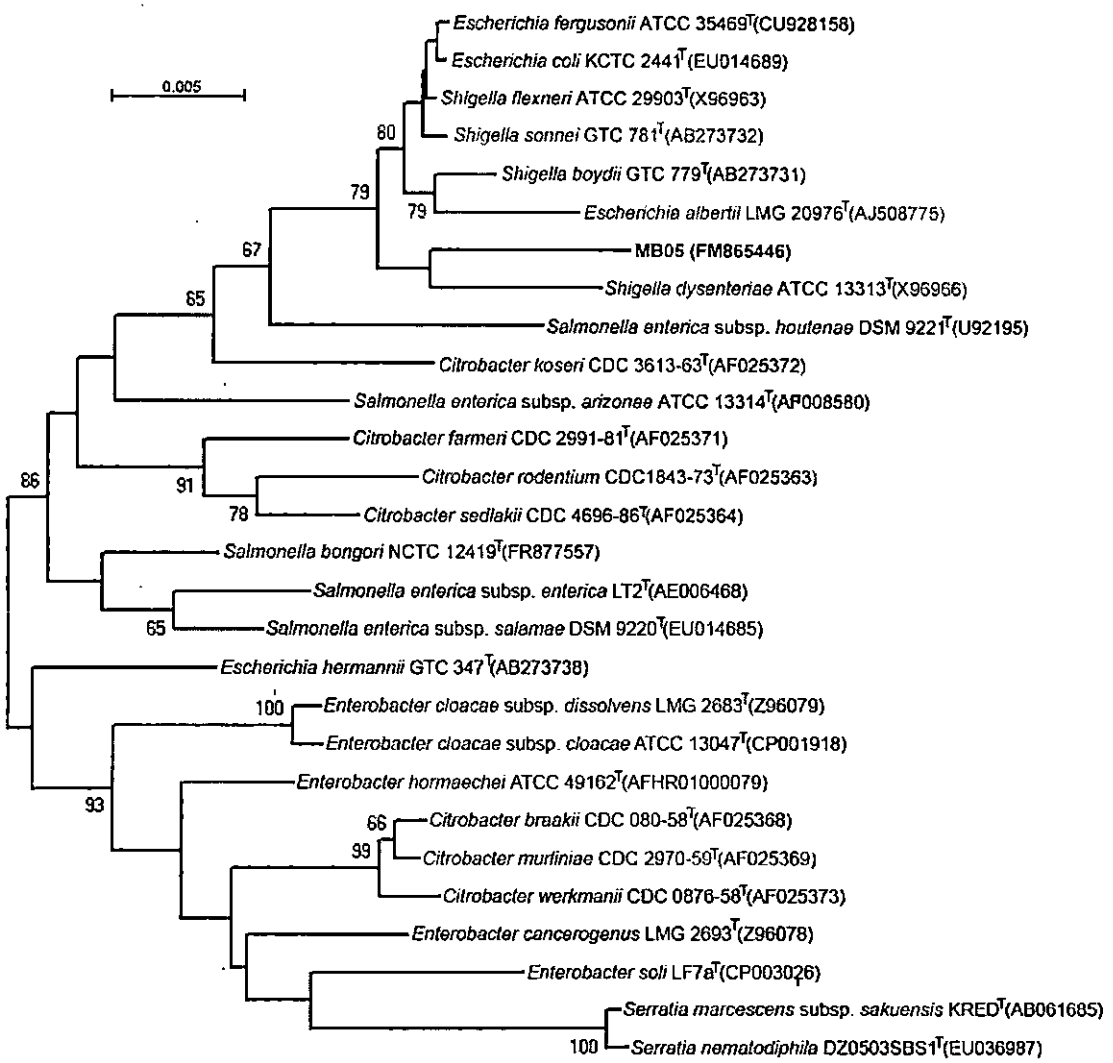


Fig. 3.9: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB-05 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

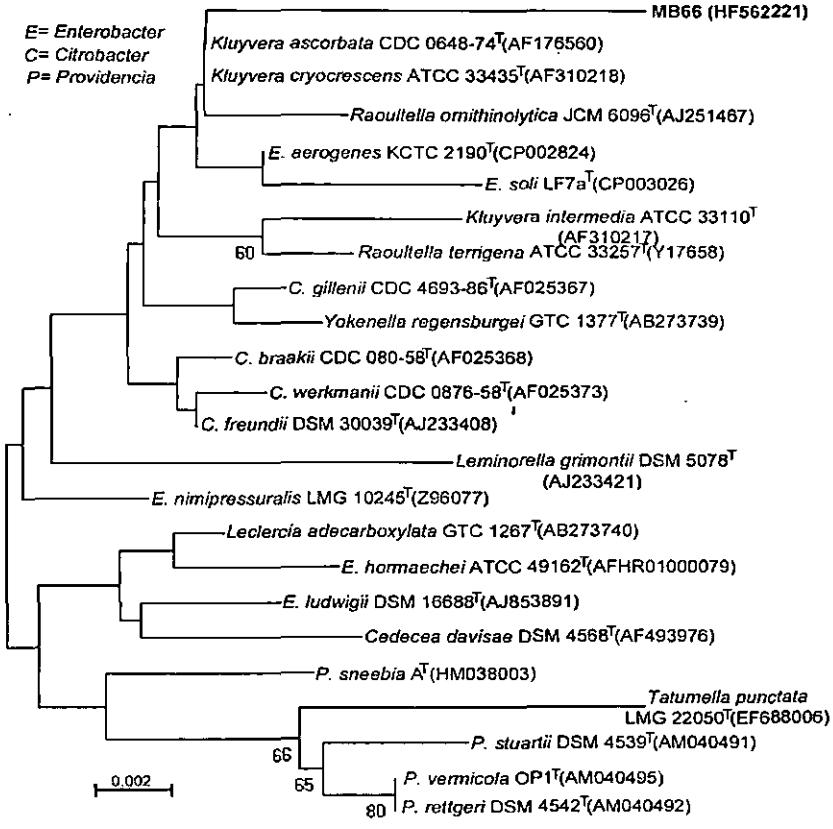


Fig. 3.10: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB66 (Bold face) within the members of family *Enterobacteriaceae.* Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

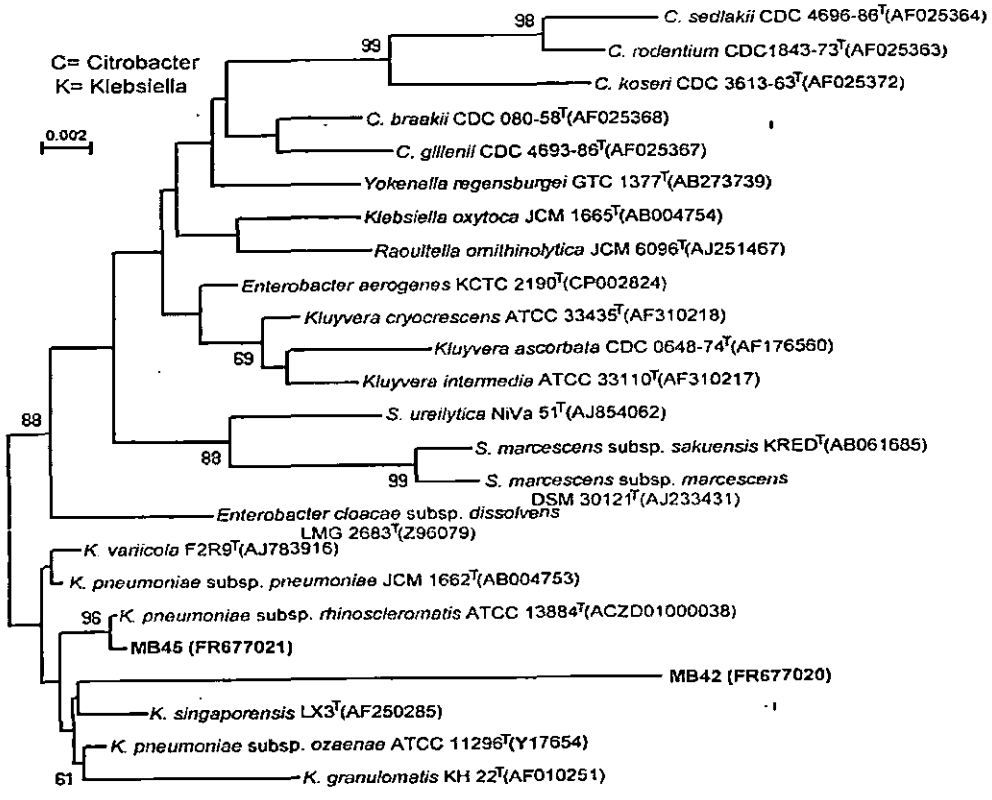


Fig. 3.11: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the positions of strains, MB42 and MB45 (Bold face) within the members of family *Enterobacteriaceae.* Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

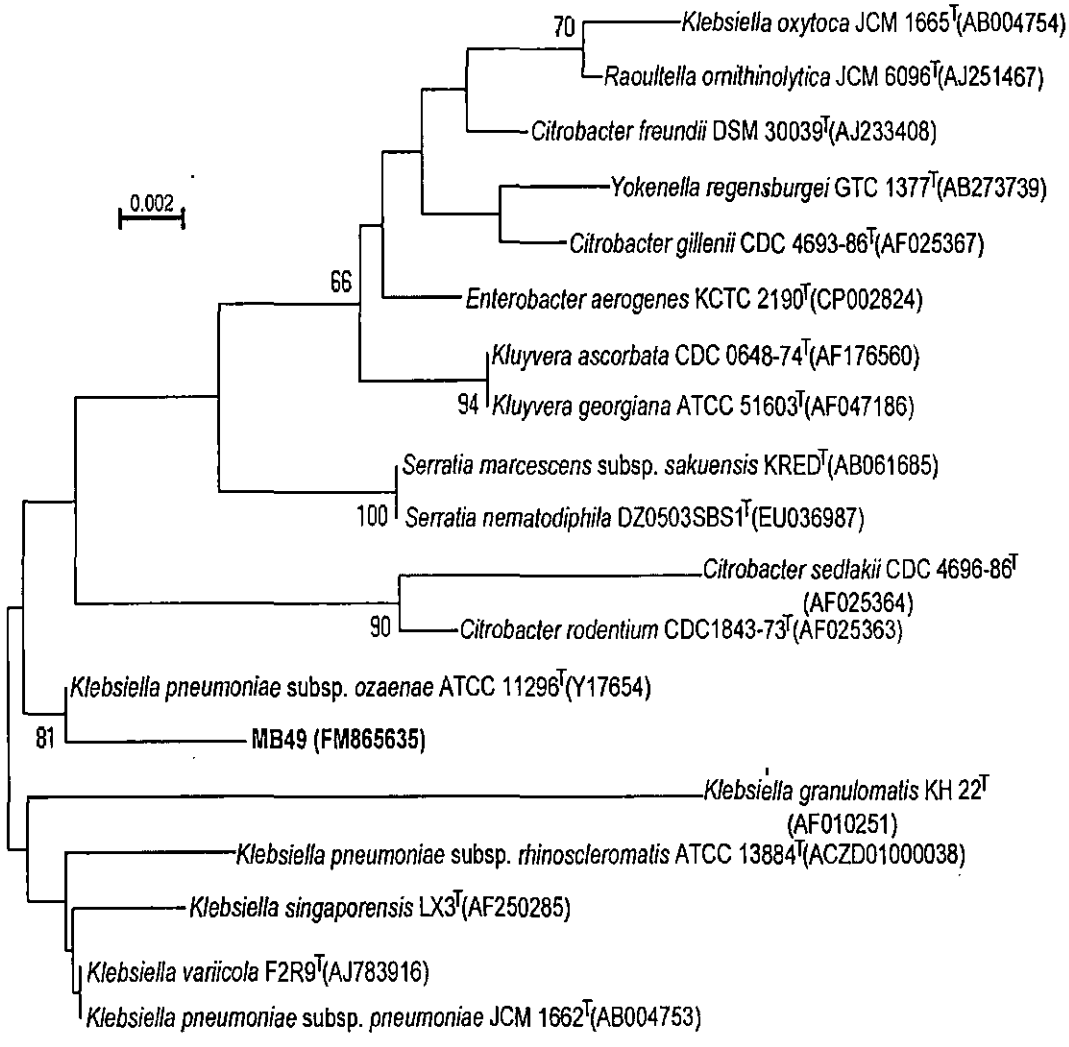


Fig. 3.12: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB49 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

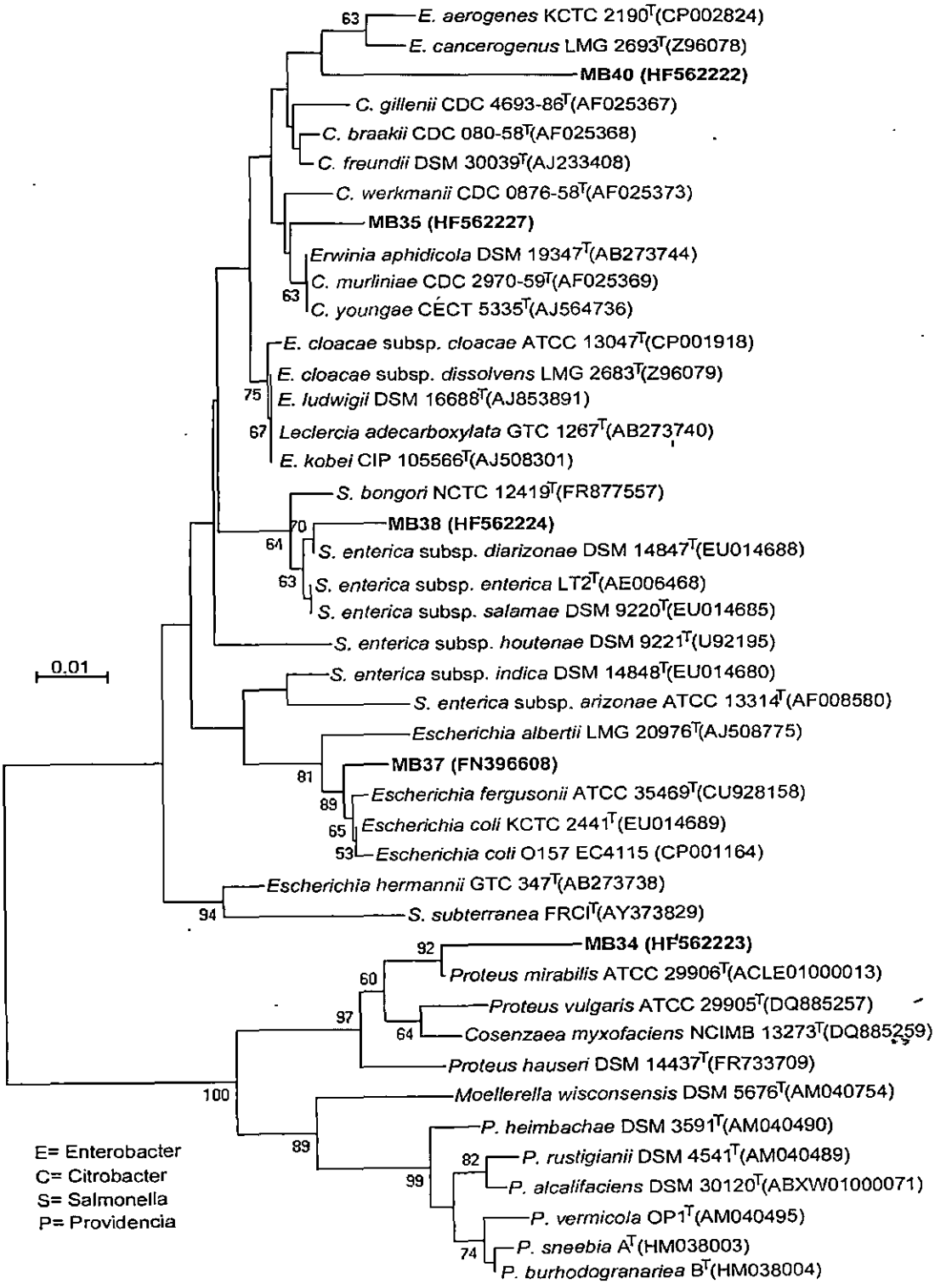


Fig. 3.13: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the positions of strains, MB34, MB35, MB37, MB38, and MB40 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide.

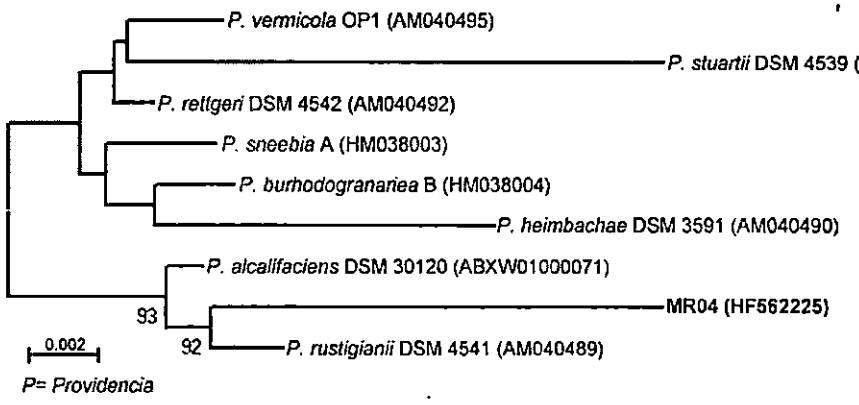


Fig. 3.14: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MR-04 (Bold face) within the members of genus *Providencia*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are indicated at branching nodes. Accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

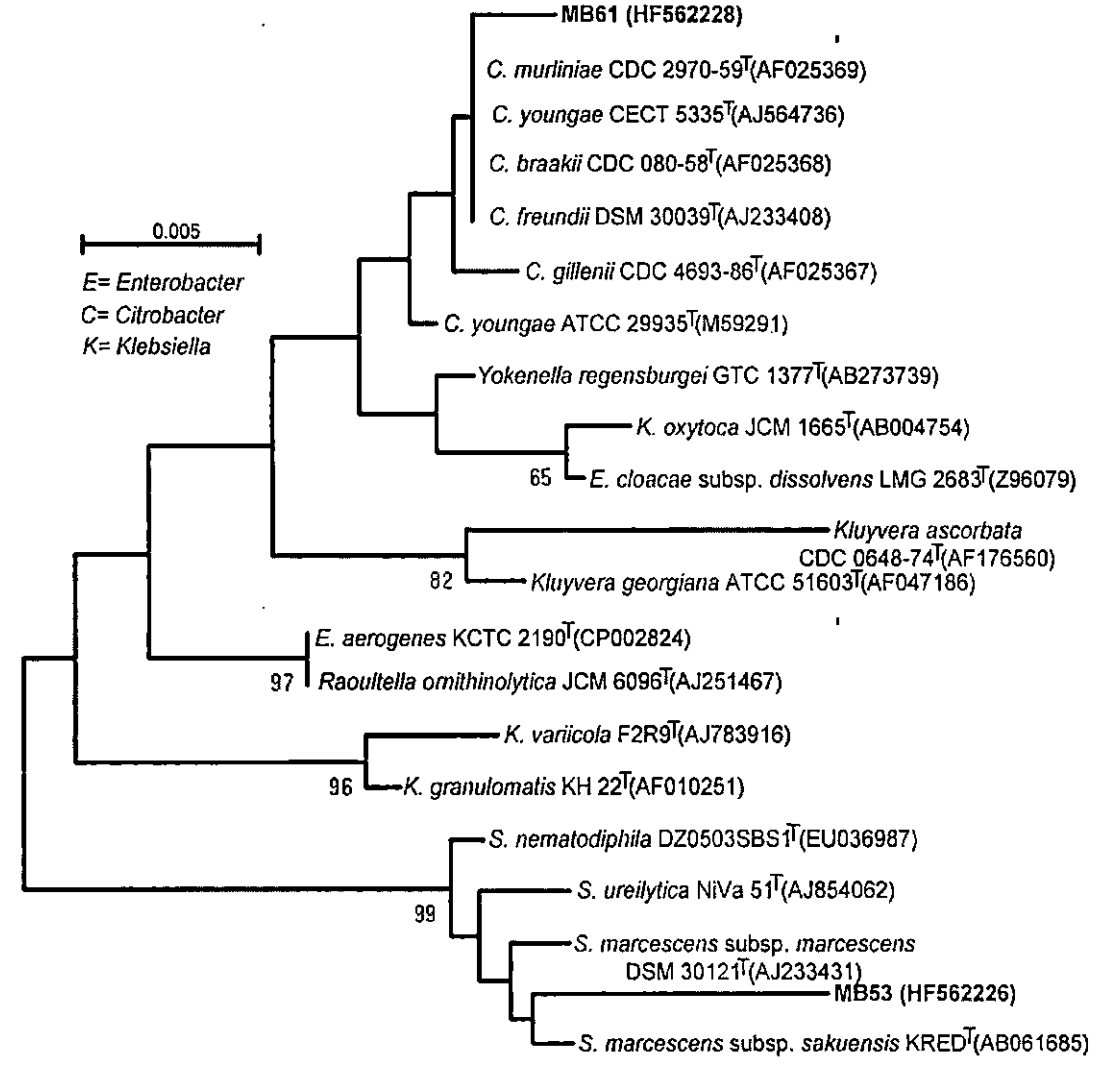


Fig. 3.15: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the positions of strains, MB53 and MB61 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

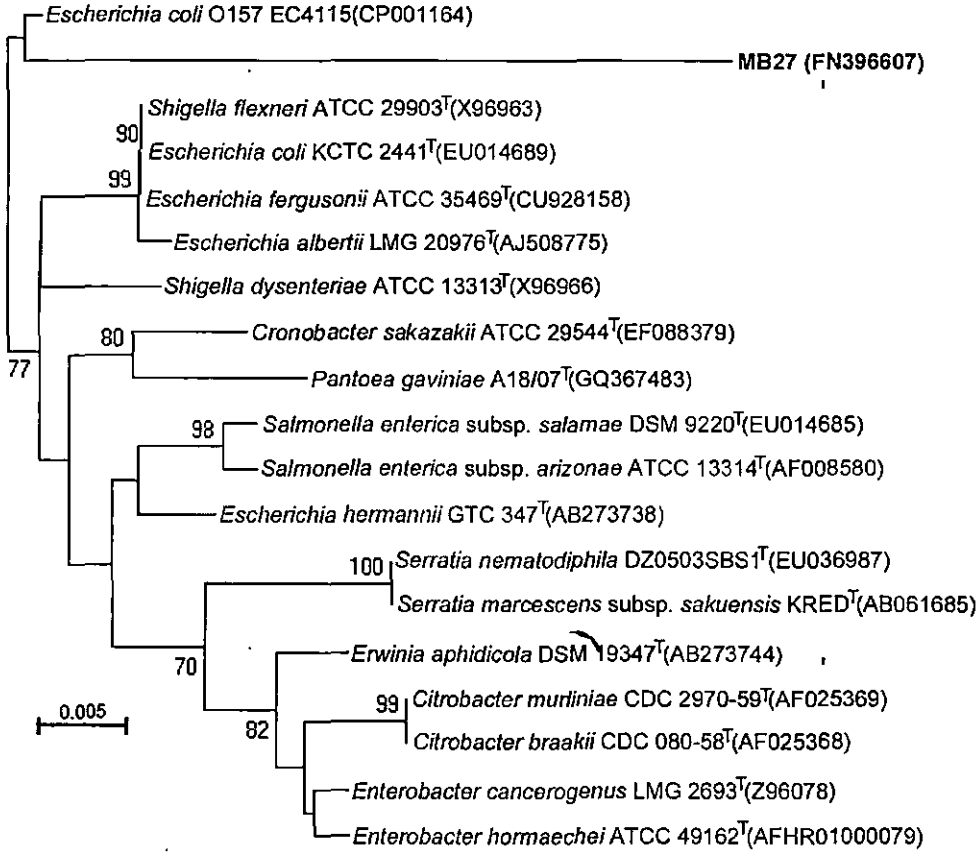


Fig. 3.16: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB27 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

