STUDIES ON ANTIBIOTIC RESISTANCE PATTERNS OF BACTERIAL POPULATION OF TORSA RIVER WATER AND MOLECULAR CHARACTERIZATION VIS-À-VIS ASSESSING THE POTENTIAL FOR GENETIC EXCHANGE OF RESISTANCE PLASMIDS

Thesis submitted for the Degree of Doctor of Philosophy in Science (Botany) of the University of North Bengal

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Certificate of Supervisor

This is to certify that the work presented in the thesis entitled *'Studies on Antibiotic Resistance patterns of Bacterial Population of Torsa River Water and Molecular Characterization vis-à-vis Assessing the Potential for Genetic Exchange of Resistance Plasmids'* is the original work of Smt. Shriparna Mukherjee, M. Sc., under my supervision at the Department of Botany, North Bengal University. The results incorporated in the thesis have not been submitted for any other degree elsewhere.

Further, I certify that Smt. Shriparna Mukherjee has followed the rules and regulations laid down by the university in carrying out the research.

16.12.05

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"Where the mind is without fear
and the head is held high;
Where knowledge is free;
Where the world has not been broken
up into fragments by narrow domestic
walls;
Where words come out from the
depth of truth;
Where tireless striving
Stretches its arms towards
Perfection;
Where the clear stream of reason
Has not lost its way into the
Dreary desert sand of dead habit;
Where the mind is led forward
by thee into ever-widening thought and action-
into that heaven of freedom,
my Father,
let my country awake".

— Rabindranath Tagore

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PREFACE

The river Torsa is the second largest river of Northern West Bengal and also an international river, intersecting three countries, China (Tibet), Bhutan and India. Concentrated urban developments, with increased human activity, in the nearby region of Torsa river basin have contributed to the degradation of water quality of this river. Human uses of water for almost all purposes result in the deterioration of water quality and generally limit the further potential use of water. Certain natural phenomena like torrential rainfall may also be the contributor for degrading water quality of a fresh water body. Such natural events may be frequent or occasional. Permanent factors like human activities may make water unfit for drinking or for specific uses and may serve as one of the major contributors for communicable diseases.

One periodic monitoring program was carried out to ascertain the physico-chemical characteristics of the Torsa river water as well as to determine the sanitary status by MPN determination. It was apprehended that microbiological examination should be carried out with the more definite objectives other than simply assessing the degree of fecal contamination. Therefore, another important aspect included was to explore the nature of microbial communities present in the river under study with special emphasis on the incidence and abundance of the antibiotic and metal resistant bacterial population.

Antibiotic resistant copiotrophic bacterial populations recovered from water samples of river Torsa exhibited very wide variations with large dispersion values. Significant differences in occurrence of different antibiotic resistant populations were recorded in various sampling months. Although antibiotics are needed to control bacterial infections, they can have broad, undesirable effects on microbial ecology. That is, they can produce long-lasting change in the kinds and proportions of bacteria – the mix of antibiotic susceptible types. On a large scale, antibiotic resistance that emerges in one place can often spread far and wide.

With this end in view, a further detailed study was needed that ultimately led to frame the presented research program. This study has been undertaken to provide detailed descriptive information about the antibiotic resistance patterns (ARPs) of the copiotrophic bacterial population and also to explore whether antibiotic resistance patterns among isolates from different sampling sites and seasons vary in a systematic manner or not. Isolation and molecular characterization of plasmids from the multiple-antibiotic-resistant (MAR) bacteria with an objective to evaluate their role in the horizontal transfer of resistance genes between bacteria of different species and genera have constituted another major area of this work.

General Introduction

"..... For men may come and men may go, but I go on for ever....."

-Tennyson.

The facts of history, vis-à-vis the plight of most mankind today are sufficient evidences that the problems of man and his environment are not problems of the men of individual nations. They are problems of all men and all nations. This is especially true of water. The mobility of water is one of its useful properties, but it also gives rise to serious problems, both practical and scientific, international as well as national.

River is not just a large flowing water body, but always remained in the driving seat of all human civilizations. Since the dawn of civilization, increased number of people and proliferation of their activities have depended on surmounting natural environmental restrictions, including the amount and distribution of water. Water development and water policies always have been important, as is evident from the many physical and administrative measures to control its distribution and use, beginning with the Indus valley civilization and becoming even more complex with the passage of time. Even so, water problems are becoming increasingly critical in many regions even where water is relatively abundant. The reason is that in many regions problems are less apt to relate to water quantity than to its quality. Broadly stated water problems are few but basic: distribution in space (too much or too little); distribution in time (too much in some seasons or years and not enough in others); chemical quality (too high mineralized; lacking in desirable minerals; containing deleterious minerals); and pollution.

If the liquid comprising the natural stream were pure water, the river story would differ greatly from its actuality. But, no river water is pure. The impurities of each stream are characteristic of it and for certain periods of observation, constant. Their chemistry is related to climate and terrain. The water of a very large river, composed as it is of tributary contributions from various sources, represent as nearly as possible the average of its region.

The impurities that occur in natural water fall under three groups. The first group comprised of completely dissolved substances present as separate molecules or ions, dissolved gases, dissolved minerals (salts of Na, K, Ca, Mg, Fe, Al, NH_4, Mn), heavy metals (Cu, Pb, Hg, Ag, Zn), and various organic substances (eg. phenols, formaldehyde etc.). The second group consist of colloidal substances of mineral origin such as SiO_2, $Al(OH)_3$, $Fe(OH)_3$ etc. The third group consists of suspended matters like clay, silt, sand etc. The natural impurities are derived from atmosphere, catchment areas and the soil. They are in very low amounts and normally do not pollute water and it is potable. But the fact remains that less than 0.01% of Worlds water is fresh water and <1% of that is potable water; the rest is polluted fresh water.

Adequate supply of fresh water is the basic need for all human beings on earth, yet it has been observed that millions of people worldwide are being deprived of this. Freshwater resources all over the world are threatened not only by over

exploitation and poor management but also by ecological degradation. Population growth, urbanization, unplanned development, land degradation and lack of infrastructure for waste disposals have serious and adverse impacts on fresh water bodies. All these causes have a cumulative effect leading to the rapid deterioration in water quality in majority of rivers, streams and lakes worldwide.

The pollution of rivers and streams with various contaminants has become one of the most crucial environmental problems since the 20th century. International Conference on Primary Health Care held in Alma Ata in 1978 identified the provision of an adequate supply of safe water as one of the eight components of primary health care. In most countries the principal risks to human health are associated with the consumption of polluted water. Water borne transmission is a highly effective means of spreading infectious agents to a larger portion of the population. An estimated 80% of all human diseases and over one third of deaths in developing countries are caused by the consumption of contaminated water and on average as much as one tenth of each person productive time is sacrificed to water related diseases.

In this perspective, the monitoring of river water quality is gaining importance both nationally and internationally. One of the only global attempts at water quality monitoring has been the UNEPGEMS/WATER program that examined data from 82 major river basins worldwide over a period of a decade and a half (www.gemswater.org). Yet the numbers of monitored watersheds were too sparse and the frequency and the type of measurements were too inconsistent to point a comprehensive picture of global water quality trends.

India is bestowed with many rivers and rivulets, which in turn has supported life of Indians in all possible ways. However, in the present time they are also inviting our attention to the threats they are receiving from their benefactors. Today, in India, the most significant environmental problem and threat to public health in both rural and urban, is inadequate access to clean drinking water and sanitary facilities. Of the urban population, 84.9% had access to clean drinking water in 1993 as compared to 69% in 1985, but for the rural population the figure fell from 82% in 1985 to 78.4% in 1993 (World Resource Institute, 1995) (www.wri.org). According to water demand projections, the domestic and industrial sectors are expected to experience a 3 fold and 2-fold increase, respectively, in water demand by 2025.

India has had environmental legislation dealing with water pollution in the Indian penal code since 1960. However this law and similar laws over the next 100 years would be dealing with only public springs and reservoirs used for the purpose of drinking. The water quality was considered legally as an environmental issue after implementation of 1974 water (Prevention and Control of Pollution) Act. This act along with the environmental pollution act (which followed in 1986), empowered the Central Pollution Control Board to lay down and maintain ambient water standards, to demand information regarding effluent emission, to shut down pollution activities and to prevent new discharges of effluent and sewage. The water quality monitoring of rivers is being done under the national water quality-monitoring programme.

The state of West Bengal is geographically networked with a variety of perennial rivers. Broadly speaking, West Bengal has two natural divisions - the magnificent belt of the Himalayas in the northern part and alluvial plain in the southern part. The sub Himalayan Northern West Bengal has variety of renewable and non-renewable resources. A variety of swift flowing rivers along with their tributaries are flowing through this region from time immemorial. Among which four major snow-fed rivers are Teesta, Torsa, Jaldakha and Mahananda. Of late, this repository of natural wealth has been subjected to a virtual plunder. In consequence, the local ecosystems are fast loosing resilience and regenerative capacity. The ever-increasing influx of population, simultaneously by keeping pace with the changes in the land use pattern, has affected the region in a dynamic way. Such natural effectors are serving as the major causes of environmental hazards of this particular region. Since for centuries, rivers are being used as the dumping grounds for all these types of effluents, this naturally has acted as the sole cause to change the original character of the water. These changed characteristics of the river waters, therefore, demand thorough scientific investigations.

Torsa is the second largest river of North Bengal and also an international river. It traverses four countries - China (Tibet), Bhutan, India and Bangladesh. Torsa is called as Amo-Chu in Bhutan. It rises in Tibet, where it drains the Chumbi valley and enters Bhutan, north of the frontier settlement of Pasha. The river flows rapidly and follows a confined valley between precipitous mountains. She cuts across in a southeasterly direction and passes by the market town of Phuntsholing on the Indo-Bhutan border. In the plains of North Bengal it is familiar as Torsa. As it leaves the foothills of Bhutan and enters undulating Dooars plain in the northern part of West Bengal, it widens into a braided channel. Phuntsholing, the southern terminus of the Indo-Bhutan road, lies on the left bank of the Torsa. To the west of the river, Buxa-dolomites formed striking ridges, which is observable from Phuntsholing. Concentrated urban development and establishment of cement factories in the nearby region must have contributed to the degradation of the water quality of this river. River Torsa and its tributaries are of considerable importance because they drain the Jaldapara Wild Life Sanctuary, as well as the Totopara (where the endangered and anthropologically high valued Toto lives). Torsa flows through human settlements, tea gardens and agricultural lands. As a consequence of pest control practices in the tea gardens, use of nitrogen fertilizers in the agricultural fields and several point and nonpoint sources of sewage disposals, the hazards of persistent pollutants might have increased in the river water. These might have caused serious health problems and there are reports on several outbreak of water borne diseases in this region. It was therefore important to know whether the quality of the water remained satisfactory or not, over the entire length of the river in the Indian province. It was also pertinent to know if the river water is suitable even for drinking purposes by direct surface intake, after a simple clarification, filtration and chlorination. Water quality monitoring coupled with hydrological parameters of rivers of North Bengal has generally been overlooked for various reasons. Those are mainly resource and manpower constraints, institutional inertia and public apathy due

to lack of awareness. Water quality directly influences many issues in this part of West Bengal. Human health, agriculture, aquaculture, industry and tourism, all are vital to regional economy as well as national development. So a comprehensive monitoring and water quality control strategy is needed to serve an explicit purpose, be it for environmental impact assessment, project planning and design, project evaluation, or for regulatory requirements.

"Water quality" is not to be defined objectively. It should be defined socially, as to the desired use of water. Water quality monitoring has evolved along two different lines. One of which is a river basin approach. Another approach is an integration of chemical and biological parameters to measure condition. One potential problem with river basin level approaches is that such basins often cross-state and national boundaries necessitating the need for a high level of governmental and intergovernmental coordination and monitoring activities.

Monitoring of the chemical quality is usually undertaken in two different directions, one includes the routine monitoring of known problem substances while the other includes periodic quality assessment. The later type of monitoring is either routine or non-routine assessment of water quality done on a relatively infrequent basis (annual or greater). Such assessments are likely to include a wider analytical range. They are used to provide regular comprehensive assessments of water quality. It also assists in long-term water source and supply management, and long-term trend analysis.

In certain instances, monitoring of microbiological quality of water is much more important than monitoring of chemical quality and chemical testing. Because health hazards caused by chemicals are chronic rather acute. Changes in water chemistry tend to be long term unless a specific pollution event has occurred. On the other hand, microbiological monitoring is a practical method to determine the potential health risk of water exposure.

Natural waters are rich in bacteria, algae, protozoa, worms and other organisms. The greater the amounts of organic nutrients in water, the faster the biological contamination of water. Bacteria are the most frequently occurred microorganisms. They take an active part in the formation of all aquatic populations. The composition of the micro flora or fauna of a stream or a river serves as a good indicator to determine the extent of pollution. Along with their indicative property, such natural microbial population, in turn, function as the vectors, causing rapid and widespread dissemination of the water borne diseases leading to high rates of morbidity and mortality.

Since their first isolation from feces in the late 19th century the coliform group of bacteria has been used as an indicator of the bacteriological safety of water (APHA, 1989). Total coliforms as a general group are not particularly useful in terms of estimating human health risks because they can also be found in soil and plants naturally. The fecal coliform group comprises the genera *Escherichia* and to a lesser extent, *Klebsiella* and *Enterobacter*. High levels of fecal indicator bacteria in rivers and streams can indicate the possible presence of pathogenic (disease causing) microorganisms. Cholera, typhoid

fever, bacterial dysentery, infectious hepatitis and cryptosporidiosis are some of the well known waterborne diseases that spread through water contaminated with fecal matter. However, in many fresh water systems, fecal bacteria are of little numerical significance despite the fact that they are discharged into almost all inland waters.

A number of bacterial enteropathogens, namely *Campylobacter jejuni/coli, Salmonella, Shigella, Plesiomonas, Aeromonas, Vibrio cholerae* and *Escherichia coli,* were isolated from river water sources (Obi *et al.* 2002). These enteric bacterial pathogens are variously incriminated in cases of diarrhoea, which accounts for a substantial degree of morbidity and mortality in different age groups world wide (Black *et al.* 1993, Nath *et al.* 1993, Prado and O'Ryan 1994, Obi *et al.* 1997, 1998, El-Sheikh and El Assouli 2001). Isolation of pathogens from water sources connotes a serious public health risk for consumers. To further compound this problem, enteric pathogens have been widely reported to demonstrate resistance to several antibiotics (Cooke 1976, Kelch and Lee 1978, Hoge *et al.* 1998, Obi *et al.* 1998, Boon and Cattanach 1999, McArthur and Tuckfield 2000, Engberg *et al.* 2001, Ash *et al.* 2002, Lin *et al.* 2004). For example, in 1984, 82% of *Campylobacter* strains from Lagos, Nigeria were sensitive to erythromycin and 10 years later 20.8% were sensitive (Coker and Adefoso 1994). In Thailand ciprofloxacin resistance among *Campylobacter* species increased from 0% before 1991 to 84% in 1995 (Hoge *et al.* 1998). Strains of *Salmonella typhi* with multiple resistances to chloramphenicol, ampicillin and trimethoprim have led to several outbreaks (Rowe *et al.* 1997). Presence of antibiotics and the bacteria resistant to them in various environmental compartments like surface water, ground water, soils etc. may pose a serious threat to public health in that more and more infections may no longer be treatable with known antibiotics. A recent study (Ash *et al.* 2002) showed that several rivers in the United States of America have become a major reservoir for antibiotic resistant microbes.