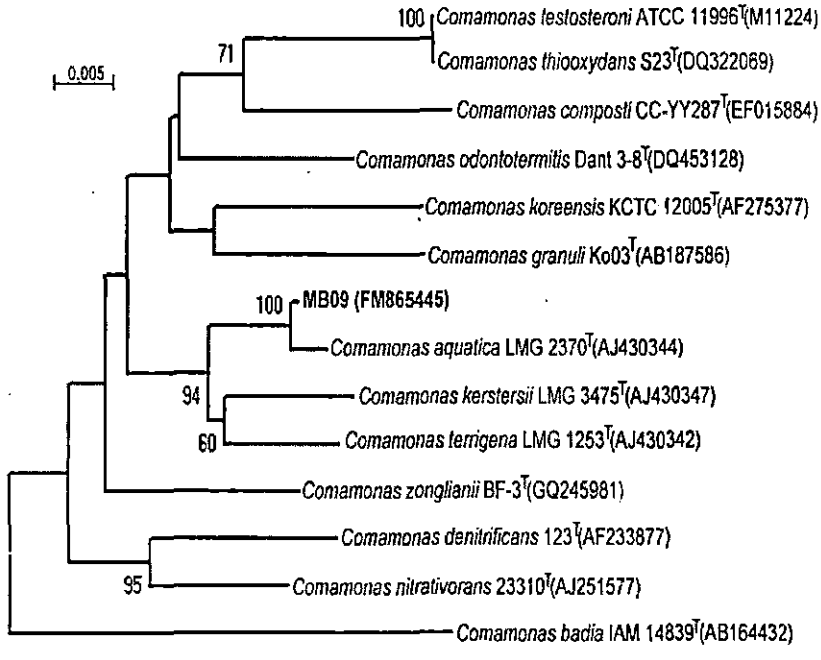


Fig. 3.17: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB-05 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (≥60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

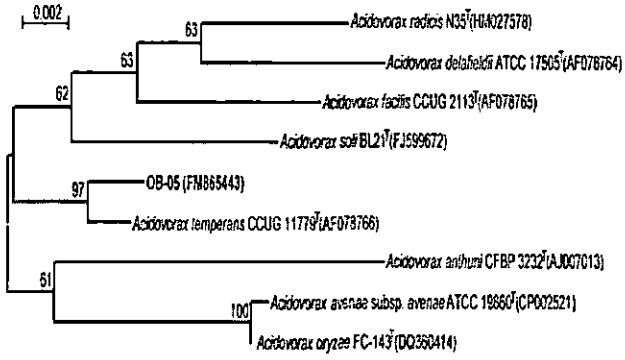


Fig. 3.18: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain OB-05 (Bold face) within the members of genus *Acidovorax*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

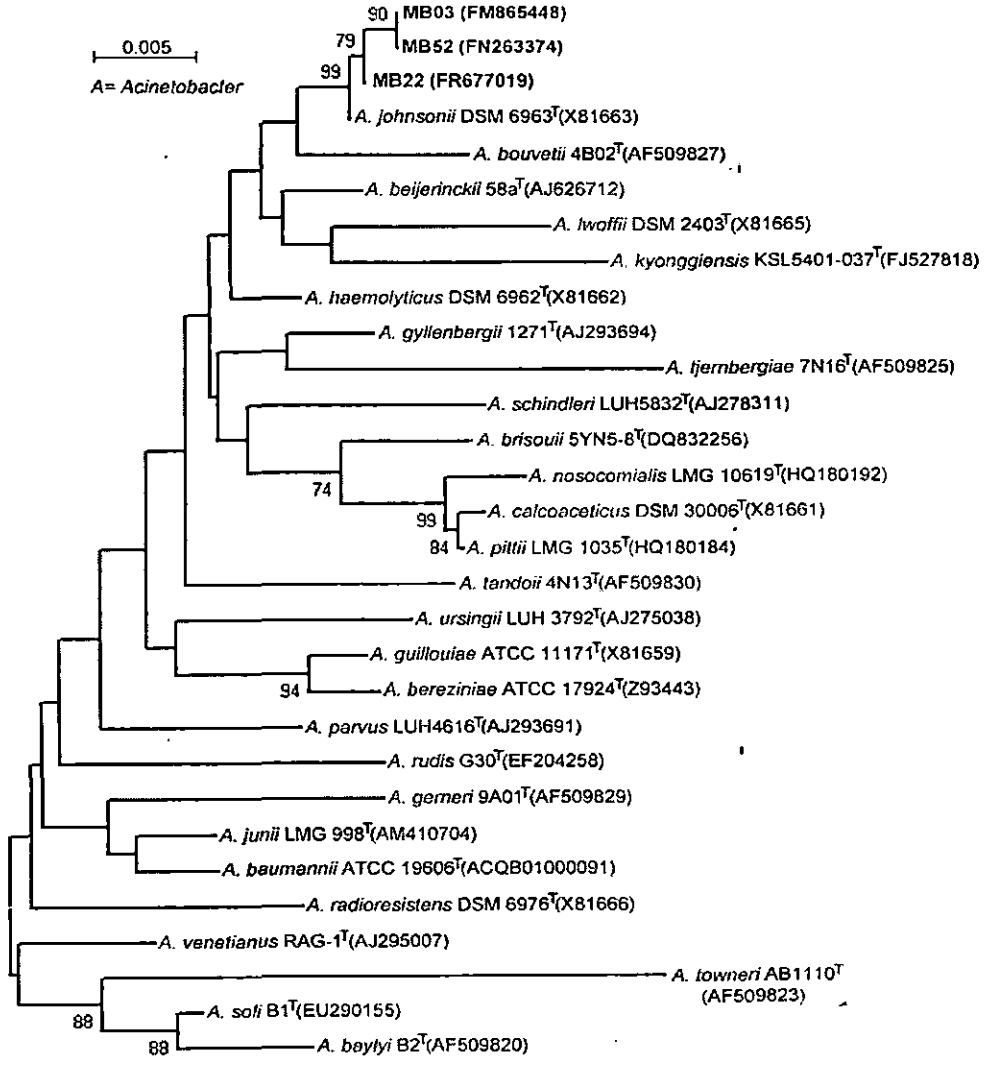


Fig. 3.19: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strains, MB03, MB22, and MB52 (Bold face) within the members of genus *Acinetobacter*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

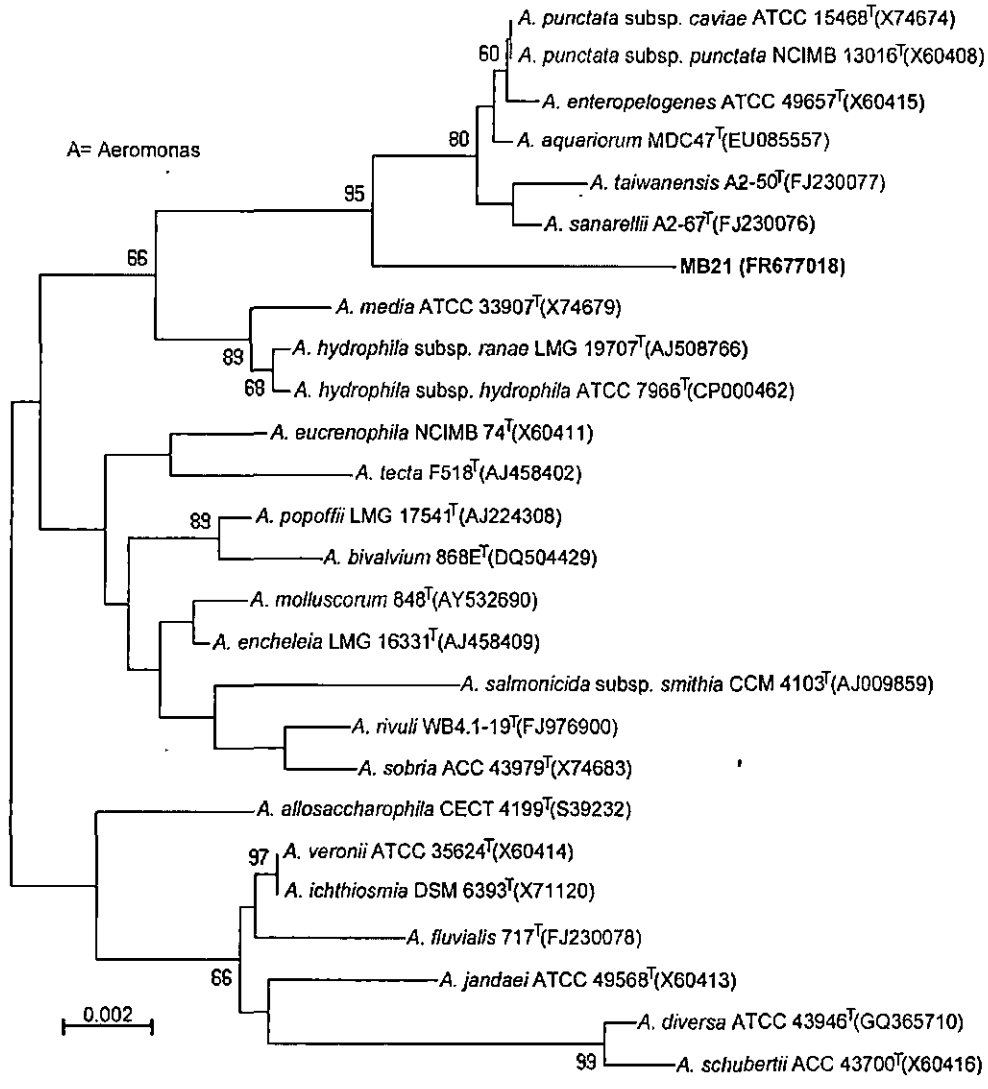
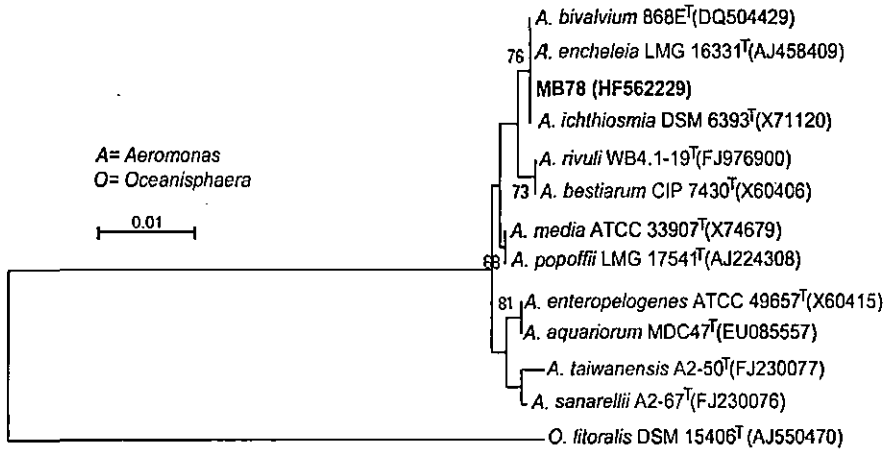


Fig. 3.20: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB21 (Bold face) within the members of genus ***Aeromonas***. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

Fig. 3.21: Rooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB-78 (Bold face) within the members of genus *Aeromonas*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. *Oceanisphaera litoralis* DSM 15406^T was used as out group. Accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotides.



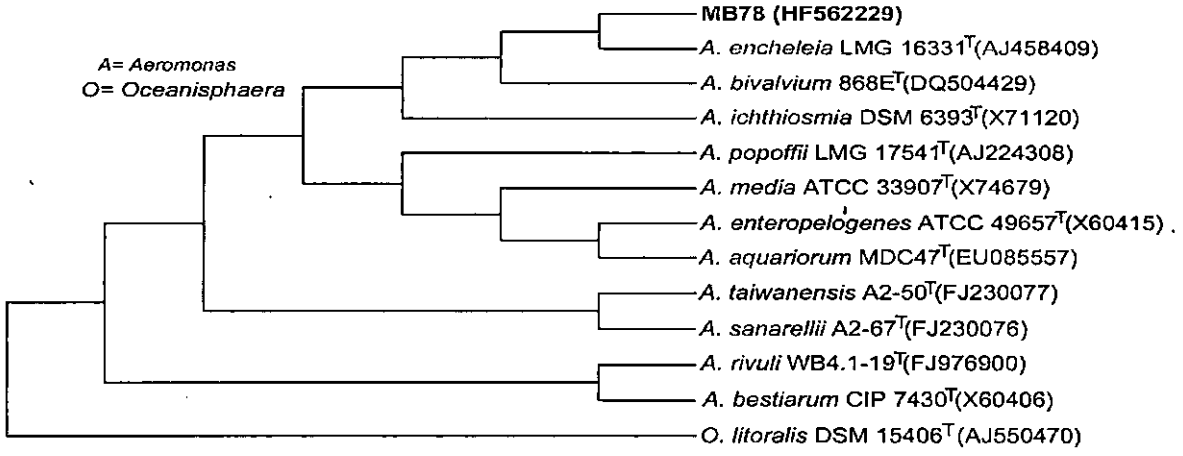


Fig. 3.22: Maximum parsimony tree (based on 16S rRNA gene sequences) showing the position of strain MB-78 (Bold face) within the members of genus *Aeromonas*. *Oceanisphaera litoralis* DSM 15406T (Ac. No. AJ550470) was used as an out group.

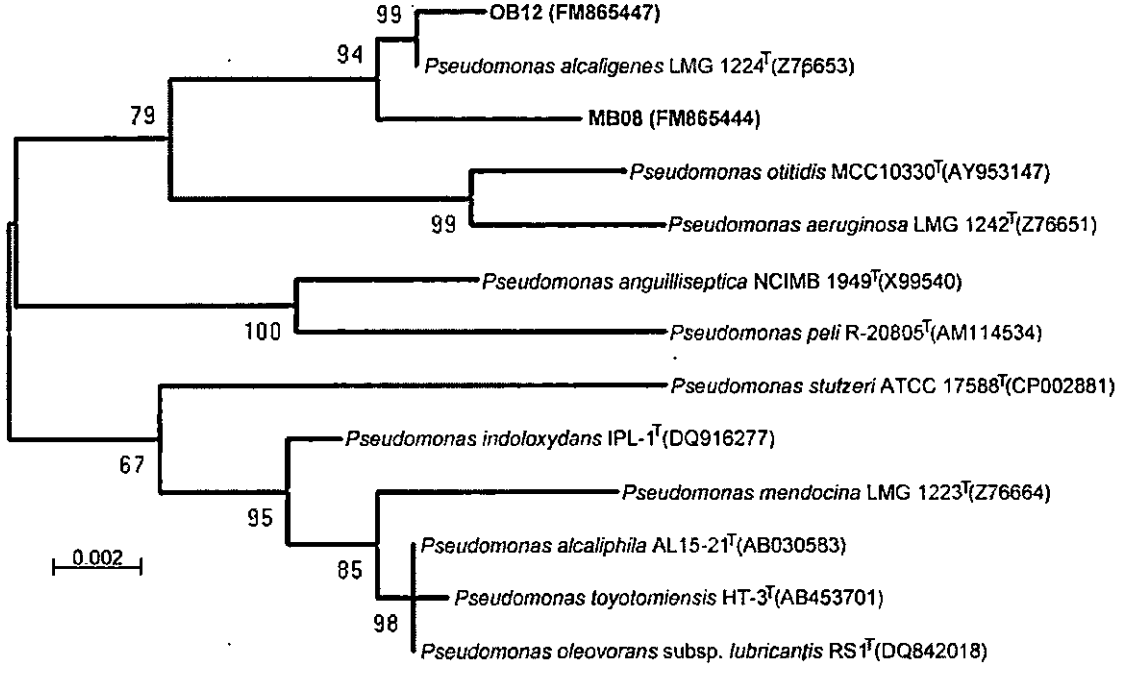


Fig. 3.23: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strains OB-12 and MB08 (Bold face) within the members of genus *Pseudomonas*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

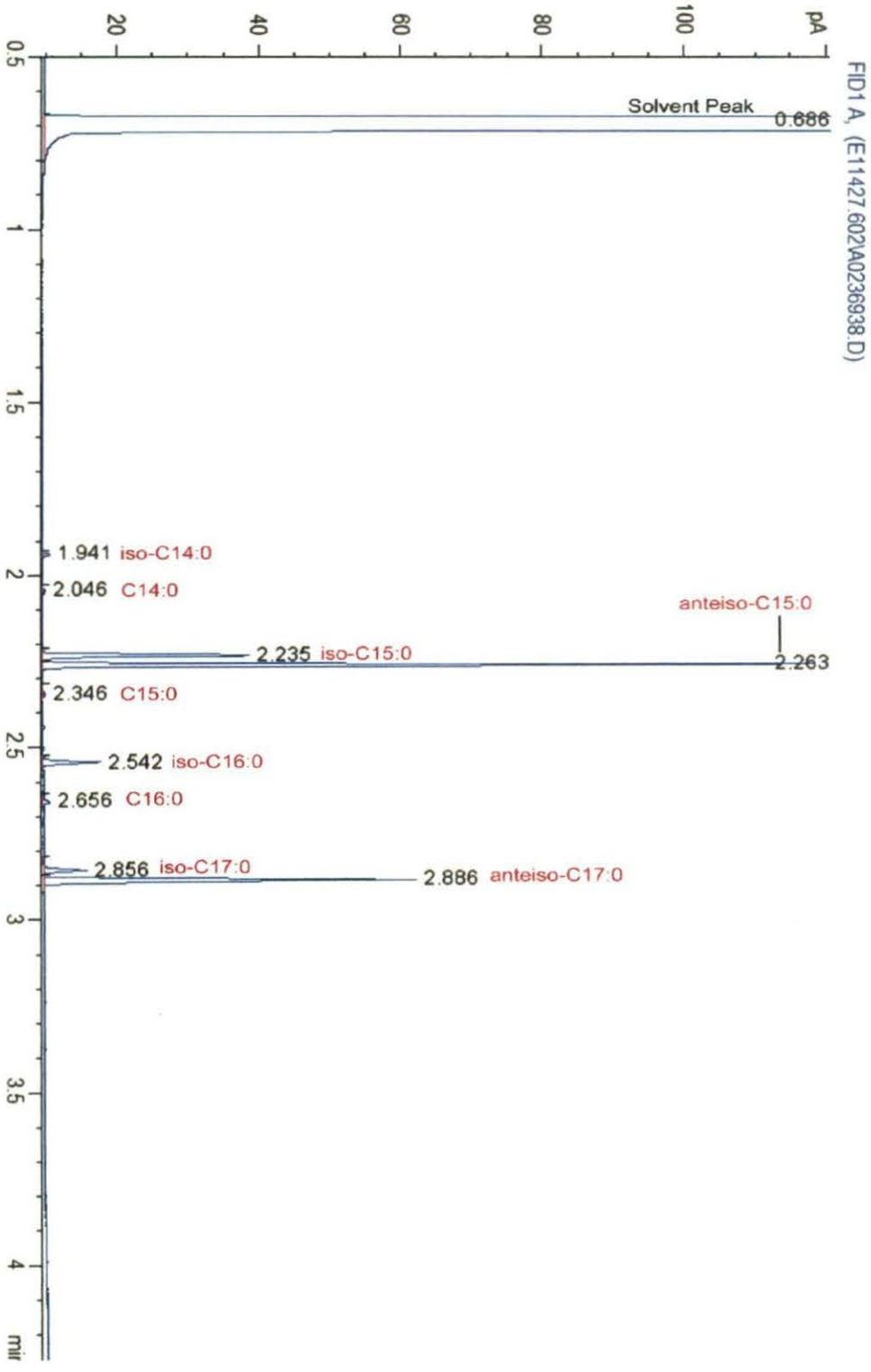


Fig. 3.31: Fatty acid composition analysis of *Brevibacterium siliguriense*. Fatty acid methyl esters (FAMEs) were analyzed by gas chromatography with flame ionization detection. The analyzed fatty acids corresponding to the FAME peaks are indicated with red text.

Table 3.1: Phenotypic characteristics exhibited by class 1 integron bearing facultatively oligotrophic bacteria.

Tests	Isolates									
	OB05	OB12	MB03	MB05	MB08	MB09	MB16	MB18	MB19	MB20
Indole	-	-	-	+	-	-	-	-	-	+
MR	-	-	-	+	-	-	-	-	-	+
VP	-	-	-	-	-	-	-	+	+	-
Citrate	-	+	+	-	+	-	-	+	+	+
Catalase	+	-	+	+	-	-	+	+	+	+
Casienase	-	+	-	-	+	-	-	-	-	-
Gelatinase	-	-	-	-	-	+	-	+	-	+
Amylase	-	-	-	-	+	-	-	-	-	-
H2S	-	+	-	-	+	-	-	-	-	-
Oxidase	+	+	-	-	+	+	+	-	-	-
Acid from										
Dextrose	-	-	-	-	-	-	-	-	+	+
Dulicitol	-	-	-	+	-	-	-	-	-	-
Adonitol	-	+	-	-	+	-	-	-	-	+
Cellobiose	-	-	-	-	-	-	-	-	+	-
Melibiose	-	-	-	-	-	-	-	-	+	+
Mannose	-	+	-	-	+	-	-	+	+	-
Trehalose	-	-	-	-	-	-	-	-	+	+
Maltose	-	-	-	-	-	-	-	-	+	+
Sorbitol	-	+	-	-	+	-	-	-	+	+

Table 3.1: continue.....