Antibiotic pollution has been a growing concern as it has the potential for several human and environmental health impacts. The source from where the antibiotic resistant microorganisms are originating is of less importance. But it is alarming that gradually the rivers are becoming reservoirs of antibiotic resistant bacterial population. As rivers are one of the major sources of water, directly or indirectly, for human and animal consumption, this pollution may contribute to the maintenance and even spread of bacterial antibiotic resistance. Since these antibiotic resistant bacteria are significant environmental contaminants, calls have been made for antibiotic resistance to be considered when establishing bacteriological water quality criteria. The results of the studies that have been carried out on the different rivers all over the world, deduce that monitoring of river waters on the ground of microbiological analysis by analyzing the antimicrobial resistance pattern of the native bacterial population is gaining importance day by day. The relevance of information obtained on the resistance of bacteria to antibiotics is to appreciate the magnitude of the problem and establish baseline for taking action. The survey of antibiotic resistances in the microbial flora of fresh waters allows detection of hidden uses that contribute to the increase of bacterial resistances and

thus limit the efficacy of these drugs in the treatment of human and animal infections. Simultaneously, monitoring resistance patterns in commensal bacteria could also provide valuable clues about non-antibiotic selection pressures.

The study of the phenomenon of antibiotic resistance is an active area of investigation that covers many aspects of gene transfer mechanism, including the biology of plasmids. Thus, there is interest in, not only the biochemical mechanism by which the determinants express their resistance, but also in the distribution, origin and dissemination of resistance mechanisms. Plasmid mediated resistance to antibiotic was discovered in Japan because of the unexpected appearance of multiple drug resistance during an outbreak of bacillary dysentery. Ever since this time, unexpected appearance of a new or unusual drug resistance marker or unusual pattern of multiple drug resistance has been a clue that plasmids might be involved as carriers of the resistance genes. Most studies on genetic recombination in bacteria have been conducted *in vitro* and there are few data showing that gene transfer occurs *in situ*. Antibiotic resistant bacteria containing conjugative R plasmids have been isolated from sewage-impacted waters in the US (eg. in Hudson river, the New York Bight and in Chesapeake Bay). Many of these strains contained plasmids that conferred resistance not only to antibiotics but also to heavy metals and to other antibacterial agents such as the algal product, chlorellin. It has been suggested by several authors that the selective pressure exerted upon the bacterial flora of animals by antibiotics, gives rise to large populations of resistant microorganisms. The organisms are then postulated to enter the human population either through agricultural practice or via the food chain. Once in contact with man, the resistant bacteria could presumably cause disease directly or transfer their resistance to organisms more pathogenic for humans.

The study relating to the distribution of antibiotic resistance bacteria in river water is not only important, at the same time it is also very much essential to identify the nature of the elements responsible for the development and spread of antimicrobial resistance to far and wide. In the event, that antibiotic resistance is spread from nonpathogenic to pathogenic bacteria, epidemics may also result. This spread represents an elegant, if not desirable, example of molecular evolution over a very short time scale. Recent studies have demonstrated that the majority of these multiple antimicrobial resistant phenotypes are obtained by the acquisition of external genes that may provide resistance to entire class of antimicrobials. A number of these resistance genes have been associated with various gene transfer elements namely plasmids, transposons, integrons and so on, which take a very active part in the development of multi-drug resistance and rapid dissemination of resistance genes among different bacterial genera and species. Loss of efficacy through the emergence and transfer of bacterial antibiotic resistance is an increasing reality. Bacteria have been observed to transfer resistance in laboratory settings as well as in the natural environment. In this context, several authors have emphasized the need to review water quality standards as they relate to the spread of antibiotic resistance genes in water borne bacteria carrying transmissible R factors. Gene transfer elements can- and often do- hunt as a

pack, by interacting with each other in a variety of ways that enhance their collective ability to transfer resistance genes. This interactive capacity needs to be taken into account when considering the potential for horizontal transfer of resistance genes. Although the acquisition of new resistance genes is an important factor in the increasing incidence of resistant strains, it is only part of the resistance story.

A critical but often under appreciated feature of resistance gene transfer elements is their stability. The widespread existence of plasmids in natural isolates and their apparent stability, even where antibiotics are not present argue against the widely held belief that plasmids and other gene transfer elements are readily lost in the absence of antibiotics. Moreover the abundance of antibiotic resistant strains in environmental settings where bacteria presumably do not come into contact with antibiotics suggests that resistance genes can also be stably maintained in the absence of antibiotic selection. Much is known about the fact that plasmids carrying multiple resistance genes can be held in bacterial strains by selection for any one of the resistance genes on the plasmid but remarkably little is known about the reasons for stability of gene transfer elements and resistance genes in the absence of any known selection pressure.

Horizontal gene transfer event permits movements of alleles among bacterial lineages, increasing the opportunities for the spread of antibiotic resistance. Although the actual contribution and consequences of horizontal gene transfer remain highly debated for a long time, its importance is immense as it leads to the "evolution in quantum leaps" (Jain *et al.* 2003). During bacterial evolution, the ability of bacteria to exploit new environments and to respond new selective pressures can often be more readily explained by the acquisition of new genes by horizontal transfer rather than by sequential modification of gene function by the accumulation of point mutations. Especially conjugal transfer itself can contribute to stable maintenance of antibiotic resistance genes in a bacterial population by continually reseeding the members of a population that have lost a resistance gene transfer element.

Keeping all these facts in mind, the assessment of the fluidity of the antibiotic resistance genes in the aquatic environment of Torsa river was done by thorough analysis of antimicrobial resistance patterns of the copiotrophic bacterial flora followed by investigating the elements and the mechanisms responsible for the development as well as spread of the antimicrobial resistance genes. The major objectives that had been set forth were:

1. To provide detailed descriptive information about the antibiotic resistances of culturable copiotrophic bacteria isolated from water samples of river Torsa.
2. To explore whether antibiotic resistance patterns among isolates from different sampling sites and seasons vary in a systematic manner.
3. To understand the molecular mechanism of spread and persistence of easy to get and hard to loose antibiotic resistance genes.
4. To explore the molecular diversity of antibiotic resistance gene cassettes.

References

American Public Health Association, Standard Methods for Examination of Water and Waste Water, 17th ed., APHA, Washington, DC, 1989.

Ash, R. J., B. Mauck, and M. Morgan. 2002. Antibiotic resistance of gram negative bacteria in rivers, United States. Emerg. Infect. Dis. **8**: 713-716.

Black, R. E. 1993. Persistent diarrhoea in children in developing countries. Pediatr. Infect. Dis. J. **12** : 751-761.

Boon, P. I, and M. Cattanach. 1999. Antibiotic resistance of native and fecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, south-eastern Australia. Lett. Appl. Microbiol. **28** : 164-168.

Coker, A. O, and A. O. Adefoso. 1994. The changing patterns of *Campylobacter jejuni/coli* in Lagos, Nigeria after ten years. East. Afr. Med. J. **71**: 437-440.

Cooke, M. D. 1976. Antibiotic resistance among coliforms and fecal coliform bacteria isolated from sewage, seawater and marine selfish. Antimicrob. Agents. Chemother. **9** : 879-884.

El-Sheikh, S. M, and S. M. El-Assouli. 2001. Prevalence of viral, bacterial and parasitic enteropathogens among young children with acute diarrhoea in Jeddah, Saudi Arabia. J. Health. Pop. Nutr. **19**: 25-30.

Engberg, J., F. M. Aerestrup., D. E. Taylor, Gerner-Smidt and Nachamkin. 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: Resistance and trends in human isolates. Emerg. Infect. Dis. **7**: 24-34.

Hoge, C. W., J. M. Gambel., A. Srijan., C. Pitarangsic, and P. Echevervia. 1998. Trends in antibiotic resistance among diarrhoeal pathogens isolated in Thailand over 15 years. Clin. Infect. Dis. **26**: 341-345.

Jain, R., M. C. Rivera., J. E. Moore, and J. A. Lake. 2003. Horizontal gene transfer accelerates genome innovation and evolution. Mol. Biol. Evol. **20**: 1598-1602.

Kelch, W. J, and J. S. Lee. 1978. Antibiotic resistance patterns of gram negative bacteria isolated from environmental sources. Appl. Environ. Microbiol. **36** : 450-456.

Lin. J., P. T. Biyela, and T. Puckree. 2004. Antibiotic resistance profiles of environmental isolates from Mhlathuze River, KwaZulu- Natal (RSA). Water SA. **30** : 23-28.

McArthur, J. V, and R. C. Tuckfield. 2000. Spatial patterns in antibiotic resistance among stream bacteria: Effects of industrial pollution. Appl. Environ. Microbiol. **66**: 3722-3726.

Nath, G., B. N. Shukla., D. C. Reddy, and S. C. Sanyal. 1993. Acommunity study on the aetiology of childhood diarrhoea with special reference to Campylobacter jejuni in a semi-urban slum of Varanasi, India. J. Diarrhoeal. Dis. Res. **11**: 165-168.

Obi, C. L., A. O. Coker., J. Epoke, and R. N. Ndip. 1997. enteric bacterial pathogens in stools of residents of urban and rural regions in Nigeria: A comparison of patients with diarrhoea and controls without diarrhoea. J. Diarrhoeal. Dis. Res. **15**: 241-247.

Obi, C. L., A. O. Coker., J. Epoke, and R. N. Ndip. 1998. Distributional pattern of bacterial diarrhoeagenic agents and antibiograms of isolates from diarrhoeic and non-diarrhoeic patients in urban and rural areas of Nigeria. Cent. Afr. J. Med. **44**: 223-229.

Obi, C. L., N. Potgieter., P. O. Bessong, and G. Matsaung. 2002. Assessment of the microbial quality of river water sources in rural Venda communities in South Africa. Water SA. **28**: 287-291.

Prado, V, and M. L. O'Ryan. 1994. Acute gastroenteritis in Latin America. Infect. Dis. Clin. North. Am. **8**: 77-106.

Rowe, B., L. R. Ward, and E. J. Threlfau. 1997. Multidrug resistant *Salmonella typhi*: a worldwide epidemic. Clin. Infect. Dis. **24**: 5106-5109.

Chapter 1

Antibiotic resistance patterns of culturable copiotrophic bacteria from river Torsa

1.1 Introduction

The antibiotic era began with the discovery of the first three significant antibiotics-tyrothricin, penicillin and actinomycin, in 1939 and 1940. Within the first 18 years of the antibiotic era, about 30 antimicrobial agents had come into use (Swartz 2000). Since their discovery, the use of manufactured antibiotics to control diseases has revolutionized medicine. It has also greatly reduced the threat of many once fatal illnesses. The use of these wonder drugs, combined with improvements in sanitation, housing and nutrition and the advent of widespread immunization programme, has led to a dramatic drop in deaths from diseases that were previously widespread, untreatable and frequently fatal. By helping to bring many infectious diseases under control, these drugs have also contributed to the major gains in life expectancy experienced during the latter part of the last century. These gains are now seriously jeopardized by another recent development: the emergence and spread of resistant microbes. 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.

1.1.1 Antimicrobial resistance in the environment

The bacterial resistance to multiple antibiotics characterizes the present decade. In a report by the UK House of Lords, it is stated: "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"(House of Lords) (Kummerer 2004). The percent occurrence of multiple antibiotic resistant bacteria (MAR) in different environmental compartments such as wastewater, surface water, ground water, sediments and soils, has been a growing concern. Resistance genes as well as resistant bacteria in the environment are increasingly seen as an ecological problem (Davison 1999). It has been evidenced that the selective pressure exerted following the widespread use and misuse of antibiotics in both medical and agricultural fields does select those bacterial strains possessing antibiotic resistance genes. These resistant bacteria, which develop, find their way into lakes and rivers. The potential for antibiotic contamination is just one part of the larger problem, an issue with both medical and environmental components.

1.1.1.1 Drinking water

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). These authors found the occurrence of resistant bacteria within the distribution network of drinking water supply systems. It was reported that the percentage of multiple antibiotic resistant (MAR) bacteria was significantly greater among isolates from distribution water samples than that of bacteria in corresponding untreated source waters (Armstrong *et al.* 1981). The study conducted by Diab *et al.* 2000 has proved the presence of antibiotic resistant gram-negative bacteria in several drinking water samples in Ismalia city of Pakistan. In agreement with these data, increased resistance rates were also detected in the drinking water from different sampling points in another study by Schwartz *et al.* (2003).

1.1.1.2 Ground water

Antibiotics are rarely found in ground water (Kummerer 2004). The presence of antibiotic resistant bacteria in ground water has been documented by several authors (McKeon *et al.* 1995). Sacher and coworkers (2001) analyzed 105 ground water wells in Baden-Wuettemberg, Germany. Among 60 pharmaceuticals tested, erythromycin-HO and sulfamethoxazole, which were the only antibiotics out of eight compounds, were detected in at least three ground water samples. Though microbial contamination of ground water sources, revealing the presence of coliforms above the permissible limits, has been recorded by Indian workers (Box: 1.1), no attempts have been made to assess the antibiotic resistance profile of the isolates.

Box : 1.1 Investigation on microbiological status of ground water in India: Classified Abstracts

Dayal, G.1992. Groundwater qualities of rural and urban settlements at Agra. J. Nature. Conserv. **4** : 89-93

A detailed investigation was carried out to ascertain the degree of contamination of groundwater in Agra, both urban and rural areas. The investigations indicated a high degree of pollution in ground waters of Agra city. Though much of the variables were within the standard limit of potable water, a few heavy metals recorded a concentration much beyond the permissible limits set by the WHO (1984). The water sources around septic tanks and sewage channels showed a high contamination of coliforms.

Somasekhara, R. K., R. L. Venkateswara., D. Padmavathy, and C. Rambabu, 1992. Groundwater quality in Challapalli Mandalam. Indian. J. Environ. Prot. **12** : 341-347.

Physicochemical and bacterial parameters of 23 bore wells and dug wells of 23 villages of Challapalli Mandal were monitored. The quality of well waters was assessed by comparing with existing standards for important parameters. Correlation coefficients among various water quality parameters were determined. It was found that there is high incidence of fluoride.

Raja Sekhar, C. R., C. Vasudeva Reddy, and B. Kotaiah .1994. Ground water pollution from unsewered sanitation-a case study in Tirupati. Indian. J. Environ. Prot. **14** : 845-847.

The pollution potential of septic tank effluents and their impact on ground water quality is assessed in an unsewered area of Tirupati. The results indicate that the septic tank effluents contain carbonaceous and nitrogenous matters in addition to phosphorous and high bacterial population. The ground water quality analysis data indicates that there is wide spread variations in the ground water.

Sharma, S, and R. Mathur. 1994. Bacteriological quality of groundwater in Gwalior. Indian. J. Environ. Prot. **14** : 905-907.

The study was carried out on the ground waters sources adjacent to the Swarna Rekha Sewage channel in Gwalior for their microbiological quality. The seasonal survey of 51 potable raw water sources revealed that the hand pumps and the bore wells are comparatively safer sources as compared to the dug wells. Unhygienic practices of the population and unsanitary conditions in the area are the reasons for poor microbial quality in the dug wells.

Narain Rai, J. P, and H. C. Sharma 1995. Bacterial contamination of ground water in rural areas of north west Uttar Pradesh. Indian. J. Environ. Hlth. **37** : 37-41.