Tests	Isolates									
	MB21	MB22	MB23	MB24	MB25	MB26	MB27	MB28	MB29	MB30
Indole	-	-	-	+	+	-	+	+	-	-
MR	+	-	+	+	+	-	+	+	-	-
VP	-	-	-	-	-	+	-	-	+	-
Citrate	+	+	+	-	-	+	-	-	+	+
Catalase	+	+	+	+	-	+	+	+	+	+
Casienase	+	-	+	-	-	-	-	-	-	-
Gelatinase	+	+	-	-	-	-	+	-	-	+
Amylase	+	-	-	-	-	-	-	-	-	-
H2S	-	-	-	-	-	-	-	-	-	+
Oxidase	+	-	-	-	-	-	-	-	-	-
Acid from										
Dextrose	+	-	+	-	+	+	+	+	+	+
Dulicitol	-	-	+	+	-	-	+	-	-	-
Adonitol	-	-	+	-	-	-	-	-	-	+
Cellobiose	-	-	+	-	-	+	-	-	+	+
Melibiose	-	-	+	-	+	+	+	+	+	+
Mannose	-	-	+	-	+	+	+	+	+	+
Trehalose	+	-	+	-	+	+	+	+	+	+
Maltose	+	-	+	-	+	+	+	+	+	+
Sorbitol	-	-	+	-	+	+	+	+	+	-

Table 3.1: continue.....

Tests	Isolates									
	MB31	MB32	MB33	MB34	MB35	MB36	MB37	MB38	MB39	MB40
Indole	+	+	+	-	-	+	+	+	-	-
MR	+	+	+	-	+	+	+	+	+	-
VP	-	-	-	-	-	-	-	-	-	+
Citrate	-	-	-	+	+	-	-	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Casienase	-	-	-	-	-	-	-	-	+	-
Gelatinase	+	+	+	+	-	+	+	+	+	-
Amylase	-	-	-	-	+	-	-	-	+	+
H2S	-	-	-	+	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	+	-
Acid from										
Dextrose	+	+	+	+	+	+	+	+	+	+
Dulicitol	+	+	+	-	+	+	+	-	-	-
Adonitol	-	-	-	+	+	-	-	+	-	+
Cellobiose	-	-	-	+	+	-	-	-	-	+
Melibiose	+	+	+	+	+	+	+	+	-	+
Mannose	+	+	+	+	+	+	+	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	-	+	+	+	+	-	-

Table 3.1: continue.....

Tests	Isolates									
	MB41	MB42	MB43	MB44	MB45	MB46	MB47	MB48	MB49	MB50
Indole	+	-	+	-	-	-	-	+	-	-
MR	+	-	+	+	-	-	-	+	-	-
VP	-	+	-	-	+	-	-	-	+	-
Citrate	-	+	+	-	+	+	+	-	+	-
Catalase	+	+	+	+	+	+	+	+	+	+
Casienase	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	+	-	-	-	+	-	-	-
Amylase	-	-	-	-	-	-	-	-	-	-
H2S	-	-	-	-	-	-	+	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	+
Acid from										
Dextrose	+	+	+	+	+	-	+	+	+	-
Dulicitol	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	+	-	-	-	+	-	-	-
Cellobiose	-	+	-	-	+	-	+	-	+	-
Melibiose	+	+	+	+	+	-	+	+	+	-
Mannose	-	+	-	+	+	-	+	+	+	-
Trehalose	+	+	+	+	+	-	+	+	+	-
Maltose	+	+	+	-	+	-	+	+	+	-
Sorbitol	+	+	+	+	+	-	-	+	+	-

Table 3.1: continue.....

Tests	Isolates									
	MB51	MB52	MB53	MB54	MB55	MB56	MB57	MB58	MB59	MB60
Indole	-	-	-	+	-	-	-	-	-	+
MR	-	-	+	+	-	-	-	-	-	+
VP	+	-	-	-	-	-	-	-	+	-
Citrate	+	+	+	-	+	-	+	-	+	-
Catalase	+	+	+	+	+	+	+	+	+	+
Casienase	-	-	+	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	+	-	-	+
Amylase	-	-	-	-	-	-	-	-	+	-
H2S	-	-	-	-	-	-	+	-	-	-
Oxidase	-	-	-	-	-	+	-	+	-	-
Acid from										
Dextrose	+	-	+	+	-	-	+	-	+	+
Dulicitol	-	-	+	-	-	-	-	-	-	+
Adonitol	-	-	+	-	-	-	+	-	+	-
Cellobiose	+	-	+	-	-	-	+	-	+	-
Melibiose	+	-	+	+	-	-	+	-	+	+
Mannose	+	-	+	+	-	-	+	-	-	+
Trehalose	+	-	+	+	-	-	+	-	+	+
Maltose	+	-	+	+	-	-	+	-	+	+
Sorbitol	+	-	+	+	-	-	-	-	-	+

Table 3.1: continue.....

Tests	Isolates									
	MB61	MB62	MB63	MB64	MB65	MB66	MB67	MB68	MB69	MB70
Indole	-	-	-	+	+	+	+	+	+	-
MR	+	-	-	+	+	+	+	+	+	-
VP	-	-	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	-	+	+	-	-	-
Catalase	+	-	+	+	+	+	+	+	+	+
Casienase	-	+	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	+	+	-	+	+	+	-
Amylase	+	+	-	-	-	-	-	-	-	-
H2S	+	+	-	-	-	-	-	-	-	-
Oxidase	-	+	-	-	-	-	-	-	-	+
Acid from										
Dextrose	+	-	-	+	+	+	+	+	+	-
Dulicitol	+	-	-	-	+	+	-	+	+	-
Adonitol	+	+	-	+	-	-	+	-	-	-
Cellobiose	+	-	-	-	-	+	-	-	-	-
Melibiose	+	-	-	+	+	-	+	+	+	-
Mannose	+	+	-	-	+	-	-	+	+	-
Trehalose	+	-	-	+	+	+	+	+	+	-
Maltose	+	-	-	+	+	+	+	+	+	-
Sorbitol	+	+	-	+	+	-	+	+	+	-

Table 3.1: continue.....

Tests	Isolates									
	MB71	MB72	MB73	MB74	MB75	MB76	MB77	MB78	MB79	MB80
Indole	-	-	-	+	+	+	-	-	+	-
MR	-	-	-	+	+	+	+	+	+	-
VP	-	+	+	-	-	-	-	-	-	-
Citrate	-	+	+	+	+	+	+	+	-	+
Catalase	+	+	+	+	+	+	+	+	+	+
Casienase	-	-	-	-	-	-	+	+	-	-
Gelatinase	-	-	-	+	+	+	+	+	+	-
Amylase	-	-	+	-	-	-	+	+	-	-
H2S	-	-	-	-	-	-	-	-	-	-
Oxidase	+	-	-	-	-	-	+	+	-	-
Acid from										
Dextrose	-	+	+	+	+	+	+	+	+	-
Dulicitol	-	-	-	-	-	-	-	-	+	-
Adonitol	-	-	+	+	+	+	-	-	-	-
Cellobiose	-	+	+	-	-	-	-	-	-	-
Melibiose	-	+	+	+	+	+	-	-	+	-
Mannose	-	+	-	-	-	-	+	+	+	-
Trehalose	-	+	+	+	+	+	+	+	+	-
Maltose	-	+	+	+	+	+	+	+	+	-
Sorbitol	-	+	-	+	+	+	-	-	+	-

Table 3.1: continue.....

Tests	Isolates									
	MB81	MB82	MB83	MR01	MR02	MR03	MR04	SR19	NV66	OD05
Indole	+	+	+	-	-	-	-	-	-	-
MR	+	+	+	-	-	+	+	-	+	+
VP	-	-	-	+	-	-	-	+	-	-
Citrate	-	+	-	+	-	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Casienase	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	+	-	-	-	-	-	-	-	-
Amylase	-	-	-	+	-	+	-	+	-	+
H2S	-	-	-	-	-	+	-	-	-	+
Oxidase	-	-	-	-	+	-	-	-	-	-
Acid from										
Dextrose	+	+	+	+	-	+	+	+	+	+
Dulicitol	-	-	-	-	-	+	-	-	-	+
Adonitol	-	+	-	+	-	+	+	+	+	+
Cellobiose	-	-	-	+	-	+	-	+	-	+
Melibiose	+	+	+	+	-	+	+	+	+	+
Mannose	-	-	-	-	-	+	-	-	-	+
Trehalose	+	+	+	+	-	+	+	+	+	+
Maltose	+	+	-	+	-	+	+	+	+	+
Sorbitol	+	+	+	-	-	+	+	-	+	+

Table 3.1: continue.....

Tests	Isolates								
	OD08	OD10	OC16	OC24	OC74	OC75	OC78	OD21	OD24
Indole	-	-	-	-	-	+	-	-	-
MR	+	+	+	+	-	+	-	-	+
VP	-	-	-	-	-	-	-	+	-
Citrate	+	+	+	+	+	-	+	+	+
Catalase	+	+	+	+	-	+	-	+	+
Casienase	-	-	-	-	+	-	+	-	-
Gelatinase	-	-	-	-	-	+	-	-	-
Amylase	+	+	+	+	+	-	+	+	+
H2S	-	+	-	+	+	-	+	-	+
Oxidase	-	-	-	-	+	-	+	-	-
Acid from									
Dextrose	+	+	+	+	-	+	-	+	+
Dulicitol	+	+	+	+	-	+	-	-	+
Adonitol	+	+	+	+	+	-	+	+	+
Cellobiose	+	+	+	+	-	-	-	+	+
Melibiose	+	+	+	+	-	+	-	+	+
Mannose	+	+	+	+	+	+	+	-	+
Trehalose	+	+	+	+	-	+	-	+	+
Maltose	+	+	+	+	-	+	-	+	+
Sorbitol	+	+	+	+	+	+	+	-	+

Table 3.2: Putatively assigned genera (on the basis of partial 16S rRNA gene sequences) of integron carrying isolates

Sl. No	Isolate	Genus	Ac. No.	Sl. No	Isolate	Genus	Ac. No.
1	[a] OB 05	*Acidovorax* sp.	FM865443	46	MB 57		-f-
2	OB 12	*Pseudomonas* sp.	FM865447	47	MB 58		-a-
3	[b] MB 03	*Acinetobacter* sp.	FM865448	48	MB 59		-e-
4	[c] MB 05	*Shigella* sp.	FM865446	49	MB 60		-o-
5	[r] MB 08	*Pseudomonas* sp.	FM865444	50	[k] MB 61	*Citrobacter* sp.	HF562228
6	MB 09	*Comamonas* sp.	FM865445	51	MB 62		-r-
7	MB 16		-a-	52	MB 63		-b-
8	MB 19		-d-	53	MB 64		-g-
9	MB 20		-g-	54	MB 65		-o-
10	[p] MB 21	*Aeromonas* sp.	FR677018	55	MB 66	*Kluyvera* sp.	HF562221
11	MB 22	*Acinetobacter* sp.	FR677019	56	MB 67		-g-
12	MB 23		-i-	57	MB 68		-o-
13	MB 24		-c-	58	MB 69		-o-
14	MB 25		-l-	59	MB 70		-a-
15	MB 26		-d-	60	MB 71		-a-
16	[o] MB 27	*Escherichia* sp.	FN396607	61	MB 72		-d-
17	[m] MB 28	Un. B	HF562231	62	MB 73		-e-
18	MB 29		-d-	63	MB 74		-g-
19	MB 30		-f-	64	MB 75		-g-
20	MB 31		-o-	65	MB 76		-g-
21	MB 32		-o-	66	MB 77		-q-
22	MB 33		-o-	67	[q] MB 78	*Aeromonas* sp.	HF562229
23	[f] MB 34B	*Proteus* sp.	HF562223	68	MB 79		-o-
24	[j] MB 35	*Citrobacter* sp.	HF562227	69	MB 80		-b-
25	MB 36		-o-	70	[n] MB 81	Un. B	HF562232
26	MB 37A	*Escherichia* sp.	FN396608	71	MB 82		-g-
27	[g] MB 38	*Salmonella* sp.	HF562224	72	MB 83	Un. B	HF562233
28	MB 39		-p-	73	MR 01		-e-
29	[e] MB 40	*Enterobacter* sp.	HF562222	74	MR 02		-a-
30	MB 41		-n-	75	MR 03		-k-
31	[d] MB 42	*Klebsiella* sp.	FR677020	76	[h] MR 04	*Providencia* sp.	HF562225
32	MB 43		-g-	77	SR 19		-e-
33	MB 44	Un. B	HF562230	78	NV 66		-h-
34	MB 45	*K. pneumoniae*	FR677021	79	OD 05		-k-
35	MB 46		-b-	80	OD 08		-k-
36	MB 47		-f-	81	OD 10		-j-
37	MB 48		-m-	82	OC 16		-k-
38	[d] MB 49	*Klebsiella* sp.	FM865635	83	OC 24		-j-
39	MB 50		-a-	84	OC 74		-r-
40	MB 51		-d-	85	OC 75		-o-
41	MB 52	*A. johnsonii*	FN263374	86	OC 78		-r-
42	[i] MB 53	*Serratia* sp.	HF562226	87	OD 21		-e-
43	MB 54		-m-	88	OD 24		-k-
44	MB 55		-b-	89	MB 12	Un. B	AM937246
45	MB 56		-a-	90	MB 18	*B. siliguriense*	AM937247

Un.B, Unclassified bacterium;

Isolates exhibiting similar phenotype: a, *Acidovorax* sp.; b, *Acinetobacter* sp.; c, *Shigella* sp.; d, *Klebsiella* sp.; e, *Enterobacter* sp.; f, *Proteus* sp.; g, *Salmonella* sp.; h, *Providencia* sp.; i, *Serratia* sp.; j and k, *Citrobacter*.; l-n, Unknown bacterium; o, *Escherichia* sp.; p and q, *Aeromonas* sp.; r, *Pseudomonas* sp.; *A. johnsonii, Acinetobacter johnsonii; B. siliguriense, Brevibacterium siliguirense; K. pneumoniae, Klebsiella pneumoniae*; rDNA, ribosomal deoxyribonucleic acid.

Table 3.4A: Detailed phenotypic characteristics of *Brevibacterium siliguriense* sp. nov strain MB18^T. Symbols: +, positive; –, negative; W, weak reaction; R, resistant; S, sensitive

Characteristics	MB18^T	Characteristics	MB18^T
NaCl tolerance (% w/v)	15	Citrate utilization	+
Oxidase	–	Utilization of D–arabinose	–
Hydrolysis of Esculin	–	Utilization of L–arabinose	–
Hydrolysis of Gelatin	+	Utilization of Sodium gluconate	–
Phenylalanine deamination	–	**Acid from:**	
Haemolysis	–	2,3 butylene glycol	–
H_2S production	–	D–Arabinose	–
Voges proskaurs	+	D–mannitol	+
Methyl Red	–	D–Xylose	–
Indole	–	D–galactose	–
Amylase	–	D–ribose	–
Lipase production	–	Lactose	–
α–Glucosidase	–	D–Maltose	–
Pyrrolidone peptidase	–	D–Sorbitol	–
Pyrazinamidase	+	D–mannose	+
Phosphatidyl-inositol-Phospholipase	–	D–Raffinose	–
Arginine Dihydrolase1	+	D–Trehalose	–
Arginine Dihydrolase 2	–	Saccharose/Sucrose	–
β–Galactosidase	+	Fructose	w
α–galactosidase	+	Dextrose	w
L–aspartate arylamidase	–	Melibiose	–
β galactopyranosidase	+	L–Arabinose	–
α–mannosidase	–	Inulin	–
Phosphatase	–	Glycerol	+
Leucine arylamidase	+	Salicin	–
L–proline arylamidase	+	Dulicitol	–
β –glucuronidase	–	Inositol	–
Ala–Phe–Pro Arylamidase	–	Xylitol	–
β –glucuronidase	+	Adonitol	–
Alanine arylamidase	+	Melezitose	–
Tyrosine arylamidase	+	Sorbose	–
D–amygdalin	–	Rhamnose	–
L–Lactate alkalization	–	DNA G+C content (mol%)	64.6
N–Acetyl–D–Glucosamine	–	**Antibiotic susceptibility test:**	
Methyl– β –D–Glucopyronside	–	Bacitracin Resistance (0.0006 mg)	R
α–methyl–D–mannoside	–	Novobiocin Reistance (0.000075 mg)	S
α–methyl–D–glucoside	w	O/129 Resistance (0.0084 mg)	S
Glucosamine	–	Optochin (0.000399 mg)	R
Pullulan	–	Ampicillin (<S/R≥, 25 mg/L)	R
Salicin	–	Cefepime (<S/R≥, 7.5 mg/L)	R
ONPG	–	Chloramphenicol (<S/R≥, 30 mg/L)	R
Lysine utilization	–	Co-trimoxazole (<S/R≥, 15 mg/L)	R
Ornithine utilization	+	Netilmicin (<S/R≥, 3.75 mg/L)	R

Table 3.4B: Detailed phenotypic characteristics of *Brevibacterium siliguriense* sp. nov. strain MB18^T. Symbols: +, positive; –, negative; W, weak reaction; V, variable reaction

Characteristics	MB18^T	Characteristics	MB18^T	Characteristics	MB18^T
α-Cyclodextrin	–	β-Methyl-D-Galactosidee	–	Succinic Acid	–
β-Cyclodextrin	–	3-Methyl Glucose	–	N-Acetyl-L-Glutamic Acid	–
Dextrin	w	β-Methyl-D-Glucoside	–	L-Alaninamide	–
Glycogen	–	α-Methyl-D-Mannoside	–	D-Alanine	–
Mannan	+	Palatinose	–	L-Alanine	–
Tween 40	+	D-Psicose	–	L-Alanyl Glycine	–
Tween 80	–	L-Rhamnose	–	L-Asparagine	–
N-Acetyl-D-Mannosamine	–	Stachyose	–	L-Glutamic Acid	–
N-Acetyl-β-D-Mannosamine	–	D-Tagatose	–	Glycyl-L-Glutamic Acid	–
Amygladin	–	Turanose	–	L-Pyroglutamic Acid	–
D-Arabitol	–	Acetic Acid	+	L-Serine	v
Arbutin	–	α-Hydroxybutyric Acid	–	Putrescine	w
D-Cellobiose	–	β-Hydroxybutyric Acid	–	2,3 butandiol	–
D-Fructose	–	γ-Hydroxybutyric Acid	–	Adenosine	–
L-Fucose	–	p-Hydroxy-Phenylacetic Acid	–	2'-Deoxy Adenosine	–
D-Galactose	–	α-Ketoglutaric Acid	–	Inosine	–
D-Galacturonic Acid	–	α-Ketovaleric Acid	–	Thymidine	–
Gentibiose	–	Lactamide	–	Uridine	–
D-Gluconic Acid	–	D-Lactic Acid Methyl Ester	–	Adenosine-5'-Momonophosphate	–
α-D-Glucose	–	L-Lactic Acid	–	Thymidine-5'-Momonophosphate	–
m-Inositol	–	D-Malic Acid	–	Uridine-5'-Momonophosphate	–
α-D-Lactose	–	L-Malic Acid	–	D-Fructose-6-Phosphate	–
Lactulose	–	Pyruvatic Acid Methyl Ester	+	α-D-Glucose-1-Phosphate	–
Maltotriose	–	Succinic Acid Mono-methyl Ester	+	D-Glucose-6-Phosphate	w
D-Melezitose	–	Propionic Acid	+	D-L-α-Glycerol Phosphate	–
D-Melebiose	–	Pyruvic Acid	+		
α-Methyl-D-Galactoside	–	Succinamic Acid	–		

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Chapter 4

Characterization of class 1 integron associated novel Trimethoprim resistance gene, dfrA28, from Acinetobacter spp. MB52

4.1 Background

The genus *Acinetobacter* comprises aerobic, non-motile, oxidase-negative, catalase-positive, gram-negative, non-fermenting cocci or cocco-bacilli that grow well on common complex media (Bouvet and Grimont, 1986). *Acinetobacter* strains are present ubiquitously in nature and have been isolated from diverse habitats like, cotton, soil, water, sewage, plants, animals, humans, insects and activated sludge (Baumann *et al.*, 1968; Carter *et al.*, 1970; Warskow and Juni, 1972; Henriksen, 1976; Bouvet and Grimont, 1986; Nishimura *et al.*, 1988; Nemec *et al.*, 2001; Carr *et al.*, 2003; Hwa *et al.*, 2009; Lee *et al.*, 2009; Rani *et al.*, 2009). It has been estimated that approximately 0.001% of the total culturable heterotrophic aerobic population of soil and water is constituted of *Acinetobacter* (Baumann, 1968). Yet, majority of the strains of described species have been isolated from clinical specimens (Carr *et al.*, 2003). An earlier study to assess the natural habitats of the different members of the genus, by studying the colonization with *Acinetobacter* species of skins of hospitalized patients and healthy volunteers, has putatively identified human skin as one of the natural habitats of *A. lwoffii*, *A. johnsonii*, *Acinetobacter* DNA group 3, and probably *A. radioresistens* (Seifert *et al.*, 1997). Interestingly, the most important species associated with hospital infection and epidemic outbreaks, *A. baumannii*, was found rarely on human skin (Seifert *et al.*, 1997). Another species, *A. ursingii*, isolated from seriously ill hospitalized patients were recovered from patients with diagnosed bacteremia or septicemia, while strains of *A. schindleri* were isolated from non-sterile body sites of outpatients (Nemec *et al.*, 2001). *Acinetobacter* strains are capable of surviving in extremely nutritionally deficient (oligotrophic) abiotic environments of hospitals for a considerable period, for example, up to 10 days on dust and dry particles (Webster *et al.*, 2000) or for more than 4 months on both moist and dry surfaces such as polyvinyl chloride, rubber, ceramics, and various types of medical equipments (Wendt *et al.*, 1997) and they are also an important cause of infection in immunocompromised patients. The nosocomial strain, *A. baumannii* exhibits a remarkable ability to rapidly develop antibiotic resistance to several classes of antimicrobial agents that led to multidrug resistant Acinetobacter (MDRA) (Bergogne-Berezin and Towner, 1996) or to almost all currently available antibacterial agents (Van Looveren and Goossens, 2004). Genomics of MDR strain, *A. baumannii* AYE has revealed an existence of an 86-kb genomic resistance island where 45 resistance genes were found to be clustered (Fournier *et al.*, 2006). Sequence similarity and phylogenetic analyses confirmed that most of the resistance genes found in AYE have been acquired relatively recently from bacteria of the genus *Pseudomonas*, *Salmonella*, or *Escherichia coli* (Fournier *et al.*, 2006).

Conjugation, amongst the three gene transfer modes, plays the significant role in the transfer of resistance genes in *Acinetobacter* (Towner and Vivian, 1977). *Acinetobacter* develops resistance through acquisition of plasmids (Seifert *et al.*, 1994), transposons (Devaud *et al.*, 1982) or integrons (Segal *et al.*, 2003). Several classes of integrons have been reported till date, of which the most documented and well characterized are the class 1 integrons. The following are the three different segments involved: (i) 5′ conserved segment (5′ CS) containing an *int*I gene that codes for integrase and an *att*I recombination site, (ii) the 3′ conserved segment (3′ CS) containing a combination of *qac*E (antiseptic-resistance gene), *sul*I (sulfonamide resistance gene) and one or two additional

ORFs, ORF5/and or ORF6 that are hypothesized ISCR1 element for development of complex class 1 integrons (Toleman *et al.*, 2006), and (iii) a variable region between 5′ CS and 3′ CS that constitutes a class 1 integron (Hall and Collis, 1998). Very recently, class 1 integrons have been detected and characterized among carbapenem-resistant isolates of *Acinetobacter* spp. (Hwa *et al.*, 2009).

This chapter describes a novel class 1 integron-borne *dfrA28* gene, conferring resistance to trimethoprim. In addition to gene description, the chapter also dealt in details about the oligotrophic characteristics along with biochemical and molecular phylogeny of MB52 (*dfrA28* harboring strain/host).

4.2. Materials and methods

4.2.1. Sampling, isolation, and identification of the oligotrophic strain: previously described in chapter 1.

4.2.2. Antibiotic resistance determination: Antibiotic susceptibility and resistance determination was performed according to the method described in chapter 1. The bacterium MB52, was identified as MAR strain, and could resist trimethoprim 30 mg/L. The control organism (*E. coli*) was susceptible to trimethoprim at 5 mg/L.

4.2.3. Phenotypic tests of facultatively oligotrophic test strain MB52

Gram-staining, motility, and growth at different pH and aerobic/anaerobic behaviour were analyzed according to the standard procedures. Growths at different temperatures were observed in nutrient rich broth (NRB) [composition: peptone, 10 g/L; yeast extract, 5 g/L; sodium chloride, 5 g/L; pH 7.0], nutrient poor broth (NPB) [composition: peptone, 10 mg/L; yeast extract, 5 mg/L; sodium chloride 5 mg/L; pH 7.0], R2A broth, nutrient broth (NB) and tryptone soya broth (TSB) at temperatures: 4, 10, 30, 37, and 42 °C. All phenotypic tests were performed in tryptone soya broth/agar (TSB/A, HiMedia, Mumbai, India). Tests were carried out at 30 °C unless otherwise specified. The cell morphology and motility were examined under Phase contrast microscope (Olympus, Japan). To distinguish aerobic-anaerobic nature of MB52, the culture was grown in thioglycollate media (HiMedia, Mumbai, India) at 30 °C for 24 h. Gelatin hydrolysis and haemolysis were performed as described previously (Bouvet and Grimont, 1986). *Staphylococcus aureus* was taken as positive control in haemolysis test. A 1% (v/v) filter sterilized ethanol was amended to 3ml XfD media (Almeida *et al.*, 2004) and tubes were incubated for 24 to 48 h to detect ethanol as sole carbon source for energy. Catalase activity was tested using 3% H_2O_2. Urea hydrolysis, citrate utilization (Simmons citrate), indole production, oxidase activity and carbohydrate utilization/fermentation test were performed according manufacturer's instructions (HiMedia, Mumbai, India).

4.2.4. DNA extraction, amplification, cloning and sequencing of 16S rRNA gene: as described in chapter 4

4.2.5. 16S rRNA gene sequence based phylogeny

The 16S rRNA gene sequence of *Acinetobacter johnsonii* MB52 was aligned with 16S rRNA gene sequences of all the known species of *Acinetobacter* retrieved from GenBank using clustal X version1.83 (Thompson *et al.*, 1997). The 16S rRNA gene sequence of *Escherichia coli* was included as outgroup. The resulted multiply aligned sequence was corrected, edited and approximately 1334 base pair long nucleotide sequence of all the *Acinetobacter* were selected for further analysis. Rest nucleotide sequence from both the end was omitted due to alignment ambiguities. The multiple alignment file was transported to MEGA4 package and tree was constructed. Trees topology was inferred by neighbor-joining (NJ) (Saitou *et al.*, 1987). The topology of the phylogenetic tree was evaluated using a bootstrap analysis (Felsenstein, 1985) based on 1,000 replications.

4.2.6. Oligotrophic characteristics of the strain MB52

To validate oligotrophic behaviour, the growth was assessed in NPB. Inoculum was prepared by transferring a single colony of 24 h old culture into 100 ml Erlenmeyer flask containing 10 ml sterile NRB (pH 7.0) followed by incubation at 30 °C for 20 h without agitation. The culture was harvested by centrifuging at 7000 rpm for 7 min at 4 °C and washed twice with sterile saline (0.5% NaCl) water to ensure removal of traces of media. The washed bacterial cells were finally suspended in 3 ml sterile NPB. Aliquots of 0.5ml of concentrated (1.75×10^9 to 2.25×10^9cells/ml) cell suspension(s) were transferred to 250 ml Erlenmeyer flask(s) containing 25 ml sterile NPB. The flasks were kept at 30 °C (without agitation) throughout the period of investigation. Survivability of MB52 cells in NPB medium was assessed through dilution-plating of pure culture aliquots at different time intervals on fresh NRA medium.

5.2.7. DNA extraction, detection of class 1 integron, cloning and sequencing of variable region:

as described in chapter 2.

4.2.8. Sequence analysis of class 1 integron borne gene cassette and phylogeny of novel DfrA protein

The arrangement of obtained sequenced oligonucleotide, orientation, and trimming of vector sequence was done as described in chapter 3. Sequence quality was checked through the developed electropherogram. Nucleotide sequence analysis was performed using BLAST (Altschul *et al.*, 1990) search programs [National Center for Biotechnology Information (NCBI)]. Open reading frames were identified using ORF finder tool (www.ncbi.nlm.nih.gov/) and corrected manually. A phylogenetic analysis of novel DfrA protein (translated *dfrA* gene) was performed by making a phylogenetic tree based on one representative microorganism for each trimethoprim-resistance-mediating DfrA protein. The multiple alignment was done using program ClustalX (version 1.83) (Thompson *et al.*, 1997); an NJ tree was made based on this alignment, which was used as a guide tree for the final alignment used to produce the final bootstrap NJ tree.

4.3. Results

4.3.1. Phenotypic characterization and 16S rRNA phylogeny

MB52 cells were strictly aerobic, non-fermentative, facultatively oligotrophic, gram-negative, non-motile, coccobacilli. The colonies were circular, convex, smooth and slightly opaque on tryptone soya agar; colony diameter ranged between 0.5 to 1.0 mm after 23h, 1.5-2.0 mm after 40 h and 2.0 to 3.0 mm after 60 h of incubation at 30 °C. The cells could grow in a temperature range of 10 – 30 °C within

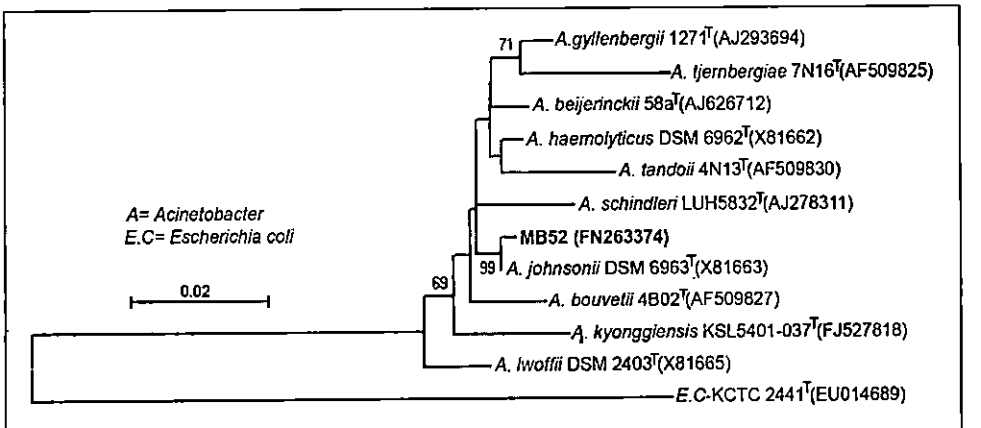

Fig. 4.1: Fig. 3. : Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB52 (Bold face) within the members of genus *Acinetobacter*. *E. coli* KCTC 2441^T (EU014689) used as an outgroup. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 2% sequence divergence. For extended NJ tree please see Chapter-3, Fig. 3.19.

24 h in UDTSB medium (optimum temperature 28 -30°C) but failed to grow at 37°C. The cells were positive to catalase, citrate (Simmons) utilization and negative to oxidase, gelatinase, urease, and hemolysis. Cells failed to reduce nitrates to nitrites and did not produce indole, γ-glutamyltransferase, and β-xylosidase. It could utilize L-arginine, L-aspartate, D-L-lactate, ethanol, malonate but could not trans-aconitate, β-alanine, DL-4-aminobutyrate, azelate, 2,3-butanediol, glutarate, histamine, L-histidine, malonate, L-leucine, D-malate, phenylacetate, and L-tyrosine. The phenotypic features of MB52 showed a high degree of similarity (~100%) with the published characteristics of *Acinetobacter johnsonii* ATCC 17909^T (Bouvet and Grimont, 1986). MB52 was found sensitive to all antibiotics used in study and recommended by EUCAST for *Acinetobacter* except cotrimoxazole [MIC, 20mg/L in correspondence to EUCAST MIC breakpoint (S≤/>R is 2/4mg/L, where S is sensitive and R is resistance)] and streptomycin [MIC, 10mg/L in correspondence to the breakpoint evaluated in this study (S</≥R is 2.5 mg/L). Sequence homology of the 16S rRNA gene sequence of the isolate MB52 produced maximum identity (99%) with the 16S rRNA gene sequence of *Acinetobacter johnsonii* ATCC 17909^T (Ac. No. Z93440). The isolate MB52 branched deeply with the *Acinetobacter johnsonii* DSM 6963^T (X81663) in the NJ phylogenetic tree constructed with 16S rRNA sequences of *Acinetobacter* spp. (Fig. 4.1).

4.3.2. Oligotrophic nature of MB52

MB52 cells were able to grow in DLB without supplementation of any other carbon or nitrogen source or growth factor (Fig. 4.2). An increase of 2.5 times the initial cell number was noted in span of 8 days. The ability of strain MB52 to survive (without reduction in viable cell number since inoculation) and multiply in a low nutrient medium explains the oligotrophic nature of the strain.

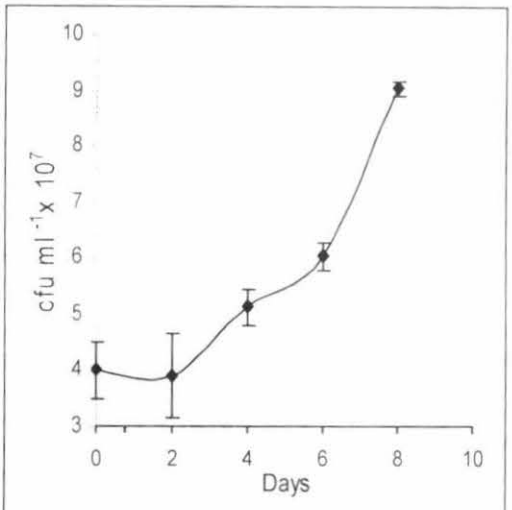

Fig. 4.2: Viability and growth of MB52 in NPB medium. Error bars show the standard errors.

4.3.3. Detection of class 1 integron borne gene cassette and characterization of novel *dfr*A 28 gene

An amplicon of 1694 bp was obtained from strain MB52 by CS-PCR. Purified CS-PCR product of MB52 was cloned in a pGEM-T easy vector was subjected to DNA sequencing and gene cassettes were identified by sequence analysis. Two consecutive gene cassettes were identified carrying one novel dihydrofolate reductase gene (*dfr*A28) conferring resistance to trimethoprim and a gene coding for aminoglycoside adenyl transferase (*aad*A1) which confer resistance to streptomycin/spectinomycin (Fig. 4.3). Both of the gene cassettes were inserted in variable region between 5′ and 3′ conserved segments of a class 1 integron and were flanked by recombination core sites. Cassettes encoded functions and antibiotic resistance phenotypes exhibited by bacterium MB52 were found significantly associated to each other.

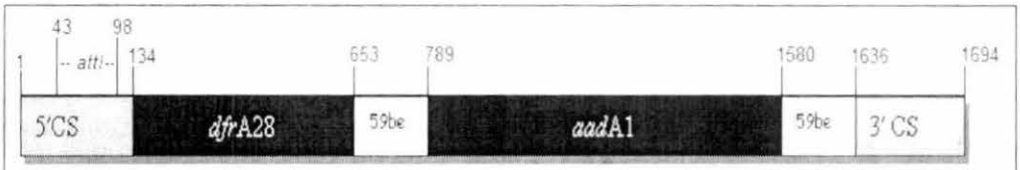

Fig. 4.3: Schematic presentation of the CS-PCR product of *A. johnsonii* MB52. Symbols: CS, conserved segment; *attI*1, attachment site; *dfr*, dihydrofolate reductase; *aad*, aminoglycoside adenyl transferase; be, base element. Numbers above the integron structure correspond to sequence positions in EMBL Ac. No. FN263373.

Gene description: The novel gene, *dfr*A28, was flanked by both a core element (GTTAACC) located 29 nucleotides 5′ to the start codon of the reading frame and differed from the consensus sequence of the core element (GTTRRRY) by a single nucleotide. An ORF of 519 bp (potentially encoding a polypeptide of 172 amino acids long) was identified at the 35 nucleotide downstream to the *attI*1 (attachment site of class 1 integron genetic element present over 5′ CS). The ORF began with an ATG start codon at positions 134 to 136 and terminated with a TAA stop codon at positions 650 to 652. The start codon was preceded by a plausible SD sequence (TGAGGAAGA). The translated polypeptide sequence was compared with other amino acid sequences of the SwissProt data base. Results revealed that the obtained sequences with the best scores were all Dfr sequences. The maximum identity (76.4%) at amino acid level derived from novel *dfr* gene sequence was found with *dfr*A1 of *E. coli* (Ac. No. AJ419168). The amino acid identities between the DfrA28 (derived from novel *dfr* gene sequence of MB52) and other Dfr(*s*) ranged between 12.9 and 70.8%; thus DfrA1 and DfrA28 cannot be placed in a monophyletic group which was also evident in the phylogenetic tree (Fig. 4.4). A 133 bp long nucleotide sequence immediately 3′ end of *dfr*A28 gene was recognized as 59-base element or *attC* (attachment site of gene cassette) (EMBL nucleotide position 653-785). The PROSITE motif scan revealed that the dihydrofolate reductase signature sequence, VIGngpdIPWsakg.EqllFkaiT, was intact in the novel DfrA28. The novel Dfr, DfrA28 clustered together with DfrA1 of *E coli* and DfrA15 of *Vibrio cholerae* (Ac. No. AB113114) (Fig. 4.3). The second gene cassette contained an ORF of 792 nucleotides starting at an ATG codon and ending with TAA stop codon (EMBL nucleotide positions 789 to 1580). This coding sequence, designated *aad*A1, shared 100% identity with the same gene (Ac. No. DQ388124). The location (based on EMBL nucleotide position) of gene cassettes and other features are presented in Fig. 4.3.

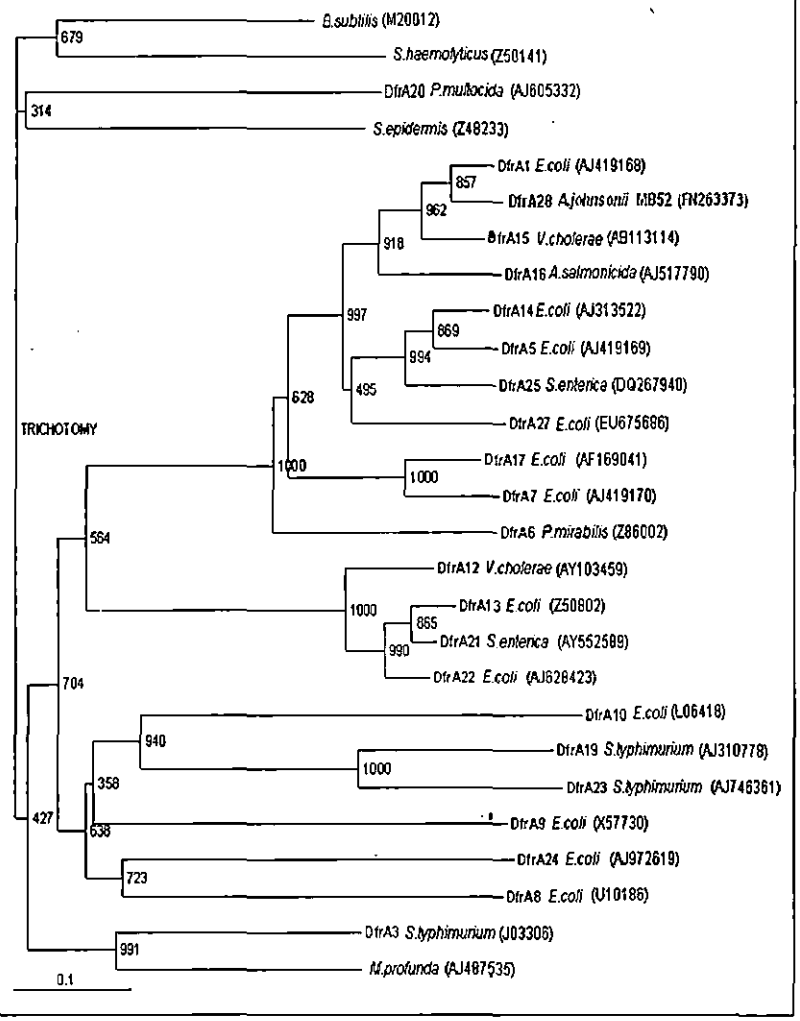

Fig.4.4: Phylogenetic tree of the DfrA proteins involved in trimethoprim resistance. The number at each major branch point refers to the percentage of times that a particular node was found in 1,000 bootstrap replications. Source and the GenBank accession (in parentheses) numbers are given for each DfrA protein. The placement of DfrA28 in the tree is indicated in bold letters. DfrB proteins, which differ distinctly from DfrA proteins in size and structure, have been excluded from this phylogenetic analysis.

4.4. Discussion

The test strain, MB52, of this study is one representative of the antibiotic-resistant population, comprised of only oligotrophic bacteria recovered on NPA medium from Mahananda. Besides water and soil, oligotrophic bacteria have also been isolated from clinical materials (Tada *et al.*, 1995). Earlier studies showed that a number of oligotrophic bacteria exhibit antibiotic resistance and can therefore be a potential reservoir of antibiotic resistance genes that can be acquired by pathogens through diverse gene transfer mechanisms (Tada *et al.*, 1995).

The strain MB52 bearing a novel *dfrA* gene was characterized as *Acinetobacter johnsonii* on the basis of phenotypic study and 16S rRNA phylogeny (Fig. 4.1). All the known bacteria under the genus *Acinetobacter* are strictly aerobic, oxidase-negative and catalase-positive (Bouvet and Grimont, 1986). They constitute a unique group of gram-negative bacteria that may be natural residents of human skin (Larson, 1981), with carriage rates of 42.5% and ~75% in healthy individuals and hospitalized patients respectively (Seifert *et al.*, 1997). Viability assay and growth assessment of *A. johnsonii* MB52 in NPB medium for more than a week by taking viable cell count (grown in nutrient deficient condition) on NRA [composition: peptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L; agar, 15 g/L) plate at harvested at different time intervals (Fig. 4.2) demonstrated that cells were capable to switch from oligotrophy (ability to survive and grow in extremely poor nutrient condition) to copiotrophy (ability to form colonies in rich medium). Such facultative nature of oligotrophy, as shown by the *Acinetobacter johnsonii* strain MB52, may contribute to the reported adaptation of remaining viable on both moist and dry surfaces such as PVC, rubber, ceramics and various types of medical equipment (Wendt *et al.*,1997; Webster *et al.*, 2000) and cause nosocomial infection in immunocompromised patients (Bergogne-Berezin and Towner, 1996). Many studies have demonstrated the presence of antibiotic resistance genes in *Acinetobacter* spp. on plasmids, transposons, and integrons and, on a resistance island (Devaud *et al.*, 1982; Seifert *et al.*, 1994; Ruiz *et al.*, 2003; Fournier *et al.*, 2006). Recently in Malaysia, class 1 integrons were detected in 31 of 39 carbapenem-resistant strains of *Acinetobacter* spp., of which two *A. calcoaceticus* strains harbored an integron-borne bla_{IMP-4} metallo-β-lactamase, 1 of which was located on a 36-kb plasmid (Hwa *et al.*, 2009). The test strain, MB52, harbored a class 1 integron which carried two gene cassettes, *dfr*A28 and *aadA*1 respectively. Several hundred (>300) of different complete cassette arrays flanked by the 5′ CS and 3′ CS have been identified in GenBank and majority arrays had two or three gene cassettes (within the limitation of CS-PCR conditions employed) (Patridge *et al.*, 2009). Most cassette array sequences in class 1 integrons with the 5′ CS and 3′ CS from gram-negative bacteria deposited in GenBank were from *Escherichia coli* (21%), *Pseudomonas aeruginosa* (19%), *Salmonella* spp. (14%), *Acinetobacter baumannii, Klebsiella pneumoniae* or *Vibrio cholerae* (each c. 6%) (Patridge *et al.*, 2009). Cassette array flanked by 5′ CS and 3′ CS had two gene cassettes in the test strain MB52 of this study (Fig. 4.3). To the best of our knowledge, *dfr*A28 gene, conferring resistance to trimethoprim, is the first novel *dfr* gene detected in *A. johnsonii*. Trimethoprim on its own or in combination with sulfamethoxazole is an antibacterial drug for the control of pathogenic gram negative organisms which inhibit the bacterial cell growth by blocking dihydrofolate reductase (DFR) (Huovinen *et al.*, 1995; Skold, 2001) which in turn fails to reduce dihydrofolate into tetrahydrofolate. Production of an additional plasmid, transposon, or cassette-borne Dfr is the most widespread mechanism of resistance to trimethoprim in bacteria which, in contrast to the chromosomal enzyme, is less susceptible to inhibition by trimethoprim (Huovinen *et al.*, 1995). More than 30 different trimethoprim-resistance-mediating dihydrofolate reductase (*dfr*) genes, subdivided into two major types, 1 and 2 (also referred as *dfr*A and *dfr*B), have been observed (White and Rawlinson, 2001). A novel trimethoprim resistance gene is claimed when the translated dihydrofolate reductase protein (Dfr) encoded by the gene have <95% identity at the amino acid level to known Dfr proteins (Skold, 2001). The phylogenetic tree (Fig. 4.4) confirmed DfrA28 to be most closely related to DfrA1. Taking into consideration the trimethoprim resistance phenotype and

the phylogenetic relationship with other Dfr type-A proteins, this gene was predicted as a novel *dfrA*, *dfrA28* gene and naming was done according to the published guidelines (Hall and Patridge, 2003) for new genes conferring resistance to trimethoprim.