Total aerobic heterotrophic bacteria (THB). total coliforms (TC), fecal coliforms (FC) and *Escherichia coli* Type-I (ECI) were estimated in fifteen well water samples collected from rural areas of Bareilly and Nainital districts. Maximum THB, TC, FC and ECI were 28,000/mL, 4460, 1480 and 305 per 100 mL of water respectively while few samples were free from ECI. However, the presence of FC and ECI revealed the unsanitary conditions of the wells.

Mitra, A, and S. K. Gupta, 1997. Assessment of groundwater quality from sewage fed farming area of east Calcutta. Indian. J. Environ. Prot. **17** : 447-447.

Groundwater of shallow and deep tube wells were collected from raw sewage irrigated farm areas of eastern fringe of the Calcutta city. At all the locations groundwater have been contaminated due to presence of high amounts of calcium, magnesium, sodium, chloride and phenolic compound. Heavy metals, like iron and manganese were also present at toxic level. Groundwater from shallow aquifer contained total and fecal coliform. Irrespective of depth the groundwater from all the locations are unsafe for drinking purpose.

1.1.1.3 Surface water

Presence of antibiotic resistant bacteria in the aquatic environment has been studied in different parts of the world. A detailed descriptive information about the antibiotic resistances of gram-negative bacteria isolated from four tributaries which enter Tillamook bay, Oregon and the bay itself, has been provided by Kelch and Lee (1978). They have also explored the correlation between the antibiotic resistances patterns exhibited by different genera involved in the study. In another study, the distribution of resistance to antimicrobial drugs among fecal coliforms in sewage, surface waters and sea water was investigated by paying attention to the effect of the species composition of the sample on the incidence of resistance and resistance patterns (Niemi *et al.* 1983). Several others have demonstrated the wide spread occurrence of such organisms in many rivers and streams (Jones 1986, Sokari *et al.* 1988, Magee *et al.* 1991, Ogan *et al.* 1993, Leff *et al.* 1993). Polluted water samples collected from the River Tigris in the vicinity of a raw sewage outfall were examined for the incidence of antibiotic resistance among coliform bacteria on three occasions during 1983. The result of the said study revealed high incidence of antibiotic resistant bacteria in natural waters that could be related to the widespread use of antibiotics in that locality (Al-Jebouri *et al.*1985). Boon *et al.* (1999) have 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). They described the analysis of various water samples for 18 antibiotic substances, representing macrolid antibiotics, sulfonamides, penicillins and tetracyclines. The study conducted by McArthur and Tuckfield (2000) have demonstrated the spatial distribution of antibiotic resistance to streptomycin and kanamycin in natural bacterial communities of two streams. The proportion of resistant bacteria was substantially higher ($P < 0.05$) in the midreaches 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. Goni-Urizza *et al.* (2000) have found a correlation between resistant bacteria in rivers and urban output.

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. The scientists of USGS have detected at least one antibiotic in nearly 50% of water samples collected across 30 different states between 1999 and 2000. Four or five different antibiotic residues, out of 22 antibiotics assayed, were present in 139 water samples collected from different streams and rivers. The most frequently detected antibiotics were erythromycin-H20 (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 has been pointed out that antibiotics used in livestock production have made their way,

via animal waste products, into the nations waterways. Studies conducted on 16 U. S. rivers revealed that rivers have started to turn into the reservoirs of antibiotic resistance genes (Ash *et al.* 2002).

Another study on Mhlathuze River (Lin *et al.* 2004) has shown that the river has become a medium for the spread of antibiotic resistance genes as well as the reservoir of resistance genes. A study on an Indian River Mahananda, has also revealed the abundance of multiple antibiotic resistant (MAR) bacteria (Mukherjee *et al.* 2005). 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). High levels of single and multiple-drug-resistant bacteria were detected, although sampling events were not correlated with clinical outbreaks and drug therapy. Antimicrobial resistance was also found to occur in marine and estuarine bacteria (Cohen *et al.* 1986, Barkay *et al.* 1995). Microbiological analyses of coastal waters polluted with sewage has revealed the presence of gentamicin resistance genes in members of Enterobacteriaceae, *Acinetobacter* spp., *Pseudomonas* spp., as well as in phylogenetically distant bacterial members of alpha and beta proteobacteria. (Heuer *et al.* 2002).

1.1.2. River water quality monitoring in the light of bacterial antibiotic resistance

For centuries, rivers have been used as the dumping grounds for the sewage of urban effluents, agricultural wastes, 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 brought about major changes in 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 rapidly 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. As rivers are one of the major sources of water, directly or indirectly, for human and animal consumption, this pollution may contribute to the maintenance and even spread of bacterial antibiotic resistance. In this perspective calls have been made for antibiotic resistance to be considered when establishing bacteriological water quality criteria (Grabow *et al.* 1974).

1.1.3. Review of water quality monitoring programs in India

Water quality monitoring of different water sources in India was largely restricted to physico-chemical analysis (summaries have been presented in Box: 1.2). Not withstanding the fact that measurement of physico-chemical characteristics is an easy way to examine the changes in water quality, but monitoring of microbiological quality of water is much more relevant in the Indian context. It is a practical method to determine the potential health risk associated with water exposure. Microbiological monitoring of different

rivers of India included mainly the determination of total and fecal coliform counts (MPN determination) (Box: 1.2). It leaves behind a paucity of information regarding antibiotic susceptibilities of native bacterial population of Indian rivers. This gap demanded a thorough study of river water bacteria in the light of antibiotic resistance profile (antibiogram) determination.

Box 1.2 Classified abstracts on water quality monitoring studies in India

A.Physico-chemical monitoring of river water

Khan Asif, A., N .Haque., A Siddiqui Intisar, and K. A Narayanan 1994. Comprehensive study on water quality parameters in the river Ganga between Narora and Kannauj, UP. Physico chemical characteristics. J. Freshwater. Bio. **6** : 295-304.

Paper presents a seasonal profile of the physicochemical and biological parameters over a period from October 1987 to March 1990 in the selected stretch of river Ganga. The physicochemical characteristics did not show marked change over the period of study. The biological features however represented no definite pattern. The utility of various parameters as water quality indices has been discussed.

Murugesan, A. G., K. M. S. A Abdul Hameed, and N. Sukumaran. 1994. Water quality profile of the perennial river Tampraparani. Indian. J. Environ. Prot. **14** : 567-572 .

River Tampraparni is the principal water source in Tirunelveli and Chidambaranar districts of southern Tamilnadu. Paper deals with monthly estimations of water quality parameters of this fluvial ecosystem with special reference to the aspects of pollution at various stations for one year. The result of the base flow quality analysis delineates variation in several physicochemical characteristics. The level of pollution is discussed with possible reasons.

Ramana Murthy, G. V., S. Venkata Mohan., P. Harishchandra, and J. Karthikeyan. 1994. A preliminary study on water quality of river Tungabhadra at Kurnool town. Indian J. Environ. Prot. **14** : 604-607.

Physicochemical characteristics of Tungabhadra river water over a stretch of 8 km near Kurnool town was studied for a period of 4 months to assess the suitability of river water for public consumption. Except at one sampling station, the water qualities of the river examined were within the permissible limit for human consumption. WQI calculated suggest treatment of river water before supplying to public.

Sharma. D., G. Chetri., J .Kalita, and A Dutta. 1994. Pollution status of the Bharalu river with special reference to physico chemical parameters. J. Freshwater. Bio. **6** : 209-213.

Paper deals with studies on the physicochemical parameters of Bharalu river water flowing through Guwahati city. The results showed high pollution status with values beyond the permissible limits DO (1.0 mg/l), Alkalinity (430 mg/l), BOD (12.2 mg/l) and COD (62.0 mg/l) etc. It has been observed that the water of Bharalu iS highly deteriorated due to various types of industrial effluents, domestic.

Srivastava, A. K, and D. K. Sinha. 1994. Water quality index for river Sai at Rae Bareli for the premonsoon period and after the onset of monsoon. Indian. J. Environ. Prot. **14** : 340-345.

Water quality index (WQI) for river Sai water at Rae Bareli at 10 different sites for the premonsoon period as well as after the onset of monsoon has been calculated to evaluate the water quality. Sixteen water quality physicochemical parameters were selected to calculate WQI. Values of WQI have been

found to be very high as compared to drinking water standard. It is suggested that discharge of wastewater and effluents play important role in determining the quality of water of river Sai. The water quality shows improvement after the onset of monsoon.

Mitra, A. K. 1995. Water quality of some tributaries of Mahanadi. Indian. J. Environ. Hlth. **37** : 26-36

Samples at five stations in streams Seonath, Jonk and Hasdeo, tributaries of Mahanadi river were analyzed at monthly intervals and the data presented. The samples were mostly alkaline, low in solute content and contained calcium, sodium and magnesium as major cations, and bicarbonate, sulphate, chloride as the major anions.

Chetana Suvarna, A, and R. K. Somasekhar. 1997. Ecological study on the riverine ecosystem of Karnataka. I. Physico- chemical Characteristics of river Cauvery. J. Env. Polln. **4** : 57-63.

The physico-chemical characteristics of the river water at three stations stretched over a distance of 20 km were studied at monthly intervals. The dissolved constituents fluctuated temporally and decreased with high flow. Turbidity and pH along with phosphates showed an increase during periods of high flow. The interrelationships between various physico- chemical characteristics are elucidated which assist in understanding the nature of intricate interactions occurring in this ecosystem.

Murthi Krishna, and S. G. Bharati. 1997 . A study on concentration of chloride of the river Kali near Dandeli, Karnataka (India). J. Env. Polln. **4** : 9-15.

Study reveals that chloride increased with the pollution load due to domestic and industrial wastes. The increase in chloride is accompanied by an increase in ammonical nitrogen and organic matter at all the sampling stations. The values of chloride were low during winter and high during monsoon. Further, the relationship of chloride with pH, major cations and anions are also discussed.

Nair. J, and S. Ganapathi 1997. Water quality of the Bhadar river basin. Indian. J. Environ. Hlth. **39** : 197-206.

EC and SAR values were determined for the surface and subsurface water of the Bhader river basin, Gujarat during pre-monsoon and two, three postmonsoon seasons. The analytical results show erratic EC and SAR values from Atkot to Navibandar, the variation mainly due to tidal ingression. The point source effluents from the dyeing and printing units show higher values, which decrease with river water dilution.

Shinde, R. S., D. G.Thorat., P.S. Gunjal, and S. R. Kuchekar. 1997. Studies on water quality of river Godavari at Nasik, Maharastra state India. J. Aquatih. Bio. **12** : 85-86.

Water samples collected from sampling stations along the stretch of river passing though Nasik city, were analyzed for a number of water quality parameters. The results reveal that most of the physic-chemical parameters were in permissible limits as recommended by ISI and in general the water is suitable for human consumption after disinfections.

Jain Praveen., S. Telang, and J. A. Khan 1998. Pollution status of Parbati river, Sehore. Eco. Env. Conerv. **4** : 71-72.

Attempt has been made to evaluate water quality of Parbati river flowing through Sehore for a period of 4 months to assess the suitability of dam water for irrigation use. The parameters observed for this study were electric conductance, percent sodium and sodium absorption ratio. These observations confirm that the dam water is suitable for irrigation.

Prasad, V. K, and R. N. Trivedi 1998. Water quality of the river basin. Int. J. Mendel. **15** : 39-40.

EC and SAR values were determined for the surface and subsurface water of the Ganga river basin, Bihar during three premonsoon and two postmonsoon seasons. The analytical results show erratic EC and SAR values from every station. The point source effluents from the dyeing and printing units show higher values, which decrease with river water dilution. However, when used for irrigation, it may affect the soil, crops, human life and the cattle. The utility of basin water for irrigation has been discussed.

Hussain, M. F, and I. Ahmad. 2002. Variability in physico-chemical parameters of Pachin river (Itanagar). Indian. J. Environ. Hlth. **44** : 329-336.

The concentration of water quality parameters in river and heavy metals in the bed sediment were measured for Pachin river for the three major flow periods. The variability in the physico-chemical parameters for different flow periods may be assigned to dilution of river water by direct runoff, human activities and organic load. The correlation study of physico-chemical parameters shows that their source of entering the river system is the same whether it may be a natural or anthropogenic or both.

Kumar Adarsh, and M. Shukla. 2002. Water Quality Index (WQI) of river Sai water at Raebareli city, U.P. J. Ecophysio. Occupl. Hlth. **2** : 163-172.

Water Quality Index (WQI) of River Sai water at six sampling stations at Raebareli city in a stretch of about 20 kms has been calculated to evaluate the water quality. Nine water quality physico-chemical parameters were selected. Values of WQI have been found to be very high as compared to drinking water standard and the river was found to be severely polluted.

Kumar Neeraj, and R .C. Sharma. 2002. Studies on the self-purification and allowable BOD load in river Krishni. Aquacult. **3** : 215-218.

The self-purification capacity of river Krishni has been calculated on the basis of dissolved oxygen and biochemical oxygen demand in the different stretch of the river at different sampling points. Along with this, allowable BOD load to be discharge in different flow of water have also been calculated.

Pathani, S. S., K. K Upadhyay, and S. K. Joshi. 2002. Some physico-chemical parameters and primary productivity of river west Ramganga (Uttaranchal). Himalayan. J. Env. Zoo. **16** : 151-158.

Paper describes physico-chemical characteristics and primary productivity of river west Ramganga at two stations Chakuhatiya and Masi Almora, Uttaranchal. DO is higher in the month of October and minimum in the month of May. Free CO_2 has been recorded higher in rainy and summer season due to high percentage of organic compounds and absence of free CO_2 in winter season. The productivity values are recorded maximum in summer and minimum in rainy season (July) due to low transparency and high velocity of the water current.

Gopalswami P.M., P. E. Kumar, and A. R. Kulandaivelu. 2003. Study on the quality of water in the Bhavani river. Asian. J. Chem. **15**: 306-310.

The Bhavani river water is being highly polluted by letting out industrial effluents, industrial wastewater, agricultural run off and sewage into the stream. The presence of inorganic ions such as hexavalent chromium, sulphate ions, etc., and biological waste has contributed to the pollution of the river water. As a result water borne diseases have become common in this area and the raw water cannot be used as such for industrial purposes. The Bhavani River water should be treated properly and disinfected before being supplied for industrial purposes and human consumption.

B. Investigation on bacteriology of river water:

Pathak, S. P., S. Kumar., P. W. Ramteke., R. C. Murthy., K. P. Singh., J. W. Bhattacharjee, and P. K. Ray. 1992. Riverine pollution in some northern and north eastern states in India. Environ. Monit. Assessment. **22** : 227-236.

Water samples from 30 rivers in northern and northeastern hilly states of India were analyzed for bacteriological and physicochemical parameters along with metals and pesticide residues. It was found that 34% of samples had > 50 coliforms/ 100 ml. while 24% of samples demonstrated > 50 thermo tolerant (fecal) coliforms/100 ml. Among the metals, iron was found to be above maximum permissible limits in the rivers of all the states, while manganese was found to be above the maximum permissible limits in the rivers of Tripura and some northern states.

Shukla, S., C., B. D. Tripathi., B. P. Mishra, and S. S. Chaturvedi. 1992. Physicochemical and bacteriological properties of the water of river Ganga at Ghazipur. Comp. Physio. Eco. **17** :92-96.

The bacteriological and physicochemical properties of the water of River Ganga were studied at four sampling sites at Ghazipur, U.P., from May 1987 to April 1988. For bacteriological analysis, samples were tested for standard plate count (SPC) and total coliform (TC) bacteria. Depletion in the dissolved oxygen, and increase in ECE, BOD, COD, pH, nitrate N, phosphate P, sodium, potassium and calcium contents of Ganga water was recorded near the area affected with sewage and industrial effluents.