4.5. Conclusion

This study demonstrated the discovery of novel *dfrA28* gene conferring resistance to trimethoprim, from a facultatively oligotrophic strain, *Acinetobacter johnsonii* strain MB52.

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ampicillin, gentamycin, netilmicin, tobramycin, chloramphenicol, cefotaxime, kanamycin and streptomycin) including a high level of trimethoprim (1500 mg/L) and was also found to be resistant to human serum. Antibiotic susceptibility specific to *Klebsiella* genus was performed following standards recommended by EUCAST (http://www.escmid.org/); and resistances were determined according to EUCAST breakpoints (those present in EUCAST list) and parameters evaluated in this study. To detect extended tolerance of trimethoprim, agar dilution method using Mueller Hinton (MH) agar as described in EUCAST definitive document, E. Def 3.1 (2000) [http://www.escmid.org/fileadmin/src/media/PDFs] was used. *Escherichia coli* ATCC 25922 was used as quality control. Criteria for susceptibility or resistance followed the EUCAST guidelines (http://www.eucast.org/clinical_breakpoints/). Resistances to the antibiotics, absent in published EUCAST breakpoints table (v 1.1 2010-04-27), were interpreted according to method described in chapter 1.

5.2.3. Phenotypic characterization of MB45

All phenotypic tests were performed in NRA/NRB medium. Tests were carried out at 37 °C unless otherwise specified. Gram-Staining and growth at different pH were analyzed according to the standard procedures. Growths at different temperatures were observed in NRB at 7, 10, 15, 25, 30, 37, and 45±1 °C. The cellular morphology and motility were examined under phase contrast microscope (Olympus, Japan). The aerobic-anaerobic nature of MB45 was discriminated by growing in thioglycollate media (HiMedia, Mumbai, India) at 37 °C for 24 h. Gelatin hydrolysis was performed as described earlier (Bouvet and Grimont, 1986). A 0.1% (v/v) filter sterilized ethanol was amended to 3 mL minimal medium (composition in g/L: 0.5 g $MgSO_4$. $7H_2O$, 0.5 g KH_2PO_4, 0.1 g KCl, 1.2 % $(NH_4)_2SO_4$; pH 6.5±0.2) containing bacterial cells and was incubated for 24 to 48 h to detect ethanol as sole carbon energy source. Catalase and oxidase activities were confirmed by method described earlier (Lee, 2006). β-galactosidase activity was determined using ONPG (onitrophenyl-β-D-galactopyranoside). Hydrolysis of casein, DL-tyrosine and xanthine was examined using the method described by Gordon *et al.* (1974). Indole production, lysine utilization, ornithine utilization, Urease production, deamination of phenyl alanine, reduction of nitrate, H2S production, citrate utilization, MR test, VP test, malonate utilization and carbon source utilization/ fermentation tests were carried out using HiBio-ID/HiCarbo system according to manufacturer's instruction (HiMedia, Mumbai, India). *E. coli* K12 (in some biochemical test) was used as control organism.

5.2.4. Determination of oligotrophic characteristics of MB45

Viability and Growth of MB45 in nutrient-poor-broth (NPB) was studied to test the oligotrophy. Inoculum was prepared according to the method described in chapter 4. Aliquots of 1.0 mL of concentrated (1×10^8 cells/mL) cell suspension(s) were added to 250 mL Erlenmeyer flask containing 25 mL volume of NPB medium. The flask was kept at 37 °C without agitation throughout the period of investigation. Survivability and growth of MB45 cells in NPB medium was assessed through dilution-plating of pure culture (aliquots harvested at different time intervals) on fresh NRA plates.

5.2.5. DNA extraction, amplification, cloning and sequencing of 16S rRNA gene

Whole cell DNA extraction, amplification, cloning and sequencing of 16S rRNA gene sequence was performed according to the method described in chapter 3.

5.2.6. 16S rRNA gene sequence based phylogeny

The organization, editing, vector trimming, quality checking of sequence, and BlastN program was conducted as described in chapter 4. The16S rRNA gene sequences of the validly published *Klebsiella* species were retrieved from GenBank and multiple sequence alignment with the 16S rRNA gene sequence of MB45 were carried out in CLUSTAL W (Thompson *et al.*, 1994) and the resulted

aligned sequences were corrected, edited and approximately 1280 base pair long nucleotide stretch of all the *Klebsiella* sp. were selected for building tree. Phylogenetic analyses were conducted using software package MEGA4 (Tamura *et al.*, 2007). Multiple alignments of sequences were done Distances were calculated according to the Jukes-Cantor. Three tree-making algorithms: the neighbour-joining (NJ) (Saitou and Nei, 1987), maximum parsimony (Eck and Dayhoff, 1966), and UPGMA (Un-weighted Pair Group Method with Arithmetic mean) methods were used to ensure consistency of the clusters formed (Sneath and Sokal, 1973). All gaps and missing data in aligned sequence were eliminated from the dataset (complete deletion option). Tree topology was evaluated by the bootstrap resampling method of Felsenstein (1985) based on 1000 replication.

5.2.7. Cloning and sequencing of the amplicon corresponding to the variable region of the class 1 integron of MB45

Whole cell DNA extraction, amplification, cloning (in pGEM-T easy vector) and sequencing were done according to method described in chapter 3 except the one modification. Beside 5′ CS and 3′ CS primer, a primer Int2F (5′-CTCGGGTAACATCAAGG-3′) was also used in combination with primer 3′ CS, to detect proximity of gene cassette to *intI1* (gene encoding class 1 integrase) gene of class 1 integron (Martinez-Freijo *et al.*, 1998). The location of primer Int2F is ~ 600 bp upstream to the position of 5′ CS primer (as it is expected to generate an additional length of ~ 600 bp compared to the corresponding product length of the CS-PCR).

5.2.8. Sequence analysis and phylogeny of the novel DfrA protein

Nucleotide sequence analysis was performed using BlastN search programs [National Center for Biotechnology Information (NCBI)]. The phylogenetic analysis of novel DfrA protein derived from class 1 integron borne gene cassette harboured in bacterium MB45, was carried out in software package MEGA4 (Tamura *et al.*, 2007). Multiple sequence alignment of translated novel *dfrA* gene derived from MB45 and other published DfrA protein sequences of each representative organisms mediating trimethoprim resistance were conducted in ClustalW (Thompson *et al.*, 1994). From the multiple aligned sequences a guide tree was constructed which was used for making final bootstrap NJ tree. Cluster consistency was evaluated by maximum parsimony (MP) algorithm. All gaps and missing data in aligned sequence were eliminated from the dataset (complete deletion option).

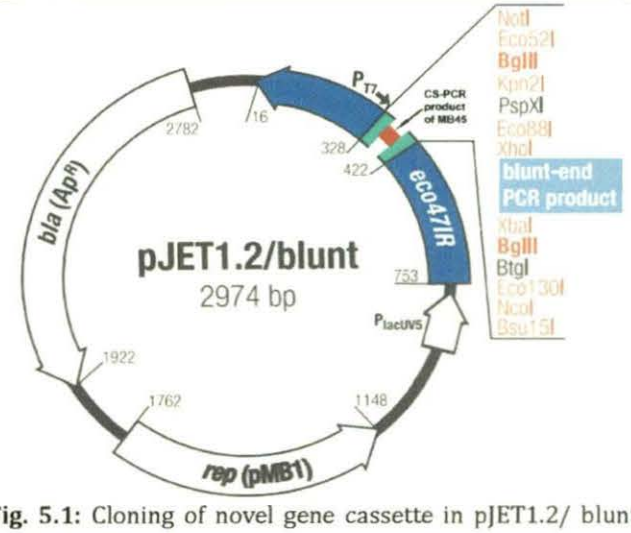

Fig. 5.1: Cloning of novel gene cassette in pJET1.2/ blunt cloning vector.

Symbols: *rep* (pMB1), replicon from the pMB1 plasmid responsible for the replication of pJET1.2 (map location, 1762-1148); initiation of replication start at 1162±1; *bla*, β-lactamase gene (selection marker) conferring resistance to ampicillin (map location 2782-1922); *eco47IR*, lethal gene *eco47IR* enables positive selection of the recombinants; P_{lacUV5}, modified P_{lac} promoter for expression of the *eco47IR* gene at a level sufficient to provide positive selection; T7 promoter, T7 RNA polymerase promoter for *in vitro* transcription of the cloned insert; MCS, multiple cloning site.

5.2.9 Expression of novel *dfrA* gene in pJET1.2 vector

For studying the expression of novel *dfrA* gene, the CS-PCR amplified variable region of class 1 integron was ligated in pJET1.2/blunt cloning vector (Fermentas Life Sciences, Vilnius, Lithuania). The map of pJET1.2/blunt cloning vector is shown in Fig. 5.1. The vector offers blunt end DNA

ligation and contains a lethal gene in it which is disrupted by ligation of DNA insert in to the cloning site and consequences of this is the only recombinant plasmids are able to propagate. The system does not require blue-white screening. Since *Taq* DNA polymerase was used for amplifying the variable region of class 1 integron which add 3′-dA overhangs by default, hence, it was necessary to remove the 3′- overhangs. To remove 3′- overhangs DNA blunting enzyme supplied by manufacturer was used. Reaction mixture for blunting the amplicon contained 2X reaction buffer (10 µl), PCR product (1 µl/ 0.15 pmol ends), DNA blunting enzyme (1 µl), and nuclease free water to make final volume 18 µl followed by incubation at 70°C for 5 minute. After blunting the DNA it was chilled on ice. Ligation reaction was setup by adding 50 ng of pJET1.2/blunt cloning vector to the blunting reaction mixture followed by addition of T4 DNA ligase according to the manufacturer's instruction. The ligation mix was incubated at 22°C for 5 minute chased by transformation into *E coli* JM109 pre-made competent cells. For transformation of heat shock method was applied. The clone (pAK45) containing plasmid with insert in proper orientation was screened on selective Luria agar plate amended with trimethoprim (5 mg/L). The clone, pAK45, was used for determination of maximum tolerance to the trimethoprim. Plasmidless *E. coli* JM109 was used as control.

5.2.10. Sequence alignment and the effect of mutation in the novel DfrA

For identification of amino acid residues which are responsible for trimethoprim resistance in the novel DfrA protein, multiple sequence alignment of DfrA proteins [novel DfrA, (Ac. No. AM997279), DfrA5 (Ac. No. AJ419169), and wild type (WT, trimethoprim sensitive) Dfr (Ac. No. J01609)] was carried out using ClustalW (Thompson *et al.*, 1994). Due to the non-availability of the trimethoprim-bound structure from *E. coli* in the Protein Data Bank (PDB) (Berman *et al.*, 2000), sequence alignment was also performed between different trimethoprim-bound Dfr available in PDB. The protein from the *Mycobacterium avium* (PDB ID: 2W3V) gave the highest score and was used for the analysis of ligand binding. Pymol http:// www.pymol.org was used for molecular visualization.

5.3. Result

5.3.1. Phenotypic characterization and 16S rRNA gene sequence phylogeny

Cells of the strain MB45 were rod shaped, capsulated, gram negative, aerobic (facultative anaerobic) and non motile. On nutrient rich medium (Luria agar), colonies were circular with entire margin, convex, translucent, mucoid, sticky and off-white in color with diameters of 2-3 mm after 3 days incubation at 37 °C . Growth occurs in PY medium (medium devoid of NaCl, detail described in chapter 4) amended with 0-6 % NaCl (optimum 0 %). Cells were able to grow at the temperature range 20-45 °C (optimum 30-37 °C) and pH range 3-12 (optimum pH 7) within 24 h in LB medium. Cells could utilize citrate (simmon's citrate agar) as sole carbon source. Cells were positive for catalase (weak), voges proskauer, lysine decarboxylase, β-galactosidase and ornithine decarboxylase but negative to oxidase, indole production, H2S production, methyl red, urease, casienase and gelatinase. Nitrate was reduced to nitrite. Acids were produced from lactose, xylose, maltose, fructose, dextrose, galactorse, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose, glycerol, inositol, sorbitol, mannitol, ribose, rhamnose, cellobiose, melezitose, D-arabinose and sacchrose but not from inulin, dulicitol, adonitol, sodium gluconate, salicin, glucosamine, α-methyl-D-galacotside, xylitol and sorbose. Gas was not produced from lactose at 44 °C . Cells could utilize citrate, ethanol and malonate as sole carbon source of energy. Differential phenotypic and biochemical characteristics of strain MB45 and nearest strains are given in Table 6.1. MB45 was resistant to antibiotics recommended by EUCAST for *Enterobacteriaceae* ampicillin (R> is 8 mg/L), gentamycin (S≤/R> is 2/4 mg/L), tobramycin (S≤/R> is 2/4 mg/L), netilmicin (S≤/R> is 2/4 mg/L), cotrimoxazole (S≤/R> is 2/4 mg/L) and trimethoprim (S≤/R> is 2/4 mg/L) and antibiotics absent in EUCAST kanamycin (S</R≥ is 5 mg/L), streptomycin (S</R≥ is 2.5). The MB45 cells were able to

resist trimethoprim (>1500 mg/L) and ampicillin (>5000 mg/L). The 16S rRNA homology and phylogenetic analysis revealed MB45 to be a member of genus *Klebsiella*. Almost complete 16S rRNA gene sequence (1503 bp, Ac. No. FR677021) was amplified, purified, cloned, and sequenced. The pair-wise alignment exhibited 99.6, 99.4, 99.3, 98.3, 98.2 and 97.5% identity with its nearest neighbors named as *Klebsiella pneumoniae* subsp. *rhinoscleromatis* ATCC 13884T (AF130983), *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 13883T (AF130981), *Klebsiella pneumoniae* subsp. *ozaenae* ATCC 11296T (AF130982), *Klebsiella granulomatis* KH 22T (AF010251), *Klebsiella singaporensis* DSM 16265T (AF250285) and *Klebsiella alba* LMG 24441T (EF154517) respectively.

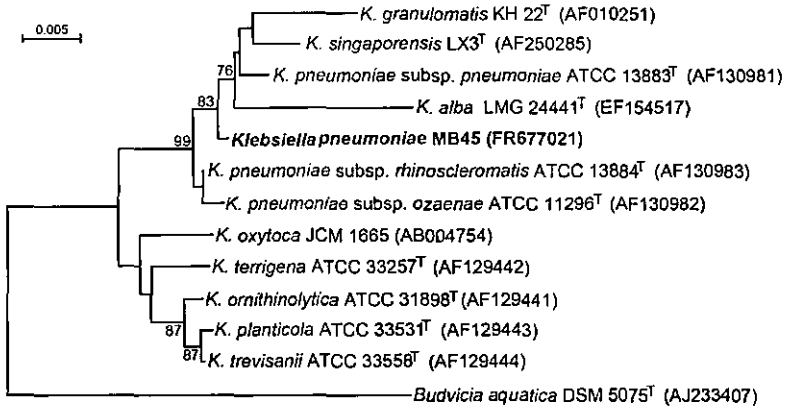

Fig. 5.2: NJ phylogenetic tree based on 16S rRNA gene sequences showing, the position of *Klebsiella* sp. MB45 (Bold face) within the members of genus ***Klebsiella***. *Budvicia aquatica* DSM 5075T (AJ233407) used as an outgroup. Bootstrap values (>70%), expressed as a percentage of 1000 replications, are given at branching points. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

In the NJ tree (Fig. 5.2) constructed with 16S rRNA gene sequences, strain MB45, formed a tight clade with a bootstrap support of 83% with the cluster comprising the four *Klebsiella* strains named as *Klebsiella pneumoniae* (subsp. *rhinoscleromatis; pneumoniae* and *ozaenae), Klebsiella granulomatis, Klebsiella singaporensis* and *Klebsiella alba*. Similar output was obtained from MP and UPGMA clustering (Fig. 5.3 and 5.4).

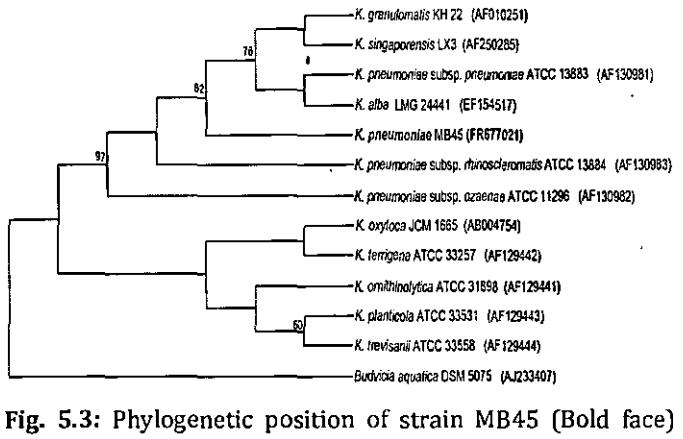

Fig. 5.3: Phylogenetic position of strain MB45 (Bold face) among the members of genus *Klebsiella* according to 16S rRNA gene sequence analysis. The topology shown was obtained using the maximum-parsimony (MP) algorithm. *Budvicia aquatica* DSM 5075T (AJ233407) used as an outgroup. Bootstrap values (≥50%), expressed as a percentage of 1000 replications, and are given at branch node. EMBL/GenBank accession numbers are given in parentheses.

5.3.2. Oligotrophic nature of strain MB45

The growth of MB45 cells were observed in NPB with no additional supplementation of growth factor (Fig. 5.5). An increase of 4.6 times of the initial cell number was noted in 2 days. The ability of strain MB45 to survive (without reduction in viable cell number since inoculation) and grow in a low nutrient medium explains the oligotrophic nature of the strain.

5.3.3. Description of class 1 integron borne gene cassette and phylogeny of novel DfrA protein

The Int_2F and 3′ CS primers pair yielded an amplicon of approximate 1.3 kb from MB45. The DNA sequence analysis showed that the amplicon contained only one gene cassette, corresponding to a *dfrA* gene. The 568 bp long *dfrA* gene was 93 % identical (nucleotide-nucleotide identity) to the gene cassette of *dfrA5* (Ac. No. AJ419169).

Gene cassette description: The 5′ CS terminates at the *attI*1 core site G/TTA [point of insertion and beginning of first gene cassette located at 686 (EMBL nucleotide position); Ac. No. AM997279) was identical to those of class 1 integrons. The core site for the site-specific insertion, GTTAACC (1R), was found at position 685–691. A 471 bp long ORF (open reading frame) potentially encoding a polypeptide of 157 amino acids was identified 35 nucleotides downstream to the 5′ CS. The ORF began with the initiation codon GTG (instead of ATG) at positions 705 to 707 and terminated with the stop codon TAA (within the inverse core site) at positions 1176 to 1178. Downstream to the 3′ end of the novel *dfrA* gene, an 81 bp long structure which was recognized as *attC* site (59 base element) that began with the sequence GGTTAAC (1L, inverse core site at position 1173-1179) and terminated with the core sequence GTTAGAT (EMBL nucleotide position, 1253) (Fig. 5.6). Integrase binding domains 2L (TATGCAAT, position, 1185-1192) and 2R (ATTGATA, position, 1241-1247) within the *attC* were also identified.

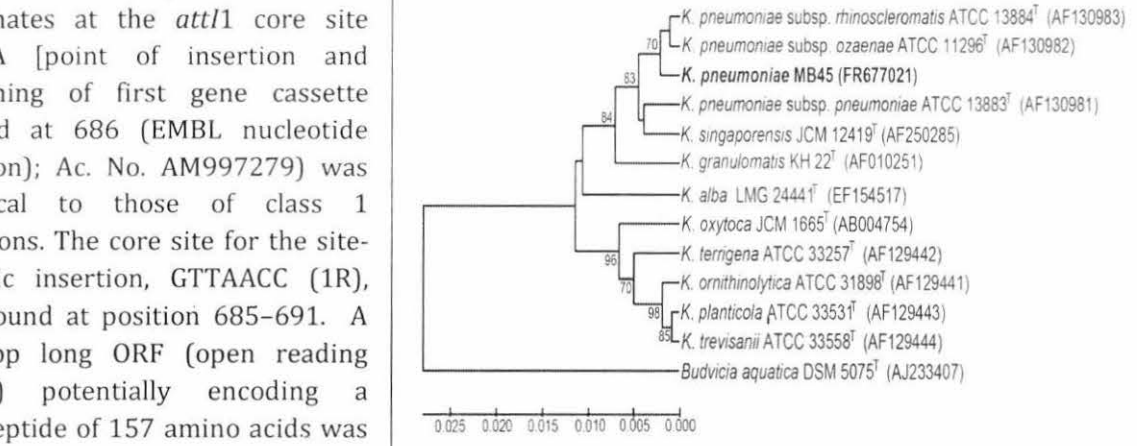

Fig. 5.4: UPGMA dendogram generated from the 16S rRNA sequences. Strain MB45 indicated in boldface among the member of genus Klebsiella. *Budvicia aquatica* DSM 5075T (AJ233407) used as an outgroup. Bootstrap values (≥70%), expressed as a percentage of 1000 replications, and are given at branch node. EMBL/GenBank accession numbers are given in parentheses. Scale shows the percentage similarity.

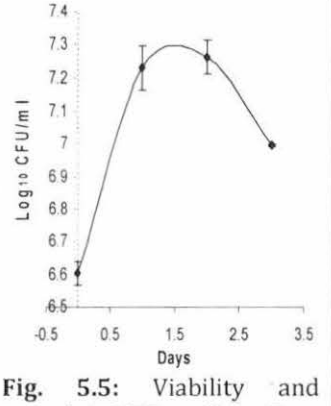

Fig. 5.5: Viability and growth in NPB medium. Bars shows standard error.