Haniffa, M. A, P. Martin, and J. Jeevaraj. 1994. Hydrobiological studies on the channels of river Tambaraparani for the assessment of water quality. Indian. J. Environ. Prot. **14** : 821-828.

The channels of river Tambaraparani are polluted by both point sources and nonpoint sources of pollutants. All the channels are facially contaminated by coliforms. Generally the most probable number (MPN) of coliform and total place count of bacteria (TPC) was very high in sediments compared to that of water. The MPN index of coliform in water and sediment was high in North Kodamelagian channel due to sewage contamination. But the MPN index was low in Kodagan channel where the fecal and sewage entries were less. The TPC was higher in south Kodamelagian channel whereas the count was less in the Kodagan channel both in water and in sediment.

Raiyani, C. V., P. B. Doctor., Y. Verma., N. M. Desai., P. K. Kulkarni., S. G. Ruparelia, and S. K. Ghosh. 1994. Magnitude of pollution of dyecontaminated river water-its physicochemical and microbial analysis. Indian. J. Environ. Prot. **14** : 252-255.

Bacteriological and physicochemical analysis of water samples of river Bhadar between Jetpur and Dhoraji were carried out. Results of analysis showed a good correlation between MPN, BOD and COD. Other parameters included pH, sulphate, nitrate, total hardness, etc. Nitrate content of the river water was always above the permissible level (l0 mg/l) as suggested by WHO.

Doctor, P. V., C. V. Ranjani., Y. Verma., N. M. Desai., P. K. Kulkarni., S. G. Ruparalia, and S. K. Ghosh. 1998. Physico-chemical and microbial analysis of dye-contaminated river water. Indian. J. Environ. Hlth. **40**: 7-14.

Magnitude and degree of pollution in river Bhadar caused by azo dye containing effluents discharge from printing cotton textile industries has been studied, both by micorbiological and physco-chemical analysis. Nitrate concentrations were above the permissible level. BOD and COD values correlated well with MPN and heterotrophic plate count. Among all the isolates *E. coli* found to be site-specific dominant microflora.

Bhadra, B., S. Mukherjee., R. Chakraborty, and A. K. Nanda 2003. Physico-chemical and bacteriological investigation on the river Torsa of North Bengal. J. Environ. Bio. **24** :125-133.

A few physico-chemical and bacteriological parameters on certain locations of the river Torsa were studied. The major characteristics of Torsa river water were high alkalinity, high concentration of free ammonia with respect to albuminoid ammonia and the presence of bacteria of fecal origin. Marked seasonal variations of the parameters were also observed.

C. Investigation on metal contamination:

Mishra, A., J. S. Datta Munshi, and M. Singh. 1994. Heavy metal pollution of river Subarnarekha in Bihar. Part I: Industrial effluents. J. Fresh Water. Bio. **6** : 197-199.

Effluents, discharged into the river Subarnarekha, Bihar during different seasons of the three-year period from five major industries, have been analyzed for Cu, Zn, Pb, Fe, Cr, and Cd. The average concentration data have been made the basis of pollution consideration. Industry wise and placewise gradations of heavy metal discharge have been made. Industries situated at Ghatsila appear to be making the highest flux of heavy metals into the river.

Shyama Sundar P. S., G. Madhu., K. Srinivasa Murthy, and V. Mangathyaramma 1994. River Krishna estimation of trace metals and their distribution. Indian. J. Environ. Prot. **14** : 654-663.

Krishna river is an important east flowing peninsular river in South India which con-flounces at Divi point in Andhra Pradesh. It has tributaries, which traverse through areas rich in industries, agricultural run off and major towns/cities in this area pointing to possible pollution. A sample base line data has been collected and reasonable interpretations were made. The biogeochemical and anthropogenic mechanisms were discussed with the data collected in this basin.

Krishnamurthy, S. R, and S. G. Bharati. 1995. Distribution of manganese in the surface water of the polluted river Kali, around Dandeli area, North Kanara district, Karnataka. Env. Eco. **13** :132-135.

The concentrations of manganese were determined at four sampling stations of the polluted river Kali near Kandeli. The values of manganese increased from unpolluted stations to polluted stations. The manganese value showed direct relationship with pH, chloride, total hardness, phosphates and dissolved organic matter content of the river water.

Krishnamurthy, S. R, S. G. Bharati. 1995. Distribution of copper in the surface waters of the polluted river Kali, around Dandeli area, Karnatakan India. Env. Eco. **13** : 192-197.

The monthly variations and yearly concentrations of copper ion at four different sampling stations of the river Kali were studied. The variations of copper ions showed inverse relationship with total hardness, total dissolved residue and alkalinity.

Krishnamurthy, S. R, and S. G. Bharati. 1995. Distribution of zinc in the surface waters of the polluted river Kali, around Dandeli area, Karnataka, India. Env. Eco. **13**: 253-257.

The distribution of zinc in the surface waters of the river Kali was investigated between June 1987 and May 1988. The average value of zinc was higher than the world mean stream concentration. Further, at all the sampling stations of the river yearly average values of zinc, chloride, and sulphate corresponded with one another.

Dwivedi, S, and I. C. Tewari. 1997. Seasonal variation in heavy metal content of river Ganga at Varanasi. Indian. J. Environ. Prot. **17** : 281-286.

The concentrations of five heavy metals namely Cu, Cd, Cr, Fe and Pb were studied in the river Ganga at Varanasi from the University ghat (the most upstream point) to Rajghat (the most downstream point). The data has been discussed with reference to flow characteristics and other hydrological aspects and the values observed have been compared with standards prescribed by various river authorities. Levels of all the heavy metals were highest during the summer season and lowest during the rainy season.

Prbha S, Selvapathy. 1998. Heavy metal pollution in Indian rivers. Indian. J. Environ. Prot. **17** : 641-649.

Paper reviews the status and trends of heavy metal pollution in the major Indian rivers. A brief review of the analytical procedures for the determination of heavy metals in water sediments is also included.

Koshy Mathew, and Vasudevan N. T. 2002. Trace metals in the sediments of river Pamba. Polln. Res. **21** : 235-242.

The trace metals in the sediments of river Pamba in the state of Kerala from ten stations were analysed for a period of one year by taking monthly samples. Copper, zinc, iron and manganese showed low concentrations as compared to other rivers. The enhanced values of the metals during pre-monsoon and post-monsoon period is attributed to the seasonal accumulation of allochthonous organic residues.

Bhosle, A. B. 2003. The iron content in the river Godavari at Nanded and its impact on river ecology. J. Ecotoxico. Environ. Monit. **12** : 193-199 .

Natural waters can be very heterogeneous vertically, horizontally and with time. This is not only to man-made pollution, but also can be caused by natural phenomena such as erosion, currents, thermo cline and precipitation washout of dust. The total iron content of river Godavari was investigated and the overall study showed the fluctuations in the iron content more than permissible limit prescribed by Indian Council of Medical Research (ICMR). The iron was maximum during November and minimum during June.

Walther, D., S. Prebha., P. Selvapathy, and D. Beck. 2003 Heavy metals from the river Adayar, India : Infiltration into the adjacent groundwater aquifer. Ambio. **32** :153-157.

The mobilization of heavy metals into greater depths and their probable effects on the groundwater body are discussed. The high concentration of heavy metals and the influent character of the river Adayar allow the mobilization of metal ions and their transport into the deeper layers of the sediment. A changing environment due to effects such as saltwater intrusion and monsoon floods is the driving force for this phenomenon.

D. Investigation on pollution of river water with urban and industrial effluents:

Siddiqi, Z. M., R.S. Panesar, and S. Rani. 1994.Biochemical effect of few sewerage disposals on the water quality of Sutlej river. *Indian J Environ Prot,* **14** : 740-743.

Due to disposing off the sewerage and industrial wastes directly into the Sutlej river, the quality of water has the high BOD and enhanced turbidity. The presence of *Escherichia coli* suggests its pathological effect on human consumption and hence its removal from water body.

Trivedy, R.K, and S. S. Nakate. 1994. Pollution of clusters of industries in the Krishna river basin. J. Indl. Polln. Contl. **10** : 119-126.

Paper reports pollution load caused by a cluster of industries. Two clusters of industries, Satara and Wai M.I.D.Cs, Maharashtra Industrial Development Corporations' Industrial Estates have been studied. It was found that majority of industries have not installed effluent treatment plants. While the pollution load generated at Wai M.I.D.C. is minimal, Satara M.I.D.C. presents an ideal case for establishing combined effluent treatment plant.

Chetana Suivarna, A, and R. K. Somasekhar. 1997. Ecological study on the riverine ecosystem of Karnataka. III. physico chemical characterisation of river Vris\tabhavathi. J. Env. Polln. **4** : 71-77.

The study was carried out to comprehend the physico-chemical characteristics of river volume Vrishabhavathi. Large of urban wastes discharged into his river influenced its physico-chemical make-up considerably. Most of the chemical components estimated were in higher concentration with the pH of water being alkaline.

Jain, C.K., K. K. S. Bhatia, and S. M. Seth. 1997. Characterization of waste disposals and their impact on the water quality of river Kali. Indian. J. Environ. Prot. **17** : 287-295.

The physico-chemical characterization of municipal waste of Muzaffarnagar city and composite industrial waste have been carried out with a view to assess the likely impact of these effluents on the quality of water of river Kali. High values of BOD and COD in the waste effluents is an indication of high degree of organic contamination in these wastes. The important characteristics associated with pollution of the river due dot the discharge of these wastes is the heavy depletion of oxygen over a small stretch of the river.

Aher, H. R., D. G. Zinjad., P. S. Gunjal, and S. R. Kuchekar. 2002. Impact of human activities on the quality of water in Pravara river basin and Pravara left bank canal. Cheml. Environ. Res. **11**: 101-104 .

Chemical analysis of water samples from Pravara river basin and Pravara left bank canal shows that the water is characterized by alkaline earth. Water samples from thirteen spots of down stream from Bhandara to Babhaleshwar were collected for analysis at an interval of ten kilometers. The results show that the physico-chemical characteristic of water changes to downstream from Bhandara to Babhaleshwar due to human activities.

Gupta, A. K, and A. K. Raghubanshi. 2002. Comparative study of enrichment of nutrients and heavy metals in river waters Ghaghra and Ganga due to anthrophogenic pressures. Polln. Res. **21** : 261-263.

For a comparative study of enrichment of nutrients in river waters of Ghaghra and Ganga due to anthropogenic activities, two sites were selected at both the river corridors and one at the confluence point of both the rivers. The findings show that, due to different anthropogenic activities, the level of nutrient enrichment varies at different sites. Similarly the heavy metal content also varies with the biotic activities. Due to nutrient and heavy metal's enrichment the water quality is adversely affected.

Kumar Neeraj, and R. C. Sharma. 2002. Water quality of river Krishni [Part-1. Physico-chemical characteristics]. J. Nature. Conservator. **14** : 273-297.

The water quality of river Krishni has been studied at eight sampling points fixed at 70 kilometers stretch. The river received about 50 to 67 cusec. of domestic and industrial waste water via three waste water channels. These waste contents of wastewater have changed the characteristics of the river water to great extent.

Pande, R. K, and A. Mishra. 2002. Impact of paper and pulp industry effluent on the water quality of river Hindon. J. Ecophysio. Occupl. Hlth. **2** : 173-184.

Hindon is a tributary of Yamuna and flows along the western district of U.P. and Uttaranchal and certain physical and chemical parameters have been studied and the effluent stress were observed to understand its possible impact on water quality of river Hindon, where effluent was discharged. It was observed that certain undesired elements had a self reducing tendency along with certain physico-chemical parameters but a few remain contaminated for a longer period of time and for longer distance in stream.

1.1.4. Importance of antibiogram surveillance

Surveillance of bacterial resistance is a key element in understanding the size of the environmental problem and disease management. One promising approach was based on the analysis of differences in antibiotic resistance by using the multiple antibiotic resistance index (Kaspar *et al.* 1990, Pillai *et al.* 1997). Wiggins (1996) used discriminant analysis of patterns of antibiotic resistance in fecal streptococci to differentiate between human and animal sources of fecal pollution in natural waters and to classify unknown isolates from polluted streams on the basis of the patterns of the unknown isolates. Multiple antibiotic resistance (MAR) analysis was used as a method for determining point and nonpoint pollution sources. Parveen *et al.* 1997, examined 765 multiple antibiotic resistant *Escherichia coli* isolates and used their antibiotic resistance profile as a possible tool to differentiate point and nonpoint sources of pollution within the Apalachicola National Estuarine Research Reserve (ANERR). The results of their study reflected the applicability of the method in facilitating management of other estuaries. Although a specific *E. coli* MAR profile may not always correlate with PS (point source) and NPS (nonpoint source) pollution in all estuaries, extensive databases may well be required to develop associations between specific MAR profiles and sources of pollution. The reliability and repeatability of antibiotic resistance analysis as a method of identifying the sources of fecal pollution in surface water and ground water was tested (Wiggins *et al.* 1999) and the study confirmed the measurable and consistent differences in the antibiotic resistance patterns of fecal streptococci isolated from various sources of fecal pollution which could be used to classify and identify the sources. The study of Harwood *et al.* 2000, described the application of antibiotic resistance analysis as a tool to differentiate between animal and human fecal isolates in subtropical surface waters of Florida. The ARPs (antibiotic resistance patterns) of fecal streptococci and fecal coliforms from known animal sources and from human-dominated sources (domestic water) were determined in order to create separate databases (fecal streptococcus and fecal coliform) to which ARPs of isolates from surface waters could be compared and categorized by probable source. The antibiograms varied with time and geographical regions. Periodic monitoring of antibiograms could, therefore, enable to document changes in resistance patterns and characterize the isolates on the basis of antibiogram updates. The multiple resistances of isolates to some antibiotic class are of great public concern and calls for caution in the indiscriminate use of antibiotics on humans and animals. There has been a growing necessity to look at how antibiotics are being used and locate

the residence of resistant strains on a global scale, because of the mobility of these organisms across countries. Good quality local data would be helpful in providing a strong basis for national and international surveillance. Exploring natural reservoirs of resistance genes may predispose the sources of transferable traits for emerging pathogens. Surveillance studies on changing pattern of antibiotic resistance in micro flora of Indian rivers were practically non-existent.

The present study is a maiden study on antibiogram surveillance of copiotrophic bacteria from a river of Northern West Bengal, India. Copiotrophs are those, which compete, well in nutrient rich environments. The Torsa is an international river, which crosses through three countries, China (Tibet), Bhutan and India. As it left the foothills of Bhutan and entered duars plain of West Bengal, it widened into a braided channel. Torsa has on its banks several tea gardens, agricultural fields and hamlets of human habitation. This river also drains Jaldapara Wild Life Sanctuary. Extensive heedless deforestation, concentrated urban development, unscientific mining, uptake of dolomite colloidal water traveling through several streams, inadequate drainage have contributed to the degradation of water quality of Torsa. The major characteristics of the river water were a high concentration of free ammonia with respect to albuminoid ammonia, high alkalinity and the presence of bacteria of both fecal and non-fecal origin (Bhadra *et al.* 2003). The aim of this chapter was to provide detailed descriptive information about the antibiotic resistances of copiotrophic bacteria isolated from this water way and the second was to analyze whether antibiotic resistance patterns vary in a systematic or random manner.