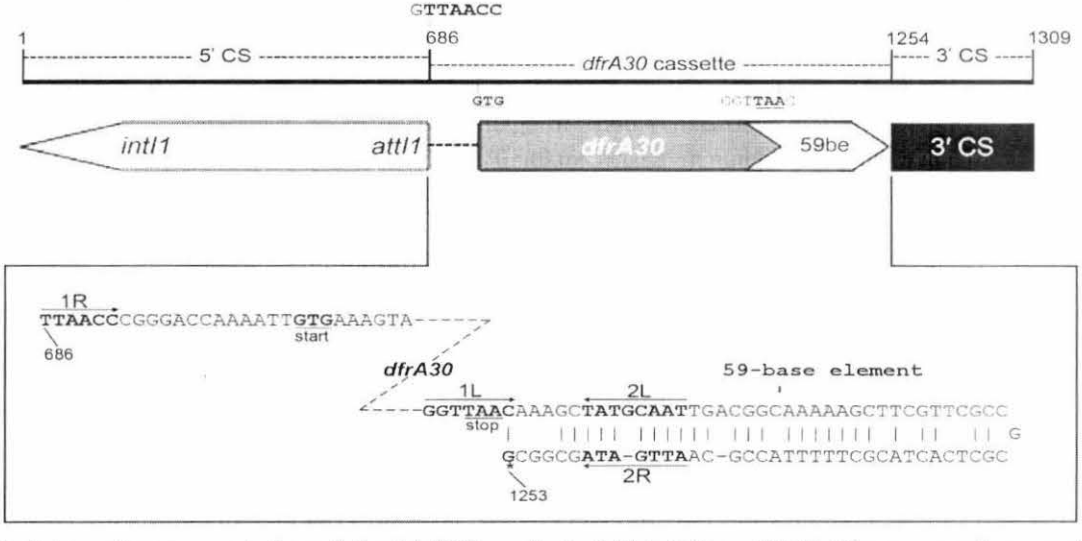

Fig.5.6: Schematic representation of the Int-PCR product of *Klebsiella* sp.MB45. CS, conserved segment; intI, integrase gene; *attI*, attachment site; *dfr*, dihydrofolate reductase; be, base element. Black thick bar shows the distribution of integron features on amplified product. The translation start (GTG) and stop (TAA) codons are in underlined bold face. In the 59 be, the putative integrase binding sites 1L, 2L, 1R and 2R are indicated by arrows. Termination of 59 be is indicated by star (*). Numbers correspond to sequence positions in EMBL Accession No. AM997279.

Phylogeny of MB45 DfrA: The translated protein sequence, DfrA protein, was made up of 157 amino acids (AA) and thus was in the same size range as of dfrA5 (157 AA) previously reported from *E coli* (Ac. No. AJ419169) and plasmid pLM020 (Ac. No. X12868). The percent identity resulted from pair-wise alignment between the predicted protein product of the said *dfrA* gene, translated from 471 bp long novel open reading frame, and the published DfrA proteins (responsible for trimethoprim resistance) varied between 15.1 and 93%. The maximum identity (93%) of MB45 DfrA was with the DfrA5 protein of *E coli* (Ac. No. AJ419169) and plasmid pLM020 (Ac. No. X12868) followed by 88.5% with the DfrA14 of *E. coli* (Ac. No. AJ313522). The least identity (15.1%) was observed with the DfrA23 derived from *S. typhimurium* (Ac. No. AJ746361). The percent identity between MB45 DfrA and *E coli* derived chromosomal *dfr* (coding for *fol*A, sensitive to trimethoprim; Ac No. J01609) was shared by 33.1%.

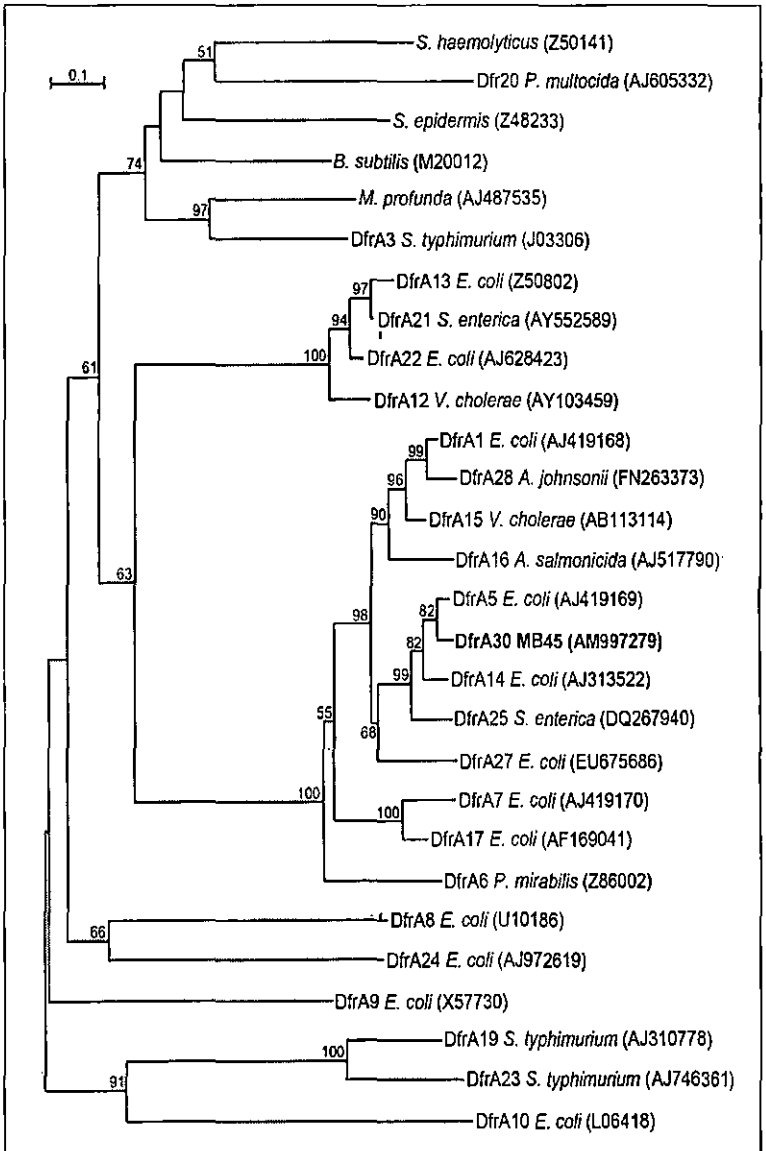

Fig. 5.7: NJ tree showing the position of DfrA29 (Bold face) within the DfrA proteins, involved in trimethoprim resistance. The number (>50%) at each major branch point refers to the percentage of times that a particular node was found in 1,000 bootstrap replications. Source and the GenBank/EMBL/DDBJ accession (in parentheses) numbers are given for each DfrA protein. DfrB proteins, which differ distinctly from DfrA proteins in size and structure, are not included in this tree.

Since the identity of translated novel *dfrA* gene [this particular sequence deposited in the GenBank was also annotated as novel by Partridge *et al.*, (2009)] derived from MB45 was 93% with its nearest variant DfrA5 and differed in characteristics described earlier to the known Dfr(s), this gene was predicted to be a new *dfrA* gene and named as *dfrA30* following guidelines for naming new trimethoprim resistance genes (Sköld O., 2001; Hall and Partridge, 2003). The detailed features of novel gene cassette are shown in Fig. 5.6.

In the NJ phylogenetic tree the novel DfrA derived from MB45 branched deeply with DfrA5 of *E coli* (Ac. No. AJ419169) constructed from amino acids of different Dfrs (Fig. 5.7). The maximum parsimony (MP) tree building algorithms also generated same output (Fig. 5.8).

5.3.4. Mutation study of DfrA30

Multiple sequence alignment of DfrA30, DfrA5 and TMP-sensitive wild type (WT) Dfr protein (encoded by *folA* gene, Ac. No. J01609)] was done. Residues constituting the binding site for TMP

(Matthews *et al.*, 1985), positions of the mutated amino acids in the active site of DfrA30 with respect to the wild type protein and also the non active site residues that are known to play a vital role in TMP binding were identified (Fig. 5.9) (Watson *et al.*, 2007). The sequence alignment between wild type (WT) and *Mycobacterium avium* Dfr (PDB ID: 2W3V) (which was used to visualize the ligand binding) is shown in Fig. 5.10. The 3-dimensional structure of Dfr (PDB ID: 2W3V) depicting the active site pocket including the two important residues (positions 28 and 94) for TMP binding was analysed (Fig. 5.11).

5.3.5. Expression of the novel *dfrA30* in *E. coli* JM109

The clone pAK45 (Fig. 5.12), containing novel *dfrA* gene cassette in correct orientation was used for determining the function encoded by gene cassette. The streaking of pAK45 clone onto Luria agar plate containing different concentrations of trimethoprim revealed that the cloned gene was functional and was producing the dihydrofolate reductase which conferred resistance to the trimethoprim. The maximum tolerance to trimethoprim was observed to be 1000 mg/L which is far greater than the settled breakpoint (S≤/R> is 2/4 mg/L) of EUCAST. The level of trimethoprim-resistance in the wild-type host strain, MB45, was noted to be >1500 mg/L. The MIC of the control strain (plasmidless *E. coli* JM109) was restricted to 5 mg/L for trimethoprim.

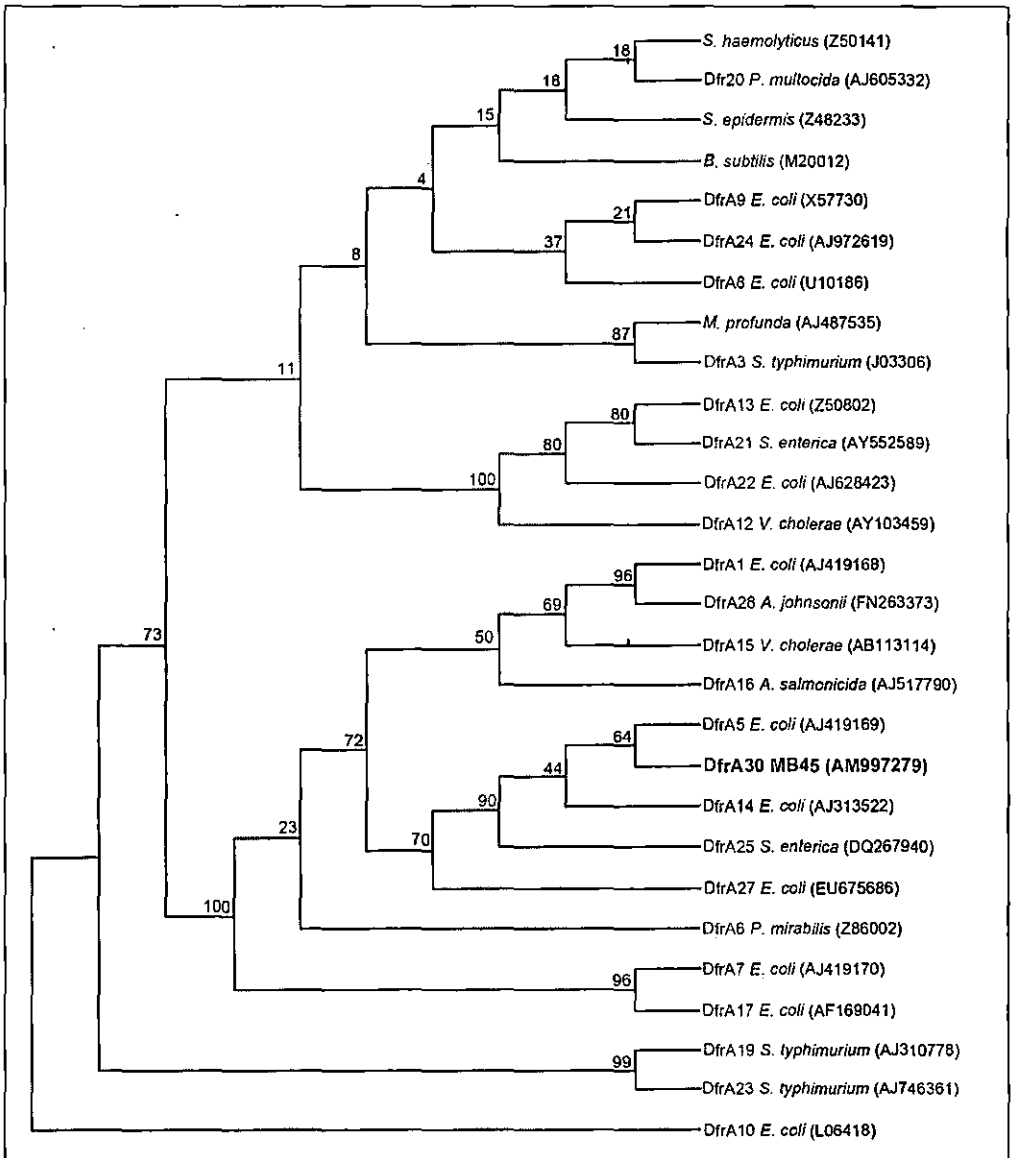

Fig. 5.8: MP tree showing the position of DfrA29 (Bold face) within the DfrA proteins, involved in trimethoprim resistance. Bootstrap values (expressed as a percentage of 1000 replications) are given at branch node. Source and the GenBank/EMBL/DDBJ accession (in parentheses) numbers are given for each DfrA protein. The Phylogeny was performed in MEGA4 package.

```
CLUSTAL 2.0.12 multiple sequence alignment


DfrA29                  VKVSLMAARARNGVIGCGPHIPWSAKGEQLLFKALTYNQWLLVGRKTFESMG-ALPNRKY 59
DfrA5                   VKVSLMAAKAKNGVIGCGPHIPWSAKGEQLLFKALTYNQWLLVGRKTFESMG-ALPNRKY 59
WT_Dfr                  -MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVVMGRLTWESIGRPLPGRKN 59
                          :**:** * : ***    :**.  .:   **  * *: :::** *:**:* .**.**

DfrA29                  AVVTRSAWTANNDNVVVFPSIEEAMGGLAKLNGHVIVSGGGEIYRETLPMASTLHVSTID 119
DfrA5                   AVVTRSAWTADNDNVIVFPSIEEAMYGLAELTDHVIVSGGGEIYRETLPMASTLHISTID 119
WT_Dfr                  IVIS--SKPGSDDRVQWVSSVEEAIAACGDV-EEIMVIGGGRVYEQFLPKAHKLYLTHID 116
                         *::  : ...:*.*  ..*:***: . ..:  .::* ***.:*.: ** * .*::: **

DfrA29                  IEPEGDVFFPN-IPNFFEVVFEQHFSSNIN----YCYQIWKKG 157
DfrA5                   IEPEGDVFFPN-IPNTFEVVFEQHFSSNIN----YCYQIWQKG 157
WT_Dfr                  AEVEGDTHFPDYDPDEWESVFSEFHDADAQNSHSYCFEILERR 159
                         * ***..**:  *: :* **.:...:: :    **::* ::
```