1.2 Materials and Methods

1.2.1 Sampling stations and sample collection from the river water

For successful monitoring of river water quality it is essential to have sufficient knowledge about the morphometric details of the subject river, selection of particular sampling site(s), sample collection methods, and preservation & maintenance of samples for parameter(s) in question. The study of morphometry, i.e., 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.

During analysis of river water quality, assortment of sampling site(s) is very important, and it should be done in the light of environmental monitoring program. The selection of actual sampling location shall depend upon the character of the water body. In case of widened region of the river many sampling sites should be selected at various corners. In monitoring the stream, which is narrow, the rapidly moving water should be thoroughly mixed laterally and vertically, hence only one sampling point needs to be selected at each location along the stream.

In an organically polluted river course at least one site should be selected above the outfall of the wastes and others should be selected downstream representing the zone of recent pollution. In places where the river is polluted by inorganic

substances, one point above and the other point below the actual point of discharge should be selected for sampling.

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.

1.2.1.1 Selection of sampling stations on Torsa River

Sampling sites on Torsa River were selected after scrutinizing topographic map of the river. For the purpose of analysis of water quality, three sampling stations were selected (Figure 1.1). The first sampling station, SS I, was located at Hasimara, Dist., Jalpaiguri of the state West Bengal. The second sampling station, SS II, was located at village- Falakata of Jalpaiguri district. The third sampling station, SS III, was located at Coochbehar Town, district Coochbehar, of the state West Bengal, India.

1.2.1.2 Sample collection

From each sampling site, three grab samples were collected from left, right and middle of the river; all three samples were mixed and, were analyzed as single sample. For sampling, sterilized water bottles 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. Samples were collected once in every month from January 2000 to December 2001, leaving the months July and August, because of heavy rainfall and flood during the monsoon which made the condition of river and roadways both equally deplorable so that the task of collecting sample became very difficult. The samples were transported to the laboratory in icebox and analyses were performed within 24 hours.

1.2.2 Preparation of antibiotic stock solutions and antibiotic plates

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. Tests with known sensitive isolates of *E. coli* indicated adequate storage stability for all antibiotics stored under these conditions. Antibiotic powders were weighed to 0.1mg accuracy; liquids were quantified by micropipette. The antibiotics used were ampicillin (100 μg ml^{-1}), chloramphenicol (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), streptomycin (100 μg ml^{-1}) and tetracycline (20 μg ml^{-1}). The desired concentrations of the antibiotics (diluted from the stock) were stirred into the melted agar at approximately 45°C and immediately poured into petridishes to minimize the exposure of elevated temperatures. These LB agar plates containing standard concentration of a respective antibiotic were stored at 4°C and were used within seven days of preparation.

1.2.3 Enumeration of total culturable copiotrophic bacteria and the fraction resistant to different antibiotics

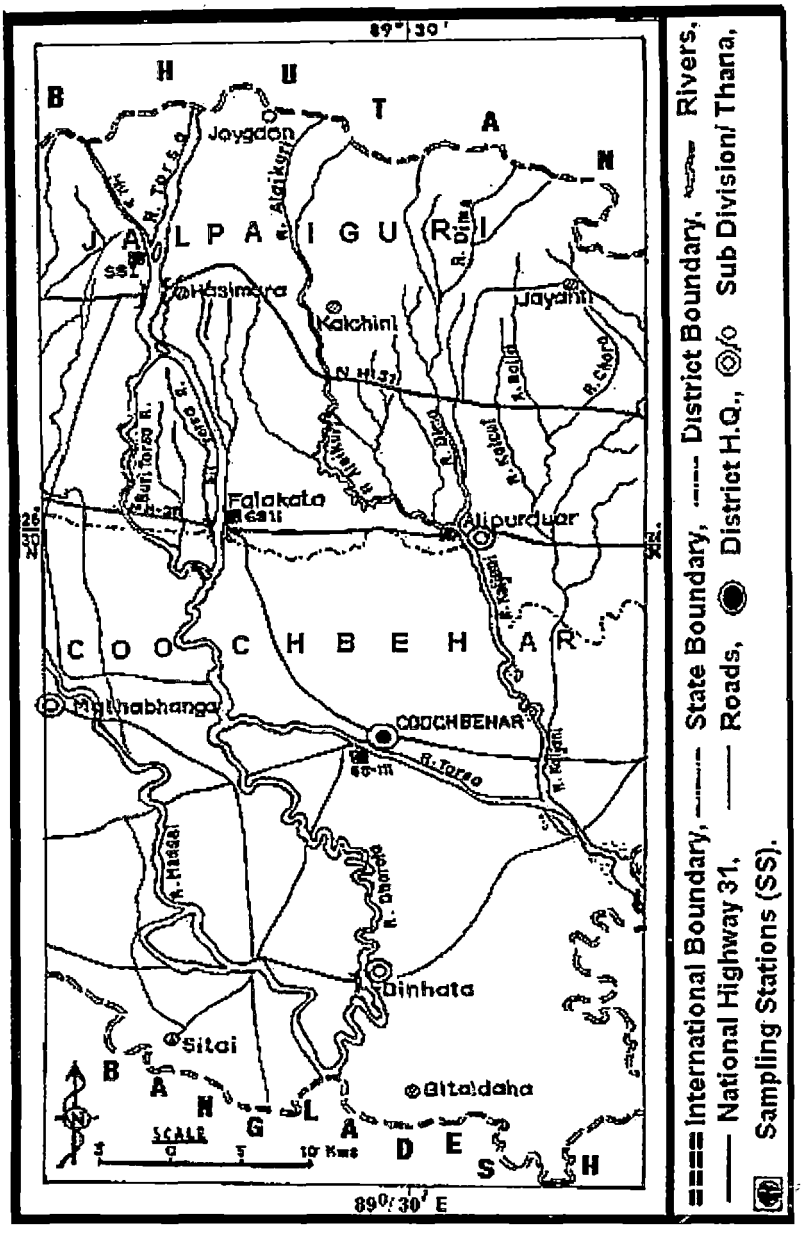


Figure 1.1. Map showing three sampling sites on river

Serial dilutions of river water samples were made in sterile 0.5% NaCl solution, which served as diluents of a known volume. Once diluted, 0.1ml of the suspension was spread uniformly on Luria-Bertani agar plates with a glass spreader. The plates were incubated at 37°C overnight. The total culturable copiotrophic bacterial count of the suspension was obtained by multiplying the number of colonies per plate by reciprocal of the dilution.

0.1ml aliquots from serially diluted tubes of river water samples (from the same dilution series of the samples as described above) was spreaded onto LB agar plates containing standard concentration of a single antibiotic and incubated overnight at 37°C for getting the total count of copiotrophs resistant to a particular antibiotic. The count obtained was finally expressed as the fraction of the total count obtained from the LB agar plates without antibiotic.

1.2.4 Determination of multiple-antibiotic-resistance (MAR) profile of copiotrophic bacterial isolates

Antibiotic resistance was determined by the method described earlier by Armstrong *et al.* (1981). The resistant bacterial colonies that appeared on LB agar plates containing single antibiotic were picked up randomly with sterile tooth picks and transferred to a gridded LB agar plate containing no antibiotic. These plates were considered as the master plates and were incubated for 24 hours at 37°C. These master plates were then replicated onto LB agar plates, each containing a single antibiotic at the concentration indicated in section 1.2.2. The final plate replicated was one of plain LB agar plate. This method allows slight differences in amount of inoculums. However, the control plate was inoculated last to confirm the successful inoculation of the preceding plates and provided a fresh master plate of cultures. The replicated plates were incubated at 37°C for approximately 24 hours and drug resistance was determined. The isolates were considered resistant to multiple antibiotics, only if their growth on the presence of antibiotic were as well developed as their growth on the control plates. Any sign of inhibition or sensitivity was considered to be indicative of nonresistance. This meant that resistance was very strictly defined so that no organism with any sign of sensitivity would be classified as resistant. This strict definition of resistance was necessary to make the interpretation of results easier and consistent.

1.2.5 Statistical analyses

A series of observations on total culturable copiotrophic bacteria and their fractions recovered on nutrient-rich solid medium supplemented singly with five different antibiotics, ampicillin, chloramphenicol, kanamycin, streptomycin, and tetracycline, from water samples collected from three different sampling stations, were recorded in different sampling months. An appropriate type of mathematical equation was selected for trend, and the constants appearing in the trend equation were determined on the basis of the given time series data. SPSS package was used to generate the trend line.

The Arcsine transformation, also known as 'angular transformation', being especially appropriate to percentages and proportions, was applied to the percentages in the original data so that the resulting transformed variates met the assumptions of the analysis.

A random sample consisting of 15 observations (per sampling month) were classified according to two factors- into 3 classes according to a factor, sampling site, and also into 5 classes according to another factor, proportions of copiotrophic bacteria resistant towards five different antibiotics. There was one observation in each of the cells corresponding to a class of factor, sampling site, and simultaneously a class of factor, fraction of copiotrophic bacteria resistant to specific antibiotics. These 15 observations (per sampling month of a particular year) were arranged in the form of a two-way table with 3 rows and 5 columns. Analyses of variance (ANOVA) on these data were performed with the help of SPSS package to determine whether there was any significant difference in the recovery of copiotrophic bacteria resistant towards specific antibiotic or between three sampling sites. It was also tested which pairs of sampling sites or antibiotic-resistant groups differed significantly, if

any. The data on occurrence of fractions of five antibiotic-resistant bacterial groups in different sampling months per site was further classified/distributed into three seasons; pre-monsoon, post-monsoon, and winter. Differences in occurrence of fractions of five antibiotic-resistant bacterial groups in three different sites or seasons as well as interaction between the factors was tested with ANOVA (two way classified data with replication). With the help of ANOVA (two way classified data with replication) it was also tested whether the occurrence of the individual antibiotic-resistant fraction differed significantly in seasons or in sampling sites, and interaction between factors, if any.

The Wilcoxon matched pairs signed ranks test, which make use of the magnitude of the differences between quantified data, was used to compare the proportion and test the significance of the difference in abundance of the five different antibiotic-resistant groups. Correlation coefficient was calculated and test on significance of correlation coefficient was done.

1.3 Results

1.3.1 Quantification of culturable copiotrophic bacteria and their fractions resistant to five different antibiotics, ampicillin, chloramphenicol, kanamycin, streptomycin, and tetracycline, in water samples collected in different months, spread over the years 2000 and 2001, from three sampling stations on river Torsa

Thirty-nine water samples, thirteen from each sampling site, were collected and studied on monthly basis from January 2000 to December 2001. The data on total culturable copiotrophic bacteria of the river Torsa in different sampling months for three sampling sites are shown in tables 1.1, 1.2 and 1.3.

Maximum and minimum occurrences of culturable copiotrophs were recorded in water samples of the month of May 2001 and January 2000 from sampling station I. The concentration of culturable bacteria remained invariant in water samples collected in the month of December of the two successive years 2000 and 2001. The fraction of copiotrophic bacterial population resistant to ampicillin occurred maximally in December 2001 followed by June 2001 and October 2000 and 2001. The lowest occurrence of the said population was recorded in March 2001. The chloramphenicol resistant copiotrophs occurred maximally during March 2001. In May 2000 also, the occurrence of chloramphenicol resistant population was recorded to be quite high. Moderately high percentage of occurrence was recorded in June 2001, November 2000 and December 2000. In September 2001, lowest occurrence for chloramphenicol resistant population was recorded. The fraction of bacterial population resistant to kanamycin was found to be present in only six of the thirteen water samples analyzed. The maximum occurrence of this particular population was observed in March 2001 and then in June and December 2001. Very low percentage of the kanamycin resisting population was found during October 2000 and 2001, as well as in December 2000. The fraction of bacterial population exhibiting resistance to streptomycin was quite low in comparison to the ampicillin and chloramphenicol resistant population. The highest occurrence of this population was recorded in September 2001 and lowest was during

Table 1.1. Total copiotrophic bacterial count (TCBC) and fraction of TCBC resistant to five different antibiotics in water samples collected in different sampling months (January 2000-December 2001) at Hasimara (SS I).

Months	Total copiotrophic bacterial count (TCBC) (c.f.u ml^{-1})	Percent resistant population (taking from TCBC value in plates without antibiotic as 100%)				
		Amp^r	Chl^r	Kan^r	Str^r	Tet^r
Jan.2000	1×10^3	34.0	16.66	0.0	0.0	0.0
May 2000	45×10^3	17.28	67.77	0.0	6.22	1.0
Oct.2000	55×10^3	41.45	22.72	0.03	3.63	1.5
Nov.2000	10×10^3	30.0	25.0	0.0	4.5	0.0
Dec.2000	80×10^3	28.75	20.7	0.84	6.97	0.0
Jan.2001	37×10^3	28.0	17.86	0.0	5.89	0.34
Mar.2001	28×10^3	4.17	89.28	5.0	8.92	4.82
May 2001	90×10^3	7.2	12.2	0.0	3.7	0.77
June 2001	9×10^3	43.47	26.08	4.78	0.02	36.95
Sept.2001	46×10^3	13.04	8.69	0.0	19.56	1.17
Oct.2001	21×10^3	35.23	15.40	0.08	10.72	1.49
Nov.2001	26×10^3	21.02	10.25	0.0	8.65	0.69
Dec.2001	80×10^3	75.0	62.5	4.6	8.75	0.93

Amp, Ampicillin; Chl, Chloramphenicol; Kan, Kanamycin; Str, Streptomycin; Tet, Tetracycline

June 2001. For tetracycline resistant bacteria, maximum occurrence was recorded in June 2001 and their occurrence was not detectable in the months of January, November and December 2000. It is interesting to note that in January 2000, only the fraction resisting ampicillin and chloramphenicol were recovered from the copiotrophic population. It was also observed that in the months where recovery of the chloramphenicol resistant populations were maximum, recovery of the other antibiotic resisting population were quite low as was observed in the month of May 2000 and March 2001.The same phenomenon was observed regarding the abundance of the recovered ampicillin resistant population compared to other antibiotic-resistant bacterial populations.

The maximum and minimum bacterial load in the water samples from sampling station II were recorded in the months of May 2000 and March 2001. The fraction of copiotrophic bacterial population resistant to ampicillin occurred maximally in May 2000 and during October 2000 and 2001. The lowest occurrence of the said population was found in March 2001. Among the recovered bacterial population, the highest fraction resisting chloramphenicol appeared in March 2001. The occurrences of the said population were quite high in the months of May 2000 and December 2001. Moderately high percentage of occurrence was recorded in June 2001, October 2000 and 2001, as well as in November 2001. In September 2001, lowest occurrence for chloramphenicol resistant population was recorded. The fraction of recovered kanamycin resistant bacterial population was lowest among all other antibiotic resisting populations (i.e, ampicillin, chloramphenicol, streptomycin and tetracycline resistant ones). Their occurrences were detected in only six of the thirteen water samples analyzed. The maximum recovery of this population took place in the month of May 2000. Very low percentage of the kanamycin resisting population was found during September 2001, October 2001, as well as in December 2001. The highest occurrence of streptomycin resisting population was recorded in May 2000 and lowest was during November 2000. For tetracycline

Table 1.2. Total copiotrophic bacterial count (TCBC) and fraction of TCBC resistant to five different antibiotics in water samples collected in different sampling months (January 2000-December 2001) at Falakata (SS II).

Months	Total copiotrophic bacterial count (TCBC) (c.f.u ml^{-1})	Percent resistant population (taking from TCBC value in plates without antibiotic as 100%)				
		Amp^r	Chl^r	Kan^r	Str^r	Tet^r
Jan.2000	50×10^3	8.75	16.25	0.0	0.0	0.0
May 2000	500×10^3	61.53	53.84	46.15	84.82	9.03
Oct.2000	40×10^3	47.50	22.50	1.25	0.85	0.375
Nov.2000	30×10^3	12.66	9.0	0.0	0.43	0.23
Dec.2000	70×10^3	9.60	7.54	0.0	0.62	0.37
Jan.2001	11.6×10^3	3.65	10.25	0.0	0.0	0.14
Mar.2001	1.9×10^3	2.5	60.25	0.0	6.71	1.5
May 2001	50×10^3	11.4	16.0	2.6	5.0	1.72
June 2001	180×10^3	22.22	27.77	0.0	0.0	16.66
Sept.2001	21×10^3	10.95	6.19	0.476	1.42	0.03
Oct.2001	150×10^3	43.25	20.9	0.672	0.85	0.47
Nov.2001	110×10^3	14.69	20.6	0.0	0.0	1.24
Dec.2001	60×10^3	21.03	42.60	0.268	0.0	0.04

Amp, Ampicillin; Chl, Chloramphenicol; Kan, Kanamycin; Str, Streptomycin; Tet, Tetracycline

resistant bacteria, maximum occurrence was recorded in June 2001 and their occurrence was not detectable in the month of January 2000. The recovery of the fraction of the bacterial populations exhibiting resistance to each antibiotic was quite high during May 2000. In the months like May 2000 and 2001, September 2001 as well as in October 2000 and 2001, all the five different types of antibiotic resistant bacteria were recovered from the copiotrophic bacterial population. In January 2000, only the fraction resisting ampicillin and chloramphenicol were recovered. Analysis of all the thirteen water samples led to the observation that only one type of antibiotic resistant population that recovered as a fraction resistant population, in a particular month, dominated over the other types. In May 2000, the maximum recovery was noted for streptomycin resistant population. It was also observed that in the months where recovery of the chloramphenicol resistant populations was maximum, recovery of the ampicillin resisting population was quite low and in months where ampicillin resistant populations excelled, the chloramphenicol resistant population declined.

For the sampling station III, the maximum occurrence of culturable copiotrophs was recorded in water sample of the month of May 2001. The concentration of culturable bacteria remained invariant in water samples collected in the months of January (lowest recovered bacterial population), as well as in November of the two successive years 2000 and 2001. The fraction of copiotrophic bacterial population resistant to ampicillin occurred maximally in September 2001. The lowest occurrence of the said population was recorded in January 2000. The chloramphenicol resistant population appeared in highest proportion during March 2001. In December and June 2001 also, the occurrence of chloramphenicol resistant population was recorded to be quite high. In January 2000, lowest occurrence for chloramphenicol resistant population was recorded. It was observed that the fractions of ampicillin and chloramphenicol resistant bacterial populations were quite high compared to the other antibiotic resistant populations among the recovered

Table 1.3. Total copiotrophic bacterial count (TCBC) and fraction of TCBC resistant to five different antibiotics in water samples collected in different sampling months (January 2000-December 2001) at Coochbehar (SS III)

Months	Total copiotrophic bacterial count (TCBC) (c.f.u ml^{-1})	Percent resistant population (taking from TCBC value in plates without antibiotic as 100%)				
		Amp^r	Chl^r	Kan^r	Str^r	Tet^r
Jan.2000	0.51×10^3	0.39	4.31	0.0	0.0	0.0
May 2000	23.6×10^3	26.09	14.68	0.0	0.0	1.52
Oct.2000	19×10^3	23.31	17.56	0.18	1.55	1.60
Nov.2000	270×10^3	16.66	12.22	0.03	2.59	5.18
Dec.2000	196×10^3	15.49	10.04	1.48	1.79	0.68
Jan.2001	0.51×10^3	0.49	15.84	0.79	0.0	0.0
Mar.2001	80×10^3	2.87	35.27	0.64	6.42	0.82
May 2001	650×10^3	5.38	13.85	0.92	0.246	3.38
June 2001	300×10^3	36.36	30.90	2.81	1.09	33.18
Sept.2001	19×10^3	47.36	28.94	0.36	1.57	0.36
Oct.2001	30×10^3	28.75	10.97	0.45	1.76	1.29
Nov.2001	270×10^3	30.59	22.68	0.69	0.79	0.48
Dec.2001	90×10^3	17.68	33.07	0.0	0.0	0.91

Amp, Ampicillin; Chl, Chloramphenicol; Kan, Kanamycin; Str, Streptomycin; Tet, Tetracycline

culturable copiotrophs. Except for three months (January and May 2000 and December 2001), fraction of kanamycin resistant population was recovered from each of the sampling months. The maximum occurrence of this particular population was observed in June 2001 and then in December 2000. In other months, recovery of the said population was quite low. The fractions of bacterial populations exhibiting resistance to streptomycin and tetracycline were quite low in comparison to the ampicillin and chloramphenicol resistant populations. The highest occurrence of streptomycin resisting population was recorded in March 2001 and lowest was during May 2001. For tetracycline resistant bacteria, maximum occurrence was recorded in June 2001 and their occurrence was not detectable in the months of January 2000 and 2001. It is interesting to note that in January 2000, only the fractions resisting ampicillin and chloramphenicol were recovered from the copiotrophic bacterial population. It was also observed that in the months where recovery of the chloramphenicol resistant populations was maximum, recovery of the other antibiotic resisting population were quite low. Similar observation was found in case of data on recovery of ampicillin resistant population.

Copiotrophic bacterial counts (CBCs) that were recorded in different sampling months displayed very wide variations and therefore larger were the dispersion (measured in terms of standard deviation) values (Table 1.4). Geometric mean values were considered because it is less affected by the presence of extremely large or small values. The CBC mean values for the water samples collected from three different sampling stations ranged from 2.64×10^4 to 4.80×10^4. The SPC GM values for SS II and SS III were very close.

1.3.2 Time series analysis: Determination of trend(s) of occurrence(s) of five different antibiotic resistant bacterial fractions of the culturable copiotrophs

The percentage occurrence of ampicillin-resistant bacteria in water samples collected from three different sampling stations, spanning a period from January 2000 to December 2001, have shown an

Table 1.4. Antibiotic resistance of copiotrophic bacteria isolated from three sampling sites of river Torsa of North Bengal

	Average SPC Density (c.f.u. ml^{-1})	Average % resistant population (taking SPC value in plates without antibiotic as 100%)				
		Amp	Chl	Kan	Str	Tet
SS I	2.64×10^4 (2.64×10^4)	22.86 (12.29)	22.96 (23.73)	1.12 (1.80)	3.25 (5.03)	1.39 (9.99)
SS II	4.80×10^4 (1.31×10^5)	14.13 (18.95)	18.79 (16.69)	1.21 (12.65)	1.66 (23.07)	1.67 (4.85)
SS III	4.18×10^4 (1.86×10^5)	10.09 (14.58)	16.44 (9.00)	1.67 (0.76)	1.28 (1.69)	1.47 (8.90)

Geometric mean of 13 samples
The standard deviation are given in the first bracket
Amp, Ampicillin; Chl, Chloramphenicol; Kan, Kanamycin; Str, Streptomycin; Tet, Tetracycline

upward trend in SS I and SS III, while a lower trend was observed in samples from SS II. There was a differential trend in case of occurrence of chloramphenicol resistant bacteria. Analysis of Site I samples revealed a lower trend while Site III showed higher trend of occurrence of the chloramphenicol-resistant bacterial population. There was a fairly constant occurrence or stagnation of the said population in water samples of SS II, over the period of time. Although the fraction of kanamycin resistant populations recovered from the water samples of each site were quite low, time series analysis has revealed an upward trend for occurrence of the said population in SS I and SS III. A lower trend was observed in samples from SS II. In case of populations resistant to streptomycin, an upward trend in SS I and a lower trend in SS II was noted. A fairly constant trend for the same population was observed in SS III. An upward trend was observed for the fraction of populations resisting tetracycline in both SS I and SS III. The said population stagnated in SS II.

1.3.3 Wilcoxon matched pairs signed ranks test

The Wilcoxon matched pairs signed ranks test, which makes use of the magnitude of the differences between quantified data, was used to compare the proportion and test the significance of the difference in abundance of the five different antibiotic-resistant groups. The significant difference in recovery of proportion of matched pairs were kanamycin and ampicillin ($p<0.001$), streptomycin and ampicillin ($p< 0.003$), tetracycline and ampicillin ($p<0.001$), kanamycin and chloramphenicol ($p<0.001$), streptomycin and chloramphenicol ($p <0.002$), tetracycline and chloramphenicol ($p< 0.001$), streptomycin and kanamycin ($p< 0.007$), and tetracycline and streptomycin ($p< 0.023$) resistant bacteria in water samples of SS I. The occurrence of the individual group of the matched pairs that were found significantly different in water samples of SS II were, kanamycin and ampicillin ($p<0.001$), streptomycin and ampicillin ($p< 0.005$), tetracycline and ampicillin ($p<0.001$), kanamycin and chloramphenicol ($p<0.001$), streptomycin and chloramphenicol ($p <0.004$), tetracycline and chloramphenicol ($p< 0.001$), and streptomycin and kanamycin ($p<0.047$) resistant bacteria. The occurrence of the individual group of the matched pairs, in the data derived from water samples of SS III, that were found significantly different were, kanamycin and ampicillin ($p< 0.001$), streptomycin and ampicillin ($p< 0.002$), tetracycline and ampicillin ($p< 0.001$), kanamycin and chloramphenicol ($p< 0.001$), Streptomycin

and chloramphenicol ($p < 0.001$), tetracycline and chloramphenicol ($p < 0.001$), and tetracycline and kanamycin ($p < 0.041$) resistant bacteria. The water samples analyzed from all the three sampling sites, SS I, II, & III, did not have any significant difference in occurrence of the proportion of ampicillin and chloramphenicol resistant fraction of the culturable copiotrophs of river Torsa.

1.3.4 Variance analysis in two-way classification

Analysis of variance (two-way without replication) for testing equality of proportions (sampling site X fraction of copiotrophs resistant to different antibiotics) for monthly data yielded the following results. In the first sampling month, January 2000, there were no significant differences between sampling sites, but the proportions of different antibiotic-resistant bacteria was not equal i.e there were significant differences ($p < 0.0124$) in the occurrences of five different antibiotic resistant fractions. Significant difference ($p < 0.05$) in occurrence of ampicillin-resistant bacteria compared with kanamycin or streptomycin or tetracycline was noted. Also, the occurrence of chloramphenicol-resistant bacteria differed significantly from the proportion of kanamycin or streptomycin or tetracycline resistant bacteria. Similar significant difference in occurrence of ampicillin-resistant/chloramphenicol-resistant bacteria compared with kanamycin or streptomycin or tetracycline was noted in the month of November 2000 ($p < 0.01$), December 2000 ($p < 0.01$), January 2001 ($p < 0.05$), and November 2001 ($p < 0.01$). Differences in the proportions of different antibiotic-resistant bacteria were most significant in the months of October 2000 ($p < 1.02$ e-5), December 2000 ($p < 0.000469$), March 2001 ($p < 0.000557$), May 2001 ($p < 0.000183$), June 2001 ($p < 0.000239$), October 2001 ($p < 0.000327$) and November 2001 ($p < 0.001104$).

In the month of May 2000, there were no differences between fractions of different antibiotic-resistant bacteria but the difference was significant ($p < 0.002$) between sampling sites. There were significant differences between SS I and SS II, and between SS II and SS III ($p < 0.05$). There were similar significant differences between sampling sites in the months of December 2000 ($p < 0.049467$) and March 2001($p < 0.019791$). Significant differences in occurrence of ampicillin-resistant/chloramphenicol-resistant bacteria compared with the all the other four (rest) of the five proportions of antibiotic resistant bacteria were noted in the month of October 2000 ($p < 0.01$).

The data of 13 samplings per site were distributed to three seasons, pre-monsoon, post-monsoon, and winter respectively and analysis of variance (two-way classified data with replication) for testing equality of proportion (Season X fraction of copiotrophs resistant to different antibiotics) site wise was performed. There were significant differences between seasons in the occurrence of fractions of different antibiotic-resistant bacteria in the sampling sites II and III ($p < 0.000835$ and $p < 0.008682$). Significant differences have been noted between winter and pre-monsoon; and pre and post-monsoon ($p < 0.01$) in site II. Similarly, the recovery of different fractions of antibiotic-resistant bacteria in the winter season was significantly different from pre or post monsoon ($p < 0.01$) in site III. Most significant differences between ampicillin-resistant/chloramphenicol-resistant

bacteria compared with kanamycin or streptomycin or tetracycline were noted in all the sampling sites, SS I ($p < 4.19e-8$), SS II ($p < 3.01e-5$), and SS III ($p < 3.5e-11$). There was no significant interaction between the factors.

ANOVA (two-way classified data with replication) for fraction of different antibiotic-resistant-bacteria for testing equality of proportions (Season X Site) was also performed to understand the interaction between factors as well as to reveal significant differences in occurrence of particular antibiotic-resistant in three seasons or sites, if any. The occurrences of particular antibiotic-resistant bacterial population in three different seasons did not differ significantly, except chloramphenicol ($p < 0.001711$) and tetracycline-resistant ($p < 0.003435$) bacterial fractions. In terms of the fractions of chloramphenicol-resistant bacteria or tetracycline-resistant bacteria there were differences in occurrence between seasons ($p < 0.01$). The fraction of chloramphenicol-resistant copiotrophs recovered in winter was significantly different from pre- or post monsoon occurrence. The occurrence of the same population in pre-monsoon significantly varied from post-monsoon. Tetracycline-resistant copiotrophic bacterial population in the winter varied significantly from the pre-monsoon. Again, the pre-monsoon occurrences of tetracycline-resistant bacteria were significantly different from post-monsoon recovery. The occurrences of particular antibiotic-resistant bacterial population in three different sampling sites did not differ significantly. No significant interaction between the factors resulted from this analysis.

1.3.5 Determination of antibiotic-resistance- patterns (ARPs)

1.3.5.1 Analysis of ARPs of the antibiotic resistant bacteria from SS I

A total of 5712 antibiotic resistant copiotrophic bacterial isolates from 13 water samples of SS I were screened for multiple antibiotic resistance phenotype (Table 1.5). Only, 5.04% of the exhibited resistance to single antibiotic and the rest 94.95% (5424) were resistant to two or more of the screening antibiotics (MAR). Among the singly resistant group, resistance to chloramphenicol, ampicillin, tetracycline and streptomycin were 2.31%, 1.31%, 1.24% and 0.17% respectively. Singly resistant bacteria to kanamycin were absent. A total of 22 different combinations of MAR phenotype were observed among the isolates. Within the group of MAR isolates, 34.48% were doubly resistant, 34.10% were triply resistant, 23.94% were quadruply resistant and 2.41% were quintuply resistant to the antibiotics that were used (Figure 1.2). Among the double resistant combinations AT (16.31%) was the most frequent one, followed by AC (13.56%) and CT (2.24%) combination. Only a single isolate exhibited CK resistance phenotype.