Fig. 5.9: Sequence alignment of DfrA30 with DfrA5 and sensitive WT-Dfr protein. The residues which form the binding site are shown in red, and the positions of mutations from the wild type (WT) protein are shown in bold letters. Residues (beyond the active site) which are known to play an important role in trimethoprim binding, and which have been mutated are marked in blue

```
CLUSTAL 2.0.12 multiple sequence alignment


WT                      ----MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVVMGRLTWESIG---RP 53
Mycobacterium           MTRAEVGLVWAQSTSGVIGRGGDIPWSVPEDLTRFKEVTMGHTVIMGRRTWESLPAKVRP 60
                           :.*: * :.. ***  . :**.:* **: **. *:.:.*:*** ****:     **

WT                      LPGRKNIVISSKP-GSDDRVQWVSSVEEAIAACGDVEEIMVIGGGRVYEQFLPKAHKLYL 112
Mycobacterium           LPGRRNVVVSRRPDFVAEGARVAGSLEAALAYAGSDPAPWVIGGAQIYLLALPHATRCEV 120
                        ****:*:*:* :*    : .: ..*:* *:* .*.     ****.::*   **:* :  :

WT                      THIDAEVEG--DTHFPDYDPDEWESVFSEFHDADAQNSHSYCFEILERR 159
Mycobacterium           TEIEIDLRRDDDDALAPALDDSWVGETGEWLASRSG--LRYRFHSYRRD 167
                        *.*: ::.   *  :.    *.* .  .*:  : :     * *.  .*
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Fig. 5.10: Sequence alignment between wild type (WT) and *Mycobacterium avium* Dfr (PDB ID: 2W3V).

5.4. Discussion

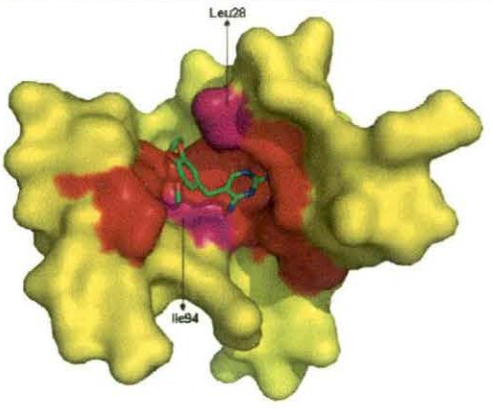

Fig. 5.11: Surface representation of TMP binding site on DFR (PDB ID: 2W3V). Residues which form the active pocket are shown in red, and the two residues that are mutated in DfrA29 are in magenta; the remaining residues are in yellow. TMP is shown in stick model.

Recent studies have shown that the frequency of antibiotic resistance has been the second highest in the genus *Klebsiella* especially in *K. pneumoniae* (next to *E. coli*) within *Enterobacteriaceae*; and the rate of occurrence has been noted to be higher in isolates from developing countries than developed countries (Adrian PV *et al*, 1993; Brolund A *et al.*, 2010; Lopes ACS *et al.*, 2010; Randrianirina *et al.*, 2010). The incidence rates of trimethoprim resistance in *Klebsiella* spp. and *E. coli* in particular have been alarming (Adrian PV *et al*, 1993; Misra R *et al.*, 2001; Motakefi A *et al.*, 2008; Penteadoa AP *et al.*, 2009). An earlier surveillance study (1987-88) on community isolates showed an increase in trimethoprim resistance from 15.2% to 24% in *Klebsiella / Enterobacter* spp. (Amyes and Towner, 1990). Trimethoprim, a broad-spectrum antimicrobial agent, either alone or in combination with sulfamethoxazole, is used globally for the treatment of enteric, respiratory, skin, and urinary tract infections (Huovinen *et al.*, 1995). Trimethoprim on binding to its target enzyme, dihydrofolate reductase (DFR), prevents growth and viability of the bacteria. DFR is an essential enzyme of all living cells which reduces dihdrofolate into tetrahydrofolate, an essential precursor in synthesis of

thymidine monophosphate which in turn serve as precursor for DNA metabolite thymidine triphosphate.

The most frequent mechanism of bacterial trimethoprim-resistance is the production of an additional trimethoprim-resistant DFR, frequently found on mobile genetic elements like plasmids, transposons, and gene cassettes (Amyes and Towner, 1990; Huovinen *et al.*, 1995). Other mechanisms of bacterial resistance to trimethoprim that have been described are impermeability (found in isolates of *Serratia, Enterobacter, Klebsiella, Pseudomonas,* and *Clostridium*) and mutational changes in the thymidylate synthase gene (Then, 1982; Amyes and Towner, 1990; Huovinen *et al.*, 1995). Nosocomial septicemia due to extended spectrum beta-lactamase producing *K. pneumoniae* and *E. coli* are a therapeutic challenge due to resistance (Mishra *et al.*, 2010). Recently, it was shown that treatment without resistance selection at the infection site with fluoroquinolone treatment can be linked to colonization of the digestive tract by *K. pneumoniae* (originally a pulmonary bacteria), followed by the emergence of resistance (Kesteman *et al.*, 2010). The present study showed that the test strain, MB45, isolated from River Mahananda at Siliguri, India, was resistant to ampicillin, cefepime, cefotaxime, chloramphenicol, netilmicin, sulfamethoxazole, cotrimoxazole, gentamycin, tobramycin and trimethoprim; and could survive in low nutrient condition (oligotrophic). The strain MB45 showed high level of resistance to trimethoprim (R> 1500mg/L). Characterization of integron-borne cassette arrays in *K. pneumonia* strains from China revealed a predominance of *dfr* and *aadA* genes that confer resistance to trimethoprim and aminoglycosides respectively (Yao *et al.*, 2007). However, the distribution of *dfr* genes in *K. pneumoniae* was not well known until a recent study, conducted on 54 trimethoprim resistant strains of *K. pneumoniae* which exhibited the most prevalence of presence of *dfrA1, dfrA5, dfrA7, dfrA8, dfrA12, dfra14, dfrA17* and rarest of *dfrA17* (Brolund *et al.*, 2010). A number of *dfr* genes conferring resistance to trimethoprim have been isolated from numerous bacteria sources (White and Rawlinson, 2001).

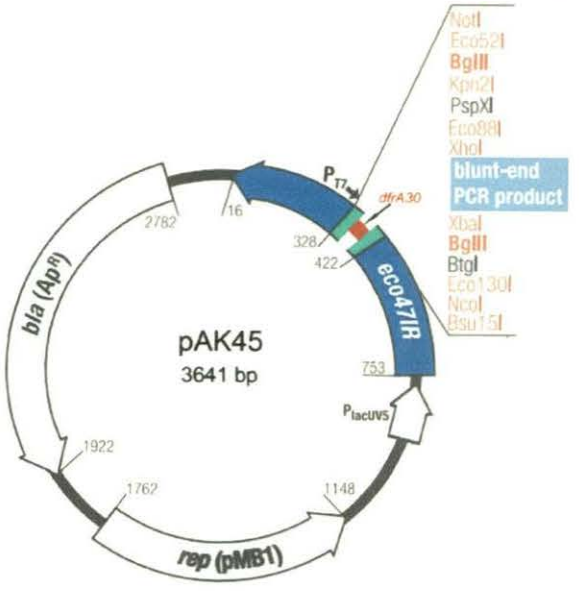

Fig. 5.12: Constructed recombinant plasmid (not in scale), pAK45, harboring novel *dfrA30* gene coding for dihydrofolate reductase which confer resistance to trimethoprim.

Symbols: *rep* (pMB1), replicon from the pMB1 plasmid responsible for the replication of pJET1.2 (map location, 1762-1148); initiation of replication start at 1162±1; *bla*, β-lactamase gene (selection marker) conferring resistance to ampicillin (map location 2782-1922); *eco47IR*, lethal gene *eco47IR* enables positive selection of the recombinants; P_{lacUV5}, modified P_{lac} promoter for expression of the *eco47IR* gene at a level sufficient to provide positive selection; T7 promoter, T7 RNA polymerase promoter for *in vitro* transcription of the cloned insert; MCS, multiple cloning site.

A gene coding for trimethoprim resistance can be claimed as novel, if, the translated dihydrofolate reductase protein possess <95% identity at the amino acid level when compared to known DFR proteins (Agerso *et al.*, 2006). On the basis percent identity (93%) between the novel Dfr and its nearest variant DfrA5 and characteristics described earlier, the gene encoding novel DfrA, was predicted as new *dfrA* gene and was named *dfrA30* following the guidelines for naming new

trimethoprim resistance genes (Sköld O., 2001). The phylogenetic trees showed that DfrA30 was making a deep branching with its neighbor DfrA5 (Fig. 5.7 to 5.8).

The strain MB45 showed high level of resistance to TMP (>1500 mg/L). Generally, a single mutation in the active site of Dfr is enough for TMP-resistance, though multiple mutations are common in clinically isolated species. The mutations in the active site residues reduce the binding affinity of the enzyme for the drug. Matthews and co-workers have identified the residues that constitute the TMP-binding site in *E. coli* Dfr (Fig. 5.9) (Matthews *et al.*, 1985). Additionally, the mutations in the active site that leads to TMP resistance in *E. coli* have been enunciated (Watson *et al.*, 2007). The mutations in DfrA30 are of the same type as those in DfrA5. In particular, two changes, glutamine for leucine at residue 28 and isoleucine to serine at 94, would change the hydrophobic/polar nature at the two opposite sides of the TMP site (Fig. 5.11), thus possibly weakening the binding. Other mutations (V10, W30 and I94) beyond the active site have also been identified in clinically isolated TMP-resistant genes (Watson *et al.*, 2007). Some of these are also found in DfrA30 (mutations V10K and W31L) (Fig. 5.9).

The facultatively oligotrophic strain used in this study was characterized as *K. pneumoniae* MB45 (ascertained from phenotype as well as from 16S rRNA phylogeny) (Fig. 5.2 to 5.4). Viability assay and growth assessment of *K. pneumoniae* MB45 cells in NPB for more than 72 h by taking viable cell count of the cell suspension on NRA at different times (Fig. 5.5) demonstrated its ability to adapt both oligotrophic (ability to survive and grow in extremely poor nutrient conditions) as well as copiotrophic (ability to form colonies in a rich medium) conditions of growth. Such facultative nature of oligotrophy, as shown by the *K. pneumoniae* MB45, may contribute to the reported adaptation of remaining viable in hospital environment (Tada *et al.*, 1995) for several days and cause nosocomial infection. Microbial contamination of working surfaces, clinical materials, and surgical devices poses a major threat in hospitals and intensive care units.

5.5. Conclusion

The present work demonstrated the characterization and expression of novel *dfr, dfrA30.* Cloning of novel *dfrA30* gene in pJET1.2 vector (clone pAK45) revealed that dihydrofolate reductase encoded by the novel dfrA gene was functional in conferring resistance to trimethoprim up to 1000 mg/L in *E. coli* JM109. Phenotypic study and 16S rRNA phylogeny showed that the strain, MB45 belonged to the genus *Klebsiella*. The strain MB45 itself was possessing resistance to trimethoprim >1500 mg/L due to possession of dfrA30 gene. In addition to trimethoprim, MB45 was also resistant to nine more antibiotics.

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An evaluation of bactericidal property of Zinc oxide Quantum Dots on a multiple-antibiotic and human serum resistant bacterium, Klebsiella pneumoniae MB45

6.1. Background

Klebsiella pneumoniae among all species of the genus *Klebsiella* derives special importance because of being pathogenic to human beings. Interestingly, they are omnipresent and have been isolated from diverse sources including animals, sewage, soils, vegetables, salt water, treated drinking water, sachet water and river water (Podschun *et al.*, 2001; Li *et al.*, 2004; Lal and Kaur, 2006; Banu and Menkuru, 2010; Xu *et al.*, 2010). The test strain MB45, described in this thesis, isolated from river water possessing oligotrophic characteristics, was resistant to ten different antibiotics. The strain MB45 was also found to be resistant to human serum. Like other *Klebsiella* strains, strain MB45 also contains polysaccharide capsule over cell which is the main determinant of pathogenicity in *Klebsiella*.

There are several reasons and mechanism (described elsewhere in the thesis) responsible for the spread of antibiotic resistance in the natural populations of bacteria. The fact remains that bacteria are becoming increasingly resistant to almost all known drugs/antibiotics invented or discovered. The prevalence of multiple antibiotic resistant bacteria among pathogens and by-standers (normally not pathogenic but becomes virulent under immunosuppressive conditions) poses a severe threat to public health worldwide. Hence, rise in incidence of antibiotic resistance among bacteria (pathogenic as well as environmental) have discouraged investment in R & D sector for searching new antibiotics. On the other hand, boom in infectious diseases caused by multiple-antibiotic resistant bacteria has created an unprecedented demand for novel antimicrobials and eradication strategies. This demand and challenge to innovate new therapies induced the researchers to look back to their civilization of the by-gone days when people used metals in the form of gold ashes for regaining health, silver for treating burns and chronic wounds or copper for making water potable (Moghimi, 2005). Gold is still used in the Indian Ayurvedas for rejuvenation and revitalization during old age under the name of Swarna Bhasma ("Swarna" meaning gold, "Bhasma" meaning ash) (Mahdihassan, 1985). Gold also has its long history of use in the western countries where it was used for revitalizing people suffering from nervous conditions (Higby, 1982). In recent years, various nanotized/nanosized (called as nanoparticles) metals like Ti, Mg, Ag, and Zn have been documented as important alternative candidate of antibiotics in controlling pathogenic bacteria (Joshi *et al.*, 2009). The potential application of nanoparticles as antimicrobials emanated from bacterial growth-inhibiting ability as well as their unique physiochemical properties. Recently, nanoparticles made out of such metallic compound are being characterised for novel physical and chemical properties; some of them are often shown to have biological effects like antimicrobials (Gajjar *et al.*, 1991). The antimicrobial activity of the nanoparticles is directly proportional to the surface area (size α 1/surface area) in contact with the microorganisms i.e. high surface to volume ratio means more interaction with the microbes (Rai and Bai, 2011) which micro- or macro-sized particles do not possess. Hence, researches on applications of nanoparticles as antimicrobials have gained momentum in the present century. The nanoparticles are broadly categorized in two groups (i) organic and (ii) inorganic nanoparticles. The latter one has received more attention due to their ability to withstand adverse processing conditions (Whitesides *et al.*, 2003). The nanosized oxide derivatives of metals are receiving increasing attention for a large variety of applications due to their superiority in terms of safety, durability and heat resistance in compare to conventional organic

antibacterial agents. Oxides of titanium (TiO2) and zinc (ZnO) nanoparticles (NPs) are included in toothpaste, beauty products, sunscreens, and textiles. Among the different oxide derivatives of nanosized metals, zinc oxide (ZnO) have been found as potent drug having antibacterial activity (Stoimenov, 2002; Hanley *et al.*, 2008; Nair *et al.*, 2008; Applerot *et al.*, 2009; Joshi *et al.*, 2009) not just because of their stability under harsh processing conditions, but also because they are generally considered as safe materials for human beings and animals (Stoimenov *et al.* 2002; Fu *et al.* 2005).

This chapter briefly describes an attempt to evaluate zinc oxide nanoparticles (ZnO-Acetate quantum dots) for their bactericidal activities. The obvious choice of bacterium (on which the Nps to be tested) was the one which could resist several different antibiotics and human serum as well. Hence, the test strain was *Klebsiella pneumoniae* MB45.

6.2. Materials and methods

6.2.1. Isolation, selection, oligotrophic characteristics, and characterization of test strain MB45: described in chapter 5

6.2.2. Antibiotic susceptibility test and determination of resistance: described in chapter 5.

6.2.3. Serum bactericidal assay

Serum bactericidal assay was basically performed according to the methods described by Sharma *et al.* (1999). Normal human serum (NHS) obtained from healthy adult volunteers (having no record of antibiotic therapy for the last one year) was used for assay. Heat inactivated serum (56°C for 30 min) (HIS) was used as control. Bacterial culture for assay was prepared by transferring a loopful culture (24 h old grown on Luria agar plate) into 10 mL sterile Brain Heart infusion broth in 100 mL Erlenmeyer flask. The inoculated medium was incubated at 37°C for 4 h without agitation. The cells were harvested by centrifugation at 8000 rpm for 5 minutes at 4°C and re-suspended in phosphate buffer saline (PBS, pH 7.3). The cell suspension was diluted with PBS and optical density (at 600 nm) was adjusted to McFarland standard No. 0.5. Cell suspensions were further diluted with PBS to achieve a cell density of 1×10^8 cells/mL. Aliquot of 0.1 mL of cell suspension ($=1\times10^7$ cells) was mixed with 0.2 mL of NHS and 0.1 mL of 10X peptone-glucose-bromothymol blue broth (composition: 10% peptone, 10% glucose and 0.075% of bromothymol blue). The volume was adjusted to 1 mL by adding 0.6 mL of sterile PBS. Control with HIS was also included. *Escherichia coli* K12 was also taken as control with NHS and HIS for testing serum susceptibility. Change in the color in NHS and HIS tubes at 5 h of incubation at 37°C indicated viability of cells as well as serum resistance while no change in color of NHS tube even at 8 h but change in HIS tube at 5 h was regarded as serum-sensitive.

6.2.4. Effect of ZnO QDs on viability and growth of MB45

The zinc oxide quantum dots (ZnO QDs) with surface adsorbed anionic species of acetate ions synthesized by wet chemical route were provided by Joshi *et al* (2009). Inoculum, for testing ZnO QDs efficacy, was prepared by the method as described in chapter 4. Aliquots of 10 µL of concentrated (4×10^6 cells) cell suspension were added to 3.0 mL of Luria broth in 150×15mm (length × width) glass tubes amended with different concentrations of ZnO-Ac QDs. The tube containing all the ingredients except ZnO-Ac was taken as negative control. Luria broth containing ZnO-Ac but lacking cells were taken as positive control. The tubes were kept at 37°C with continuous agitation at 200 rpm throughout the period of investigation. Test was performed in triplicates and standard error was measured. The optical density was measured in spectrophotometer (Model-302, Electronic India) at 600 nm at different time intervals. Growth rate constant (µ) and mean generation time (g) of MB45 in absence and presence ZnO QDs were calculated.

6.3. Results and discussion

Phenotypic and 16S rRNA phylogeny revealed that the strain, MB45, belonged to the genus *Klebsiella*. The maximum homology (99%) of MB45 16S rRNA gene sequence was shared by the *Klebsiella pneumoniae* (detailed phylogenetic analysis described in chapter 5). *Klebsiellae* (especially *Klebsiella pneumoniae* among all) are opportunistic pathogens and can spread rapidly, which often leads to nosocomial outbreaks (Nordmann *et al.*, 2009). *K. pneumoniae* although being a respiratory pathogen, the other sites of infection reported include urinary tract, digestive tract, surgical wound sites etc. The invasiveness of infection takes place generally via contaminated respiratory support equipment and urinary catheters or via other surgical/non surgical equipments. Antibiotic-resistance genes are acquired by these bacteria through horizontal gene transfer and get selected in the environment where there is extensive use of broad-spectrum antibiotics. Massive use of drugs/ antibiotics resulted in development of multiple-antibiotic-resistant *Klebsiella* strains. *Klebsiella pneumoniae* strain MB45 is resistant to ten antibiotics (cotrimoxazole, ampicillin, gentamycin, netilmicin, tobramycin, chloramphenicol, cefotaxime, kanamycin and streptomycin) and could survive and multiply in nutrient poor medium (oligotrophic). An increase of 4.6 times of the initial cell number was noted in 2 days in nutrient poor medium which explains the oligotrophic nature of strain MB45. Since the existance of oligotrophic bacteria has already been reported previously from the clinical samples (Tada *et al.*, 1995), therefore, oligotrophs are of more interest in light of human health. Such bacteria normally do not appear on conventional rich laboratory media and thus their role if any in causing infections of hospital admitted patients generally goes undetected. Besides being resistant to ten antibiotics (as stated earlier), MB45 can remain viable in an environment without nutrient-luxury. Also it has been described in chapter 5 about its bearing of a novel dihydrofolate reductase gene. MB45 was capable to resist high concentration of trimethoprim (>1500 mg/L). Besides antibiotic resistance, serum resistance in clinical isolates of pathogenic bacteria is an additional threat. The present study showed that the test strain, MB45, was also resistant to human serum. Human serum functions as bactericidal agent in healthy persons and protect from invasion of pathogens. Pathogens which are successful in causing disease are generally equipped with the capability to resist normal human serum (NHS) resulting persistent infection. In an earlier study, conducted on more than hundred clinical isolates of *Klebsiella pneumoniae* for assessing serum bactericidal activity, it was shown that 50% of the strains were resistant to 20% normal human serum collected from different healthy humans (Sharma *et al.*, 1999). The strain MB45 cells could change the color in NHS and HIS tubes at 5 h and therefore inferred as serum resistant while control, *E. coli* K12 failed to change the color of NHS tube even at 8 h, but turned HIS tube yellow at 5 h, was regarded as serum-sensitive. The strong correlation between serum resistance and the ability of a variety of gram-negative bacteria to invade and survive in the human blood stream have been studied (Taylor, 1988).

A number of nanosized metals itself or derivatives were found to be good antimicrobials. Among different oxides derivatives of metals (nano-particle), oxide derivative of zinc metal received more attention due to their long stability, effectiveness, and biocompatibility to humans and animals. The size of nanoparticles is the most

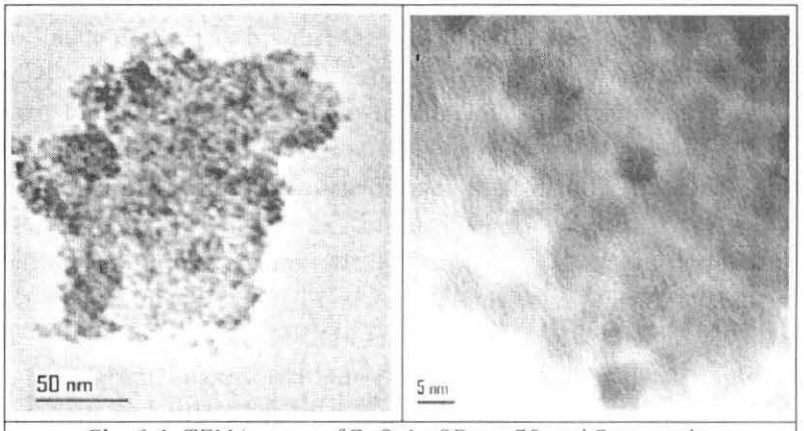

Fig. 6.1: TEM images of ZnO-Ac QDs at 50 and 5 nm scale. (Fig. adapted from Joshi *et al.*, 2009)

important factor in its effectiveness. The mechanism of action of nanoparticle is still inconclusive. However, some authors proposed their views to explain the antibacterial activity of ZnO nanoparticles; for example, Yamamoto (2001) proposed generation of hydrogen peroxide from the surface of ZnO which causes inhibition of bacterial growth. Another possible mechanism was proposed by Brayner *et al.* (2006) where it was suggested that the released Zn^{2+} ions are responsible for damaging the cell membrane leading to further lethal interaction with other intracellular contents. It is known that the physical and chemical properties of the nanoparticles change with size (coming close to nanoscale). Typically the dimension of nanoparticles fell in the range of 0.2-100 nm. The average particle size of chemically synthesized (wet route) zinc oxide quantum dots with surface adsorbed anionic species of acetate ions (ZnO-Ac QDs) was estimated around 3–5 nm (Fig. 6.1) (Joshi *et al.*, 2009). The previous study on the ZnO-Ac QDs was conducted on *E. coli K12* bacterium by Joshi *et al.*, (2009) where they found that zinc nanoparticle with surface bound acetate ions (anionic species) had superior bactericidal activity than those described earlier (Hanley *et al.*, 2008). In this study, the antibacterial potency of ZnO-Ac QDs was tested against the multiple-antibiotic and serum resistant *Klebsiella pneumoniae* MB45. The bacterial growth rate was found to be inhibited with the increase in concentration of ZnO-Ac QDs under standard cultural conditions (Fig. 6.2). The growth rate constant (μ) and mean generation time (g) in Luria broth without ZnO QDs (μ =0.019 min^{-1}, g = 36.5 min) was significantly affected on addition of ZnO QDs (μ = 0.013 min^{-1}; g = 53.3 min; at 400 mg/L). Complete inhibition of growth of MB45 is observed at concentration of 500 mg/L ZnO-Ac QDs in the medium. Hence, in future ZnO-Ac QDs could be a good nanobiotic candidate for the control of multi-drug resistant pathogens as well as in disinfecting hospital environments, external wounds, medical and surgical devices; and also in suitable format may find application in drinking water treatment plants.

6.4. Conclusion

Apart from the general direction of the search elucidated in the preceding chapters, the final chapter leaves a ray of hope to curb the menace. Zn-based nanoparticles (quantum dots) may offer a possible solution to control human-serum-tolerant pathogens resistant to multiple antibiotics.

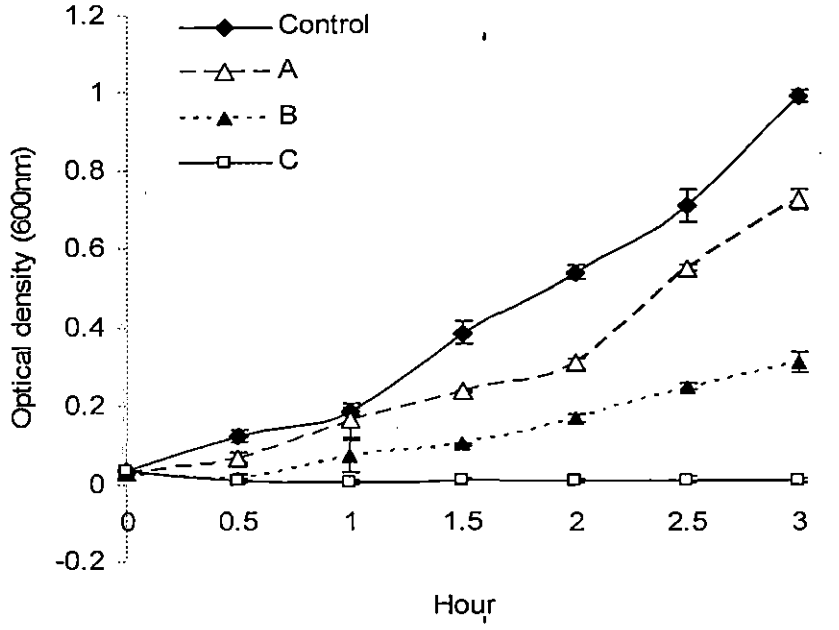

Fig. 6.2: Effect of ZnO nano-particles on the growth of MB45. Cultures were setup with an initial cell number 9×10^6 cells in Luria broth amended with different concentration (♦, Control 0.0 mg/L; Δ, A 200mg/L; ▲, B 400mg/L; □, C 500mg/L) of colloidal solution of ZnO nano-particles. Cultures were incubated at 37 °C with constant shaking at 200 rpm under normal laboratory condition. Bars shows standard deviation.

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General Discussion and Summary

Generally media used in laboratory provide ample amounts of essential elements like sugars, nitrogen, phosphorus and other vital components which are considered fundamental for the bacterial growth. Natural environments generally contain only trace amounts of nutrients as compared to nutrient concentrations in culture media used in bacteriological laboratories. It is also rare to find a true eutrophic (= copiotrophic) environment in the earth's biosphere. Bacteria, adapted for growth under low-nutrient (sometimes extremely low) conditions are called as oligotrophs (Van der Kooji and Hijen 1983; Hattori 1984; Suwa and Hattori 1984; Wainwright *et al.*, 1991). The bacteria that grow in oligotrophic condition have a number of important implications in biotechnology, bio-medical fields, and environment (Ohta and Hattori, 1983; Wainwright *et al.*, 1991; Tada and Inoue, 2000; Nagarkar *et al.*, 2001; Zhang and Huang, 2005). As for example, a soil oligotrophic bacterium was used as tool in monitoring heavy metal pollution (Tada *et al.*, 2001). Similarly HuiXia *et al.* (2007) isolated an oligotrophic bacterium SGB-5 from biological soil crust underlayer in the Xinjiang Gurbantunggut desert which was having water retention as well as sand-fixing properties. Researchers have observed that these organisms generally fail to grow on rich nutrient media used in laboratory for cultivating the bacterial cultures. However, most of the oligotrophic bacteria changed their nature on subsequent cultivation and could grow on rich-nutrient media. Scholars have devised several methods (for details please see oligotrophic bacteria: A general introduction) to isolate these bacteria from environmental and non-environmental settings; for example, some workers used media like R2A (Reasoner and Gerldreich, 1985), diluted Nutrient or Luria Bertani or Luria broth (Tada *et al.*, 1995; Oh *et al.*, 2009; Kumar *et al.*, 2010), and Ravan medium (Watve *et al.*, 2000), etc. Crude aqueous samples itself (e.g. river water/ acid mine drainage/ sea water etc.) collected from diverse niches were also used as media (Yanagita *et al.*, 1978) to recover oligotrophic bacteria from that environment. Studies exploring the phenomena of resistance to different groups of antibiotics by the oligotrophic bacteria revealed 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 (Nikitin *et al.*, 1988; Zlatkin *et al.*, 1991; Tada *et al.*, 1995; Kimura *et al.*, 1995; Miyake *et al.*, 2003; Riesenfeld *et al.*, 2004; Oh *et al.*, 2009; Kumar *et al.*, 2011). In a recent study conducted on oligotrophic bacteria isolated from Lechuguilla Cave, New Mexico, it was found that majority of recovered oligotrophic bacteria were resistant to antibiotics; even some strains were resistant to 14 different antibiotics used in the study (Bhullar *et al.*, 2012). Studies have also shown that most of the antibiotic resistance genes were plasmid-borne (Kimura *et al.*, 1995; Tada *et al.*, 1995). Very recently, two large plasmids, pREV1 and pREV2 (about 150 and 250 kb, respectively) from oligotrophic bacterium *Ancylobacter vacuolatus,* were shown not only to carry resistance genes for chloramphenicol and trimethoprim but also have been predicted to play a significant role in bacterial survival in low-nutrient condition(Zlatkin *et al.*, 2012). The genome sequence analysis of *Minibacterium massiliensis* (bacterium recovered from 0.22μm filtered ultra-pure water in a hospital setting and was found resistant to chemicals used for water disinfection), a *beta-proteobacteria,* revealed several antibiotic resistance genes on their genome including resistance to penicillin and streptomycin as well as genes coding for virulence factors (Audic *et al.*, 2007). *M. massiliensis* also contained a unique genomic island that encodes resistance to specific heavy-metal ions and metalloids that are generally used for water disinfection.

Freshwater is the fundamental requirement to sustain terrestrial life. It is a finite resource, essential for agriculture, industry and even human existence; without which sustainable development is not possible. Rivers are the main sources of fresh water. In past few decades the water quality 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 (Olimax and Sikorska, 1975; Piecznska *et al.*, 1975; Tiwari and Mishra, 1986; Vollenweidre, 1986; Milway, 1987; Reddy and Venkateswar, 1987). Deterioration of the fresh water quality is a global problem (Mahananda *et al.*, 2005). In developing countries, 90% of the sewage is being discharged directly into rivers, lakes, coastal waters without any treatment (World Resource Institute, 1996) (www.wri.org). Like that of the predecessors in this field of investigation, we pertained to the following hypothesis: "oligotrophic bacteria may be potential reservoir of novel antibiotic-resistance genes in the aquatic (riverine) environment".

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 Integron associated gene cassettes, 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. The study describes result of investigations aimed to uncover diversity of the culturable oligotrophic bacterial isolates, their antibiotic resistances phenotype, and antibiotic resistance determinants associated with class 1 integrons.

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 (chapter-1) for determination of antibiotic resistance cut-off points in case of oligotrophic bacteria by selecting proper reference strain was developed. Two international standard methods (CLSI and EUCAST) available for determining sensitivity/resistance are basically suited for copiotrophic isolates (that are grown in nutrient-rich media); hence for assessing antibiotic sensitivity of oligotrophic bacteria, sensitive reference strains from amongst the isolates were chosen for calculating LD_{50} (half lethal dose where half of the bacteria were killed in respect 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 (elaborated in chapter 1). A total of ninety composite water samples in three consecutive years (2007-2009) [each sample is a mixture of three grab samples collected per sampling site per month(except July and August, being avoided due to heavy downpour)] from river Mahananda were analyzed. The geographical location(s) of sampling sites, SS I, SS II, and SS III, are as follows: 26°44′22.62″ N, 88° 25′21.92″E (SS I), 26°44'23.20"N, 88°25'22.89"E (SS II), and 26°38′42.44″ N, 88°24′19.67″E (SS III). The oligotrophic bacterial load of river Mahananda at three sites of sampling 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. It was observed that facultative oligotrophic bacteria could survive on rich nutrient plate for longer period (more than 6 months if the plate is stored under suitable condition preventing desiccation of agar medium) compared to copiotrophic bacteria (model copiotrophic bacterium, *Escherichia coli* failed to survive beyond 20 days on Luria agar plate); owing to their adaptability to resist more adverse condition. This phenomenon also explains why oligotrophic bacteria attain adaptive fitness over copiotrophs in nutrient-scarce environment. The ability to switch from oligo to copiotrophic condition and vice-versa is an important phenomenon of facultative oligotrophs which makes them sustainable in fluctuating nutrient conditions which often prevail in an environment. 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 facultatively oligotrophic bacteria, 47% were SAR (single-antibiotic-resistant) and 53% were MAR

(Multiple-antibiotic-resistant). Surveillance programme in this study has enabled to understand the size of the problem and suggest strategies for control of antibiotic resistance. The results of this study presented in chapter 1 revealed high frequency of antibiotic-resistance in oligotrophic bacteria. Reports of such studies are very rare as compared to copiotrophic bacteria reported from several laboratories throughout the world. Hence, the present study has broadened the complexity of the problem.

A major part of this research (Chapter 2, 4 and 5) was motivated by a desire (ought to be unbiased) 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. There are several classes of integrons have been reported till date, of which the most documented and well characterized are the class 1 integrons (Martinez-Freijo *et al.*, 1998, Martinez Freijo *et al.*, 1999, Chang *et al.*, 2000, Schmitz *et al.*, 2001, White *et al.*, 2001, Gebreyes *et al.*, 2002; Thungapathra *et al.*, 2002; Jones *et al.*, 2003; Lindstedt *et al.*, 2003; Chen *et al.*, 2004; Nandi *et al.*, 2004; Betteridge *et al.*, 2011; Nardelli *et al.*, 2012; Stalder *et al.*, 2012). The following three different segments (Fig 2.1) that constitutes a class 1 integron are: (i) 5' conserved segment (CS) containing an *intI* gene that codes for integrase and an attachment site (*attI*) recombination site; (ii) the 3' CS containing a combination of antiseptic resistance gene (*qacE*), sulfonamide resistance gene (*sulI*), and one or two additional open reading frames, ORF5/and or ORF5 and ORF6 that are hypothesized to act as transposition sites of the progenitor *ISCR1* element (Toleman *et al.*, 2006) for development of complex class 1 integrons; and (iii) a variable region between 5' CS and 3' CS (Hall, 1997; Hall and Collis, 1998). Variable region is the part of class 1 integron platform where integration and excision of gene cassette (a mobile non-replicating DNA element usually bear antibiotic resistance gene but other types of genes inserted within gene cassettes also have been reported) catalyzed by *intI1* encoded integrase (Collis and Hall, 1992) (Fig. 2.2 and 2.3). Selection of a total of 2188 facultatively oligotrophic bacterial colonies/isolates was random (without any human biasness), because research randomizer tool was used which generated a random set from non-selective plates serving as an universal set of numbers (each isolated colonies bearing a number) irrespective of the knowledge of the phenotype (sensitive, single or multiple-antibiotic-resistant). These randomly selected isolates were examined for the presence of class 1 integrons, using a highly reproducible PCR strategy (CS-PCR). PCR assay showed presence of class 1 integrons in eighty nine Gram negative isolates and one Gram positive isolate. Thus 4.1% (90/2188) isolates were found to carry class 1 integron in their genome, 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) (Fig 2.4A). The amplified amplicon lengths of variable region (as the methodology itself enables to amplify the variable region of the class 1 integrons) varied from 0.15 to 3.45 kb. Cloning and sequencing of amplified class 1 integron borne gene cassettes revealed that amplicon of size ~1.0 kb was predominating and was detected in 24.4% (22/90) of the total integron positive isolates; however very short sequence like 153 bp only were also detected from two isolates, MB62 and MB63 which did not carry any gene cassette within variable region and was designated as empty class 1 integron. The finding of empty class 1 integron is as a clear indication that the isolate are ready to capture a gene cassette of desired function for better adaptability and competency in the environment where they are living. 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,* two novel *dfrA* genes, *dfrA28* and *dfrA30* and type-B: *dfr-IIe,* a single gene cassette) conferring resistance to trimethoprim. Only very recently, Bhullar *et al.*

(2012) reported that aminoglycoside antibiotic resistance was common in oligotrophic bacteria isolated from Lechuguilla cave. In this study, bacterium MB35 revealed the largest amplicon of length ~3.5 kb containing array of four gene cassettes, *blaIMP-9-aacA4-oxa10-aadA2*. Amongst dual antibiotic-resistance gene cassettes in variable region of class 1 integrons of the isolates, frequency obtained for *dfrA-aadA1* type (10%) was highest followed by *dfrA17-aadA5* (~8%). The homology modeling of DfrA1 and nucleotide binding domain of AadA1 protein derived from MB47 produced three dimensional structure(s) similar to dihydrofolate reductase and nucleotide binding domain respectively of *Haemophilus influenzae*.

Besides antibiotic resistance gene cassettes, a number of gene cassettes (~29% of the total) bearing ORFs for unrelated function than the antibiotic resistance were observed. The analyses of gene cassettes bearing ORFs of unrelated function and comparison with the existing data exhibit that most of the ORFs were coding for hypothetical proteins. The CS-PCR product sequencing of OB05 and MB83 showed that the derived sequences was 75% identical (nucleotide-nucleotide) to the part of the genome of *Acidovorax avenae* subsp. *citrulli* (Ac. No. CP000512). The feature present in that part of *Acidovorax* genome included a portion of ribosomal large subunit pseudouridine synthase B. However the predicted polypeptide of the ORF generated from OB05 DNA sequence did not produce any significant similarity with existing sequences of the protein database. Sequence analyses of the amplicons of MB05 and MB70 revealed the presence of a truncated ORF of 64 amino acids that showed 73% identity with the methyl transferase protein from *Ralstonia eutropha* (Ac no. AAZ62060). A translated polypeptide from the sequence derived from bacterium MB09, signifying type I site-specific deoxyribonuclease, HsdR family, exhibited nearly 42% identity with a protein of the same family, found in *Thiomicrospira crunogena* (Ac. No. YP390604). Partial sequencing of the CS-PCR product obtained from MB12 showed the presence of a single ORF (*livM1*) of length 154 amino acids (Fig 2.8A). BlastP analysis (Table 2.4) of this putative polypeptide product exhibited best scores with ABC type branched chain amino acid transport system with 72% identity at amino acid level to the ABC transporter permease of bacterium *Aromatoleum aromaticum* EbN1 (Ac. No. NC_006513). The multiple sequence alignment of LivM1 derived from amplicon sequence of bacterium MB12 with its neighboring ABC transporter proteins is shown in Fig. 2.8B. The transmemebrane (TM) regions prediction revealed the presence of four transmembrane regions in the putative LivM1 protein (ID: CAQ53856) of MB12 (Fig. 2.8C and 2.8D). The predicted transmembrane (TM) regions were at positions (AA position), 2-20, 50-74, 87-110, and 119-138. The conserved domain search for putative translated product obtained from translated ORF of MB12 integron sequence revealed that the predicted protein was one of the members of two TM subunits which play role in uptake of branched chain amino acids (Fig 2.8E). Mapping of signature motifs (Fig 2.8F) revealed that 19 of 19 [TM_PBP1_LivM_like, a type of transporters which consist a PBP (periplasmic binding protein), two TMs (trans-membrane subunits) and two cytoplasmic ABCs (ATP-binding cassette)] residues which were recognized on the TM-ABC transporter protein sequences of other bacterial species were also found conserved on ABC protein derived of MB12. It was assumed that in these types of transporters probably play vital roles in the import of solutes from environment. Other workers have also reported unrelated genes inserted as cassettes within class 1 integron (Nemergut *et al.*, 2004; Gillings *et al.*, 2008a, 2008b). Finding of these transporters in class 1 integrons has a greater significance since oligotrophic bacteria thriving in low nutrient milieu may require improved transporter molecules to concentrate and channelize nutrients from outside to the interior of the cells. The theoretical pI and instability index of the putative ABC transporter of MB12 was computed 9.3 and 19.13. The protein was predicted as stable protein with an estimated half life of 10 h (*in vivo* in respect to *E coli*) with a high aliphatic index of 127.21. MB16 was found to carry gene cassette carrying *appA* gene encoding bacterial extracellular solute binding protein. ProtParam computing showed that the extracellular binding protein derived from bacterium MB16 was stable and its instability index was computed 18.94. The GRAVY (grand average hydropathicity),

aliphatic index and theoretical pI was computed -0.087, 78.5, and 9.26 respectively. Psortb, a tool for subcellular localization prediction showed that the translated product of *appA* gene was periplasmic with localization score of 9.44.

Sequence analysis of amplicon obtained from MB 19 has shown the presence of a unique hybrid DNA sequence. In the 867 nucleotide long sequence, a continuous stretch of 242 nucleotides (from 3 to 244) produced 96% identity with a vertebrate (*Lepilemur dorsalis)* genomic fragment (Ac. No. AJ244007) and residual 623 nucleotide stretch produced (from nucleotides 245 to 867) 74% identities with *Burkholderia xenovorans* LB400 genomic DNA. This genomic DNA region of *B. xenovorans* coded for the putative reverse transcriptase maturase protein. MB 19 sequence yielded an ORF of 227 amino acids that has shown 65% identity with reverse transcriptase maturase protein from *Burkholderia cenocepacia* HI2424 (Ac. No. YP833935). An ORF of 164 amino acid residues (495 nucleotides) obtained from amplicon sequence of MB 22 shared 81% amino acid identity with phosphoribosylformylglycinamidine synthase (FGAM synthase) from *Acinetobacter baumannii* (Ac. No. YP001712860). The partial sequence of MB 28 (408 nucleotide) was 86% identical with *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 (Ac. No.CP000647) genomic DNA. The amino acid sequence (truncated protein) derived from amplicon of bacterium MB28 was encoding a bifunctional putative transcriptional regulator protein, this truncated protein of 84 amino acids shared homology of 96.3% with the similar protein present in *Klebsiella pneumoniae* (Ac. No.CP000647). The Complete sequence of 704 bp amplicon of MB 40B (Table 2.3B) yielded no significant homology with any nucleotide sequence available in the database. The same sequence yielded a complete ORF of 102 amino acids and BlastP analysis of this protein sequence revealed 58% identity with helicase domain protein of *Verminephrobacter eiseniae* EF01 (Ac. No. ABM585806). The sequences derived from amplicons of OB 12, MB 08, MB41, MB 44, MB 48, MB 51, MB 54, MB55, MB80 and MB81 did not produce any significant homology with any protein sequences available in the database. All of them were characterized as hypothetical proteins. All these polypeptides shared very low level of identity (2.03 to 25.19%) among either with each other or to pre-deposited hypothetical protein sequences. The translated product (Fig. 2.9A) of bacterium MB54 revealed a conserved domain belonging to the UPF0153 superfamily (Fig. 2.9B) when subjected to the BlastP search. However the function of proteins of this family is still unknown; since the proteins of this family contains 8 conserved cysteines hence it was expected that they may constitute a metal binding site and therefore might play a role in metal sequestering like Fe-S cluster as part of oxido-reductase complex. It is apparent that such predicted protein, although putative, in oligotrophic bacteria may play a role in iron acquisition. It is also speculated that there may be other multiple specific systems or mechanisms/and or a systems with multiple functions for transporting microelements and nutrients which are limiting in oligotrophic environment.

Protein homology and CDD search of 178 amino acid long protein derived from the MB 56 isolate was found to carry a conserved protein domain, encoding hemolysin activator/ secretion protein involved in intracellular trafficking and secretion. This hemolysin activator protein of MB56 produced 55% identity with the hemolysin activator protein of *Acinetobacter* sp. (Ac. No. YP045656). Translation of DNA sequence obtained from MB 58 and MB71 yielded a truncated ORF of polypeptide of 181 amino acids. The BlastP analysis of the said truncated polypeptide yielded best score (97% identity) with transposase protein of *Acidovorax* sp. (Ac. No. YP987142). Expression of such genes may enable the bacterium to be an opportunistic pathogen.

Despite other important findings, two novel dihydrofolate reductase genes, *dfrA28* and *dfrA30* were also detected and characterized (chapter 4 and 5). The expression study of *dfrA30* showed that the novel *dfrA, dfrA30* was functional and could resist trimethoprim resistance up to the level 1000 mg/L. The resistance level was too high with respect to the settled breakpoint (sensitive ≤/resistance >is 2/4 mg/L) of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) parameter.

It was observed that the integron load was highest at sampling site II, which may be due to selective pressure of high anthropogenic activity and diverse pollutant concentration at this site. An important relation between calculated resistance index (no. of antibiotics rendered resistant / no. of antibiotics tested) and frequency of the presence of class 1 integron structure. It was found that isolates corresponding to resistance index (RI) between 0.5-0.9 yielded amplicons maximally in the CS-PCR assay. Therefore, isolates with RI 0.5-0.9 has the maximum propensity to carry class 1 integrons supporting recommendation for selection of isolates for short-term snap-shot study aiming at to understand diversity of class 1 integron borne antibiotic-resistance-gene cassettes.

The taxonomic characterization of integron-positive isolates presented in chapter 3 has revealed the diversity of class 1 integron bearing bacteria of the Mahananda River at Siliguri. For classification and identification, phenotypic tests including total protein profiling, and genotypic tests including 16S rRNA gene sequence analysis were done. UPGMA dendogram constructed using binary data generated from phenotypic characters of all the isolates corroborated with the protein profiling data. The 16S rRNA gene sequences of integron positive bacteria fell under two main classes, *Betaproteobacteria* and *Gammaproteobacteria*. Only two isolates belonging to the genus *Comamonas* and *Acidovorax* were identified in class *Betaproteobacteria*. Both the said genera were comprised by single family, *Comamonadaceae*. Other isolates were identified under super class *Gammaproteobacteria*. The representative genera of class *Gammaproteobacteria* were constituted by families, *Moraxellaceae, Pseudomonadaceae, Aeromonadaceae* and *Enterobacteriaceae*. Similar to previous reports published on presence of integrons in copiotrophic bacteria, majority of the identified integron positive oligotrophic bacteria of super class *Gammaproteobacteria* were detected from the family *Enterobacteriaceae*. (Mukherjee and Chakraborty, 2006; Chen *et al.*, 2011; Han *et al.*, 2012; Su *et al.*, 2012). 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. On the other hand, the 16S rRNA gene sequences of few isolates (the isolates which could not be assigned any genus) poorly shared with the known genera of different classes. However from phenotypic data, these unidentified isolates clustered with members of family *Enterobacteriaceae* (Fig 3.3). One novel Gram positive bacterium belonging to the genus *Brevibacterium* was finally assigned a status of a novel species employing polyphasic approach including16S rRNA phylogeny, chemical taxonomy, lipid analyses cellular carbohydrate analyses, cell wall amino acid detection, fatty acid methyl ester analysis, DNA base composition and a large number of physiological and biochemical tests. The bacterium was named as *Brevibacterium siliguriense* sp. nov.

Evaluation of antibacterial property of zinc oxide nanoparticles/Quantum dots with surface adsorbed acetate ion (ZnO-Ac) having size 3-5 nm was conducted using a multiple-antibiotic-resistant (resistant to ten antibiotics: cotrimoxazole, ampicillin, gentamycin, netilmicin, tobramycin, chloramphenicol, cefotaxime, kanamycin, streptomycin and trimethoprim) facultatively oligotrophic, and human serum-tolerant isolate of the genus *Klebsiella, K. penumoniae* strain MB45; results of which have been presented in Chapter 6. In recent years, a number of nanosized metals (called as nano-particles) like Ti, Mg, Ag, and Zn have been documented as important alternative candidate of antibiotics in controlling pathogenic bacteria (Joshi *et al.*, 2009). Among the different oxide derivatives of nanosized metals, zinc oxide have been observed as effective drug possessing antibacterial activity (Stoimenov, 2002; Nair *et al.*, 2008; Hanley *et al.*, 2008; Applerot *et al.*, 2009; Joshi *et al.*, 2009) not just because of their stability under harsh processing conditions, but also because they are found as safe materials for human beings and animals (Stoimenov *et al.*, 2002; Fu *et al.*, 2005). Results revealed that growth of *K. penumoniae* strain MB45 was completely arrested at concentration of 500 mg/L ZnO-Ac QDs. Apart from the general direction of the search elucidated in the preceding chapters, the final chapter leaves a ray of hope to curb the menace.

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Publications/ Abstract/ Proceedings, Culture deposition and Sequences deposited to databases

(A) Publications

1. Mandal AK, Yadav KK, Sen IK, **Kumar A,** Chakraborti S, Islam SS and Chakraborty R. 2012. Partial characterization and flocculating behavior of an exopolysaccharide produced in nutrient-poor medium by a facultative oligotroph *Klebsiella* sp. PB12. *Journal of Bioscience and Bioengineering.* PMID: 22944200. doi: 10.1016/j.jbiosc.2012.08.006.

2. **Kumar A,** İnce İA, Kati A and Chakraborty R. 2012. *Brevibacterium siliguriense* sp. nov., a novel facultatively oligotrophic bacterium, isolated from river water. *International Journal of Systematic and Evolutionary Microbiology* (in press). PMID: 22523163. doi:10.1099/ijs.0.038281-0.

3. **Kumar A,** Chakraborti S, Joshi P, Chakrabarti P and Chakraborty R. 2011. A multiple antibiotic and serum resistant oligotrophic strain, *Klebsiella pneumoniae* MB45 having novel *dfrA30*, is sensitive to ZnO QDs. *Annals of Clinical Microbiology and Antimicrobials,* 10:19. PMCID: PMC3118321.

4. **Kumar A,** Mukherjee S and Chakraborty R. 2010. Characterization of a novel trimethoprim resistance gene, *dfrA28*, in class 1 integron of an oligotrophic *Acinetobacter johnsonii* strain, MB52, isolated from River Mahananda, India. *Microbial Drug Resistance*, 16: 29-37. PMID: 20192821.

(B) Symposia/conference/Congress

1. **Kumar A,** and Chakraborty R. 2012. Nanobiotic solution to control a potentially pathogenic β-Hemolytic *Aeromonas* sp. strain MRA221, resistant to Human Serum and Multiple Antibiotics. *In* abstract volume of "*National conference on Biology and Bioinformatics of Economically Important Plants and Microbes*" held at from 17-19 February, organized by, DRS Department of Botany, Bioinformatics facility, University of North Bengal, Siliguri, West Bengal, India. (Oral).

2. **Kumar A,** Chakraborti S, Chakrabarti P and Chakraborty R. 2011. A potentially pathogenic oligotrophic *Aeromonas* strain MB21 is sensitive to ZnO Quantum Dots. In abstract volume of "*4th Congress of European Microbiologists*, FEMS 2011 held at Geneva, Switzerland. June 26 -30. (Poster No. 126).

3. Chakraborty R, **Kumar A,** Bhowal S, Mukherjee S, Sarkar S, Kumar D, Mandal AK, Tiwary BK, Singh P. 2011. Fluidity of gene cassettes in antibiotic sensitive and resistant oligotrophic bacteria of river Mahananda at Siliguri, India. In abstract volume of "*4th Congress of European Microbiologists*", FEMS 2011 held at Geneva, Switzerland. June 26 -30. (Poster No. 6).

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5. Mandal AK, **Kumar A,** Chakraborti S and Chakraborty R. 2011. Testing the Efficacies of ZnO-QDs as Potential Antibacterial Agent for Multi-Drug Resistant Enteropathogens. *In* abstract volume of "*Young Researchers conference 2011*", held at Institute of Chemical Technology, University of Mumbai during January 13-14. (Poster).

6. Mandal AK, **Kumar A,** and Chakraborty R. 2010. Towards Explaining the Molecular physiology and Resistance to Hydrogen Peroxide and Nano-Particle Killing of A Gram Negative Facultative oligotrophic Strain PB12, Isolated from the River Mahananda, Siliguri, India". *In* abstract volume of "*Diversity and Prospects of Microbial Resources*" from 26-28 February, organized by, Department of Microbiology, University of North Bengal, Siliguri, West Bengal, India. (Poster).

7. **Kumar A**, Mukherjee S, Bhowal S and Chakraborty R. 2008. Diversity of oligotrophic riverine bacteria carrying gene cassette capturing genetic devices in their genome. *In* abstract volume of "*Diversity and Functionality of Plant and Microbes*" held at Jan 24-25, 2008 at Dept. of Botany, University of North Bengal, W.B, India. (Poster).

(D) Bacterial cultures deposited in culture Banks:

Kumar A, İnce İA, and Chakraborty R. 2010. *Brevibacterium siliguriense* strain MB18^{T} deposited under following accession numbers provided by respective culture collection centers-

- **BCCM^{TM}/LMG** Bacteria Collection, University Gent-Laboratorium voor Microbiologie, Gent Belgium, Ac. No. **LMG 25772,** and
- **DSMZ** Inhoffenstraße 7 b 38124 Braunschweig Germany, Germany, Ac. No. **DSM 23676**.

(E) Nucelotide Sequences submitted to GenBank/ EMBL database:

Accession numbers: AM937241 to AM937247, AM997271 to AM997282, AM991326 to AM991334, FM179325 to FM179328, FM955254, FM955483, FM955255, FM958478, FM998811, FM865443 to FM865447, FM865448, FM865635, FN178516, FN178517, FN178520, FN263373, FN263374, FN396607, FN396608, FR677018, FR677019, FR677020, FR677021, HE650979 to HE650986, HF562221 to HF562233, HM989848.

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