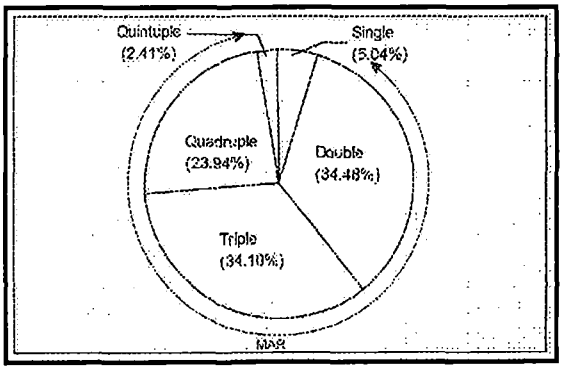


Figure 1.2. Frequency of singly resistant and MAR phenotypes among antibiotic resistant copiotrophic bacterial isolates from SS I.

Table 1.5: Antibiotic resistance patterns (ARPs) of the antibiotic resistant copiotrophic bacterial populations in different sampling months (January 2000 to December 2001) at SS I

	Jan. 2000	May 2000	Oct. 2000	Nov. 2000	Dec. 2000	Jan. 2001	Mar. 2001	May 2001	June 2001	Sep. 2001	Oct. 2001	Nov. 2001	Dec. 2001
A	48.27 (14)	0.75 (7)	1.76 (5)	0.59 (1)		0.33 (1)			0.74 (5)	0.85 (3)	1.39 (10)	2.93 (19)	1.69 (10)
C	10.34 (3)		1.06 (3)	3.55 (6)		27.0 (81)		0.69 (4)	1.18 (8)		0.41 (3)	1.38 (9)	2.54 (15)
K													
S			1.06 (3)	0.59 (1)		0.33 (1)							0.84 (5)
T		1.60 (15)	0.70 (2)	14.20 (24)				1.04 (6)	0.14 (1)	3.14 (11)		1.69 (11)	0.16 (1)
AC	34.48 (10)		1.06 (3)			9.66 (29)		20.0 (115)	12.75 (86)	9.71 (34)	2.09 (15)	39.66 (257)	38.3 (226)
AK		0.10 (1)						4.69 (27)			0.69 (5)		
AS			0.35 (1)	1.18 (2)						2.0 (7)		6.17 (40)	
AT		23.28 (217)	10.60 (30)	1.77 (3)				14.6 (84)	17.80 (120)	15.71 (55)	10.62 (76)	26.38 (171)	29.83 (176)
CK											0.13 (1)		
CS			2.82 (8)								0.41 (3)		
CT		0.53 (5)	5.30 (15)	5.32 (9)	3.58 (8)	1.66 (5)		2.43 (14)	8.16 (55)		1.81 (13)	0.61 (4)	
KS													
KT									1.33 (9)				
ST			3.53 (10)	3.55 (6)					2.22 (15)				
ACK										5.42 (19)	1.25 (9)	1.54 (10)	0.50 (3)
ACS			0.35 (1)	2.95 (5)								0.15 (1)	
ACT		44.74 (417)	36.04 (102)	29.58 (50)	4.03 (9)	51.0 (153)	75.89 (170)	40.0 (230)	27.44 (185)	24.28 (85)	15.38 (110)	17.59 (114)	20.50 (121)
AKT		0.21 (2)						2.60 (15)					
AST		3.54 (33)	2.82 (8)	7.69 (13)						3.42 (12)			
CKT						0.33 (1)		2.08 (12)	1.78 (12)		2.37 (17)		
CST		0.10 (1)	0.70 (2)	3.55 (6)	1.34 (3)	0.66 (2)		1.21 (7)	0.59 (4)		0.55 (4)		
ACKS										2.28 (8)			
ACKT		0.21(2)					4.46 (10)	7.82 (45)	11.57 (78)	22.28 (78)	18.88 (135)		1.18 (7)
ACST		24.35 (227)	31.09 (88)	24.26 (41)	91.03 (203)	9.0 (27)	19.64 (44)		7.56 (51)	3.14 (11)	22.23 (159)	0.77 (5)	1.01 (6)
AKST			0.35 (1)						2.96 (20)		13.42 (96)		
CKST								0.34 (2)			3.35 (24)		
ACKST	6.89 (2)	0.53 (5)	0.35 (1)	1.18 (2)				2.43 (14)	3.70 (25)	7.71 (27)	4.89 (35)	1.08 (7)	3.38 (20)
Total	29	932	283	169	223	300	224	575	674	350	715	648	590

A, Ampicillin; C,Chloramphenicol; K, Kanamycin; S, Streptomycin; T, Tetracycline
The numbers in parenthesis denotes the actual number of isolates

The KS combination was not found among the screened population. ACT (30.56%) and ACST (15.09%) were the most frequent patterns observed within the triply and quadruply resistant group.

In the month of January 2000, a total of 29 antibiotic resistant bacteria from SS I were screened. It was found that the 58.64% of the screened population were single resistant, whereas, 41.37% exhibited resistance to two or more antibiotics (multiple-antibiotic resistant). The population resisting only ampicillin (48.27%) was the most dominant among the singly resistant group. In this month only two different MAR phenotypes – AC (34.48%) and ACKST (6.89%) were observed. A total of three hundred bacterial isolates were screened in January 2001. Among them, 27.66% were single resistant, 11.32% were doubly resistant, 51.99% were triply resistant and 9.0% were quadruply resistant. In both the years, populations resisting only kanamycin or tetracycline were not present among the screened population. The most dominant combination found was ACT (51.0%). The MAR combinations like AK, AS, AT, CK, CS, KS, KT, ST, ACK, ACS, AKT, AST, ACKS, ACKT, AKST and CKST were absent in January 2000 and 2001.

In May 2000, a total of 932 isolates were screened. Only a small fraction (2.35%) of the population exhibited single resistance. Majority of them (97.59%) were multi-resistant. Isolates exhibiting resistance to three different antibiotics were most common among the multi-resistance group (represented by 48.59% of the population). ACT (44.74%) and ACST (24.35%) were the most frequent combination present among triply and quadruply resistant group. Similar MAR combinations were observed among the antibiotic resistant isolates screened during May 2001. None of the isolates exhibited resistance only to ampicillin, kanamycin or streptomycin. AC (20.0%), ACT (40.0%) and ACKT (7.82%) combinations were the dominant ones among the doubly, triply and quadruply resistant group.In May 2000, 0.53% and in May 2001, 2.43% were quintuply resistant. It was found that in both the years, 2000 and 2001, the water samples of the month of May, contained no isolates demonstrating multiple antibiotic resistance combinations like AS, CK, CS, KS, KT, ST, ACK, ACS, CKT, ACKS and AKST.

In two successive years, 2000 and 2001, the numbers of antibiotic resistant bacteria screened for multi-resistance in the month of October were 223 and 715 bacteria respectively. The occurrences of number of different resistance-combinations in the said month, in both the years, exceeded from all other data from other sampling months. Thirteen and fifteen different resistance-combination was noted in the year 2000 and 2001 respectively. In October 2000, 39.91% of the populations were triply resistant, 31.44% were quadruply resistant, 23.66% were doubly resistant, 4.58% were singly resistant and 0.35% was resistant to all the five antibiotics tested. ACT (36.04%) was the most dominant MAR phenotype followed by ACST (31.09%) combination. Somewhat different picture was observed during October 2001 where quadruply-resistant group was most dominant, comprising 57.88% of the total antibiotic-resistant bacteria screened. Among the quadruply resistant group, ACST (22.23%) was the most frequent combination, followed by ACKT, AKST and CKST

patterns. The incidences of other resistance-groups were: 19.55%, triply resistant; 15.75%, doubly resistant; 4.89%, quintuply resistant; and 1.8%, singly resistant. AT (10.62%) and ACT (15.38%) were the most frequent combinations among the doubly and triply resistant groups.

In November 2000, 169 antibiotic resistant bacteria were screened. 81.03% of the population exhibited resistance to two or more antibiotics. Among them 43.77% were triply resistant, 24.26% were quadruply resistant, 11.82% were doubly resistant and only 1.18% were quintuply resistant. 93.95% of the 648 isolates that were screened during November 2001 exhibited multiresistance, of which 72.82% were doubly resistant, 19.28% were triply resistant, 1.08% was quintuply resistant and 0.77% was quadruply resistant. During November 2000 and 2001, populations exhibiting resistance to only ampicillin, chloramphenicol or tetracycline were present. The populations resisting only streptomycin were found during November 2000 but not during 2001. Strikingly, only kanamycin resistant populations were absent in both the years in the month of November. In November 2000, isolates having ACT (29.58%) combination were more frequent followed by the occurrence of ACST (24.26%). The resistance combination, AC (39.66%) and AT (26.38%) constituted the most frequent ones in November 2001.

Cent percent of the screened population exhibited multi-resistant phenotype during December 2000, dominated by the occurrence of ACST (91.03%). A total of 590 antibiotic resistant bacteria were screened during December 2001. Within the population, 68.13% were doubly resistant, 21.0% were triply resistant, 5.23% were single resistant and 3.38% were quadruply resistant. Among the doubly resistant group, AC (38.3%) pattern was most frequent followed by AT (29.83%). Another dominant combination was ACT (20.50%).

In March 2001, single as well as doubly resistant was absent among the screened population from the same site. Three different MAR phenotypes were observed in the said month, of which ACT combination was present among 75.89% of the population screened. Rest of the population exhibited ACKT (4.46%) and ACST (19.64%) phenotype. In the month of June 2001, 674 isolates were screened and 12 different MAR combinations were observed among 97.86% of them. Among the resistance-groups, 42.26% were doubly resistant, 29.81% were triply resistant, 22.09% were quadruply resistant and 3.70% were quintuply resistant. The occurrence of ACT (27.44%) was most dominant among the different MAR combinations. Of the antibiotic resistant bacteria in September 2001, 95.95% exhibited multi-resistance phenotype. Of them 33.12% were triply resistant, 27.7% were quadruply resistant, 27.42% were doubly resistant and 7.71% were quintuply resistant. ACT (24.28%) was the most common ARP found among the multi-resistant isolates.

The maximum occurrence of singly resistant bacteria toward ampicillin was noted in January 2000. The occurrence of bacteria resistant to only chloramphenicol was highest in the month of January 2001 while isolates resisting only ampicillin was least occurring. The number of bacteria exhibiting only tetracycline resistance was highest in November 2000. Although

kanamycin appeared in different multi-resistance combinations, populations exhibiting only kanamycin resistance or kanamycin-streptomycin in combination were completely absent. Among the double resistant combinations, AT was the most frequent one showing highest occurrence in December 2001, followed by November 2001 and May 2000 samplings. During October 2000 and 2001, only 1.06% and 2.09% of the population exhibited this phenotype. The second highest occurrence was noted for AC. ACT represented the maximally occurred ARP among the combinations of three different antibiotics. The highest occurrence of this pattern was noted in March 2001. 44.74 and 40.0% of the population during May 2000 and 2001 exhibited the said phenotype.

1.3.5.2 Analysis of ARPs of the antibiotic resistant bacteria from SS II

From sampling site II, a total of 4429 antibiotic resistant copiotrophic bacterial isolates from 13 water samples were screened (Table 1.6). The distribution of singly, doubly, triply, quadruply and quintiply resistant bacteria have been presented in Figure 1.3. Altogether 9.73% of the screened population exhibited single resistance of which chloramphenicol resistance was most frequent (6.29%), followed by ampicillin (1.80%), tetracycline (0.69%), streptomycin (0.51%) and kanamycin (0.40%) resistance. Among 23 possible different ARPs, combinations like CK, KT and CKST were completely absent. 32.89% of the MAR population exhibited resistance to two different combinations of antibiotics. Within this group, most frequent occurrence was observed for the combination AC (20.61%). The frequencies of occurrence of other doubly resistant combinations were 7.83% for AT and 3.68% for the CT. 29.69% of the populations were triply resistant of which 24.02% exhibited ACT phenotype. ACKT (6.11%) was the most frequent pattern observed within the quadruply resistant group.

During January 2000, 145 antibiotic resistant bacteria were screened. Among the screened population, 40.0% were triply resistant, 39.29% were singly resistant and 20.67% were doubly resistant. Quadruply and quintuply resistant combinations were not found. Singly resistant group was represented by 39.28% of the population, whereas, 60.67% of the population were multi-resistant. In this month four different combinations of MAR phenotypes were observed of which the ARP like ACS (40.0%) was the most dominant. During January 2001, a total of 338 bacteria were screened. In the said month, among the bacterial isolates, ARP of five different antibiotics was not present. Chloramphenicol resisting populations were the sole representatives of the singly resistant group. Among the multi-resistant group, 44.65% were doubly resistant, 32.83% were triply resistant and 2.95%

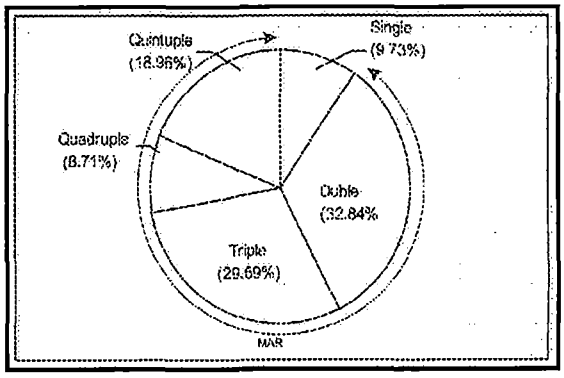


Figure 1.3. Frequency of singly resistant and MAR phenotypes among antibiotic resistant copiotrophic bacterial isolates from SS II.

Table 1.6 Antibiotic resistance patterns (ARPs) of the antibiotic resistant copiotrophic bacterial populations in different sampling months (January 2000 to December 2001) at SS II

	Jan. 2000	May 2000	Oct. 2000	Nov. 2000	Dec. 2000	Jan. 2001	Mar. 2001	May 2001	June 2001	Sep. 2001	Oct. 2001	Nov. 2001	Dec. 2001
A	13.79 (20)	1.47(15)		2.88 (3)			5.20 (14)	2.13 (7)	4.12 (9)	0.63 (2)	1.39 (8)	0.46 (2)	
C	24.82 (36)	0.49(5)		10.57 (11)	100.0 (62)	19.52 (66)				2.22 (7)	4.34 (25)	6.29 (27)	33.33 (40)
K											1.73 (10)	1.86 (8)	
S	0.68 (1)	1.08 (11)					1.48 (4)			1.58 (5)			1.66 (2)
T		0.29 (3)		2.88 (3)				0.60 (2)	2.29 (5)			2.56 (11)	5.83 (7)
AC	14.48 (21)	0.029(3)	73.52 (375)	55.76 (58)		5.91 (20)		29.26 (96)			6.95 (40)	61.7 (265)	29.16 (35)
AK											0.17 (1)		
AS							0.74 (2)				0.52 (3)		
AT		3.63 (37)		3.84 (4)			25.27 (68)	21.03 (69)	40.82 (89)		8.0 (46)	7.92 (34)	
CK													
CS	5.51 (8)	0.09 (1)	0.39 (2)			0.88 (3)							4.16 (5)
CT	0.68 (1)	0.09 (1)		7.69 (8)		37.86 (128)			11.46 (25)				
KS							1.48 (4)						
KT													
ST			0.58 (3)				0.74 (2)						
ACK										7.93 (25)			0.83 (1)
ACS	40.0 (58)		0.78 (4)			12.72 (43)							1.66 (2)
ACT		3.44 (35)	20.58 (105)	11.53 (12)		16.27 (55)	40.14 (108)	41.15 (135)	35.77 (78)	34.92 (110)	57.39 (330)	17.71 (76)	16.66 (20)
AKT		0.29 (3)					5.57 (15)	1.52 (5)					
AST		0.19 (2)	0.19 (1)				9.29 (25)			5.71 (18)			
CKT						0.29 (1)	1.85 (5)						
CST			1.96 (10)			3.55 (12)	7.43 (20)	0.30 (1)					
ACKS		0.09 (1)											
ACKT		12.58 (128)						2.43 (8)		30.47 (96)	6.26 (36)		2.5 (3)
ACST		0.88 (9)	1.37 (7)	1.92 (2)		2.95 (10)			4.58 (10)	11.74 (37)	2.60 (15)	0.46 (2)	
AKST											3.82 (22)		
CKST													
ACKST		74.92 (762)	0.58 (3)	2.88 (3)			0.74 (2)	1.52 (5)	0.91 (2)	4.76 (15)	6.78 (39)	0.93 (4)	4.16 (5)
Total	145	1016	510	104	62	338	269	328	218	315	575	429	120

were quadruply resistant. CT (37.86%) was the most dominant MAR phenotype.

In the month of May 2000, 74.92% of the total screened population (1016 isolates) exhibited resistance to all the five antibiotics tested. Among the other multi-resistance combinations, quadruply resistant group were represented by 13.55% of the population, 3.92% were triply resistant and 3.81% were doubly resistant. Within the singly resistant group (represented by 3.33% of the population), populations exhibiting resistance to only ampicillin, chloramphenicol, streptomycin and tetracycline were present. During May 2001, 328 antibiotic resistant bacterial isolates were screened. Among them 50.29% were doubly resistant, 42.97% were triply resistant, 2.73% were singly resistant, 2.43% were quadruply resistant and only 1.52% were quintuply resistant. The populations exhibiting resistance to ACT (41.45%) in combination were the most frequent ones. The ARPs like AK, AS, CK, KS, KT, ST, ACK, ACS, CKT, AKST and CKST in the MAR population were absent both during May 2000 and 2001.

The numbers of antibiotic resistant bacteria screened for multi-resistance in the month of October of 2000 and 2001 were 510 and 575 respectively. In October 2000, cent percent of the screened population exhibited multi-resistance, dominated by the occurrence of doubly resistant combinations (74.49%). AC (73.52%) represented the most frequent ARP among doubly resistant MAR isolates. Among the other MAR groups, 23.51%, 1.37% and 0.58% were triply, quadruply and quintuply resistant respectively. A different picture was observed in October 2001. The triply resistant combinations (57.39%) dominated the multi-resistance group. 15.64% represented the doubly resistant group, 12.68% were quadruply resistant and 6.78% were quintuply resistant. The isolates having ACT (57.39%) combinations were more frequent. The populations exhibiting resistance to only ampicillin, chloramphenicol and kanamycin were present but only streptomycin and tetracycline resisting populations were not found. During both the years, ARP like CK, CT, KS, KT, ACK, AKT, CKT, ACKS and CKST among MAR bacteria were absent.

In November 2000, 104 isolates were screened. 16.33% of the screened populations were resistant to a single antibiotic. The population resisting only chloramphenicol (10.57%) was the most dominant among the singly resistant group. Incidences of doubly, triply, quadruply and quintuply resistant bacteria were 67.29%, 11.53%, 1.92% and 2.88% respectively of the screened population. AC (55.76%) and ACT (11.53%) were most frequent ARPs among the doubly and triply resistant combinations. In November 2001, 429 bacteria were screened- of which 88.72% were multi-resistant. Among the multiresistant group, 69.62% were doubly resistant, 17.71% were triply resistant, 0.46% was quadruply resistant and 0.93% was quintuply resistant. The AC (61.7%) combination was the most frequent ARP among MAR isolates. In water samples of November 2000 and 2001, populations exhibiting resistance to only ampicillin, chloramphenicol or tetracycline were present. The isolates exhibiting only kanamycin resistance were found in November 2001 but not in 2000. Isolates exclusively resistant to streptomycin were absent in both the years in the month of November.

In December 2000, the entire screened population (a total of 62 isolates) exhibited resistance singly to chloramphenicol. A completely different picture of antibiotic resistance combinations was found during December 2001. In the said month, a total of 120 antibiotic resistant bacteria were screened. Singly resistant isolates was found among 40.82% of the screened population. Although, isolates exhibiting resistance singly to chloramphenicol was the most dominating one (33.33%), small fractions of the population also exhibited resistance singly to streptomycin and tetracycline. Multi-resistance was observed among 59.13% of the screened population. Among them, 33.32% were doubly resistant, 19.15% were triply resistant, 2.5% were quadruply resistant and 4.16% were quintuply resistant. In this month seven different ARPs of MAR isolates were found. The most frequently occurring ARPs were AC (29.16%) and ACT (16.66%).

In the month of March 2001, a total of 269 antibiotic resistant bacterial isolates were screened to check their antibiotic resistance profile. Among the singly resistant population (6.68%), only ampicillin resisting population was the most prevailing one. 64.28% of the screened population exhibited resistance to three different antibiotics, 28.23% were doubly resistant, 6.68% were singly resistant and only 0.74% was resistant to all the five antibiotics. No quintuply resistant populations were recovered. The most dominant ARP among MAR isolates was ACT (40.14%), followed by AT (25.27%). In June 2001, 218 bacteria were screened. Among them 52.28% were doubly resistant, 35.77% were triply resistant, 6.41% were singly resistant, 4.58% were quadruply resistant and 0.91% was quintuply resistant. Among the most populous MAR groups, the frequency of occurrence of AT (40.82%) and ACT (35.77%) were exceedingly higher than the other combinations of the respective groups. In the month of September 2001, a total of 315 bacteria were screened, of which 48.56% were triply resistant, 42.21% were quadruply resistant, 4.76% were quintuply resistant and 4.43% exhibited resistance to any one of the tested antibiotics.

The maximum occurrence of singly resistant bacteria toward ampicillin was noted in January 2000. The occurrence of bacteria resistant to only chloramphenicol was highest in the month of December 2000. In that particular month there was complete absence of other antibiotic resistant phenotypes either singly or in multi combinations. The occurrences of only tetracycline resisting population were highest in December 2001. Very few bacterial isolates, compared to that of ampicillin or chloramphenicol resisting populations, exhibited single resistance to either kanamycin or streptomycin. The most dominated ARP among doubly resistant isolates was AC, showing highest occurrence in October 2000, followed by November 2001 and 2000. During May 2000 only 0.029% of the population exhibited this phenotype. The second highest occurrence was noted for AT (maximum in June 2001). Among the triply resistant group, ACT represented the most dominant ARP. The highest occurrence of this phenotype was noted in October 2001 and lowest in the month of May 2000. ACKT was the most frequent ARP among the quadruply resistant group.

1.3.5.3 Analysis of ARPs of the antibiotic resistant bacteria from SS III

A total of 4254 antibiotic resistant copiotrophic bacterial isolates from 13 water samples of SS III were screened for multiple antibiotic resistance phenotype (Table 1.7). The distribution of singly, doubly, triply, quadruply and quintuply resistant bacteria have been presented in Figure 1.4. Single resistance was exhibited by 304 isolates. Among the singly resistant group, resistance to Chloramphenicol (5.82%) was most frequent, followed by occurrence of isolates resistant to ampicillin (0.68%), tetracycline (0.32%), streptomycin (0.25%) and kanamycin (0.04%). Twenty-one different ARPs among MAR isolates were found. The combinations like CK and KS were absent. Among the most populous MAR group, doubly resistant, of SS III, the frequency of occurrence of AC (23.57%) was exceedingly higher than the other combinations of this group. The frequency of occurrence of AT was 16.12%. Likewise, ACT (20.54%) and ACST (9.44%) were the most frequent ARPs observed within the triply and quadruply resistant group. 5.33% of the population were quintuply resistant.

In the month of January 2000, a total of 75 antibiotic resistant bacteria from SS III were screened. Among the screened population, 54.66% were doubly resistant, 26.66% were singly resistant and 18.66% were triply resistant. The population resisting only chloramphenicol (20.0%) was the most prevailing one among the singly resistant group. Three different ARPs, AC (54.66%), ACT (14.66%) and ACS (4.0%) were found in this month. Hundred isolates were screened during January 2001. All of them were found to exhibit resistance either to a single or to two different antibiotics. The maximum percentage of the population exhibited resistance to only chloramphenicol (50.0%). A single ARP, AC (45.0%), was observed in the said month. It was found that, in January 2000 and 2001, the populations resistant to four or five different antibiotics were completely absent. Moreover, in January 2001, triply resistant populations were also absent.

In May 2000, a total of 364 isolates were screened. Cent percent of the screened population were multi-resistant. Isolates exhibiting resistance to four different antibiotics were most common among the multi-resistance group (represented by the 57.14%) of the population. ACKT (54.12%) and ACKST (32.96%) were the dominant ARPs among quadruply and quintuply resistant group. In May 2001, a total of 330 isolates were screened, of which 3.33% were singly resistant. The doubly resistant isolates constituted 54.22% of the population and 29.99% were triply resistant. The quadruply and quintuply resistant isolates represented 9.69 and 2.72% of the screened population. AC and AT combinations among the doubly resistant group and ACT combination among the triply resistant group were the dominant ones.

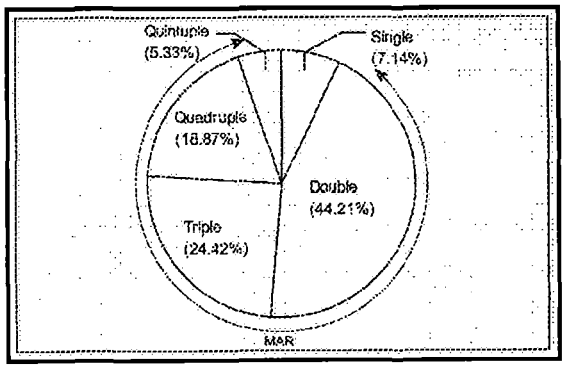


Figure 1.4. Frequency of singly resistant and MAR phenotypes among antibiotic resistant copiotrophic bacterial isolates from SS III.

Table 1.7 Antibiotic resistance patterns (ARPs) of the antibiotic resistant copiotrophic bacterial populations in different sampling months (January 2000 to December 2001) at SS III

	Jan 2000	May 2000	Oct. 2000	Nov. 2000	Dec. 2000	Jan. 2001	March 2001	May 2001	June 2001	Sep. 2001	Oct. 2001	Nov. 2001	Dec. 2001
A	4.0 (3)		0.93 (4)	1.07 (5)	0.44 (2)	5.0 (5)					0.81 (3)	2.02 (7)	
C	20.0 (15)		8.15 (35)	8.35 (39)	19.91 (89)	50.0 (50)		3.03 (10)		1.27 (5)	1.35 (5)		
K												0.28 (1)	0.43 (1)
S	2.66 (2)							0.30 (1)		0.25 (1)	1.08 (4)	0.86 (3)	
T			0.46 (2)						0.5 (2)	1.02 (4)	0.81 (3)	0.86 (3)	
AC	54.66 (41)	1.09 (4)	50.58 (217)	38.3 (179)	7.60 (34)	45.0 (45)		23.93 (79)	14.0 (56)	9.46 (37)	2.43 (9)	54.2 (187)	50.0 (115)
AK								4.84 (16)			0.81 (3)		
AS										4.34 (17)	1.89 (7)	4.34 (15)	
AT				10.06 (47)	7.82 (35)		28.66 (88)	23.03 (76)	24.5 (98)	17.64 (69)	30.60 (113)	18.84 (65)	41.30 (95)
CK													
CS			0.46 (2)	0.21 (1)						0.25 (1)			
CT			0.23 (1)		12.9 (58)			2.42 (8)	9.25 (37)	0.76 (3)	1.89 (7)		0.86 (2)
KS													
KT									1.0 (4)				
ST									2.5 (10)				
ACK		0.54 (2)		0.85 (4)						3.32 (13)		0.57 (2)	
ACS	4.0 (3)		0.23 (1)							0.25 (1)		0.57 (2)	
ACT	14.66 (11)	8.24 (30)	12.58 (54)	16.9 (79)	21.25 (95)		27.36 (84)	29.69 (98)	22.25 (89)	24.55 (96)	51.49 (190)	11.01 (38)	4.34 (10)
AKT							4.56 (14)						
AST				2.78 (13)	6.04 (27)		20.19 (62)						
CKT									4.5 (18)				
CST			0.46 (2)					0.30 (1)					
ACKS										0.51 (2)			
ACKT		54.12 (197)					5.86 (18)	7.27 (24)	8.0 (32)	14.06 (55)	1.62 (6)	3.47 (12)	
ACST		3.02 (11)	25.87 (111)	20.5 (96)	23.93 (107)				7.0 (28)	12.27 (48)	0.27 (1)		
AKST							10.09 (31)		2.0 (8)		1.89 (7)		
CKST								2.42 (8)			0.27 (1)		
ACKST		32.96 (120)		0.85 (4)			3.25 (10)	2.72 (9)	4.5 (18)	9.97 (39)	2.71 (10)	2.89 (10)	3.04 (7)
Total	75	364	429	467	447	100	307	330	400	391	369	345	230

A, Ampicillin; C,Chloramphenicol; K, Kanamycin; S, Streptomycin; T, Tetracycline
The numbers in parenthesis denotes the actual number of isolates

During 2000 and 2001, in the month of May, none of the isolates exhibited resistance to only kanamycin or tetracycline. The ARPs like AS, CK, CS, KS, KT, ST, ACS, AKT, AST, CKT, ACKS and AKST were absent in both the years during the same month. Although in May 2000, only six different ARPs among MAR isolates were found, nine different ARPs were noted in MAR isolates of May 2001.

A total of 429 and 369 isolates were screened for multiple-antibiotic-resistance in the month of October during the years 2000 and 2001 respectively. In October 2000, 51.27% of the populations were doubly resistant. The incidences of other resistance groups were: 25.87%, quadruply resistant; 13.27%, triply resistant; and 9.54% were singly resistant. Quintuply resistant populations were not found. AC (50.58%) and ACST (25.87%) were the most frequently occurring ARPs among MAR isolates. During October 2001, 51.49% of the populations were triply resistant, followed by next higher incidence of the doubly resistant group (represented by 37.62% of the population). Singly resistant isolates comprised 4.05% of the total antibiotic resistant isolates screened. The rest 4.05 and 2.71% of the population were the quadruply and quintuply resistant isolates. The most occurring ARP of the MAR isolates in the said month was ACT (51.49%) followed by AT (30.6%). Eleven different ARPs among MAR isolates were found in October 2001 whereas only seven ARPs were encountered in analysis of water samples of October 2000. Isolates exhibiting resistance to only kanamycin were absent in both October 2000 and 2001. The water samples in the month of October, in both the years 2000 and 2001, contained no isolates demonstrating ARPs like CK, KS, KT, ST, ACK, AKT, AST, CKT and ACKS.

In November 2000, 467 antibiotic resistant bacteria were screened. 90.45% of the populations were MAR. Among the MAR isolates, 48.57% were doubly resistant, 20.53% were triply resistant, 20.5% were quadruply resistant and 0.85% was quintuply resistant. The most frequent occurrences were encountered for the ARPs like AC (38.3%) and ACST (20.5%). A total of 345 isolates were screened during November 2001. Of the population 77.38% were doubly resistant, 12.15% were triply resistant, 4.02% were singly resistant, 3.47% were quadruply resistant and 2.89% were quintuply resistant. AC (54.2%) was the most frequently occurring ARP among the MAR isolates. During November 2000, there was complete absence of singly resistant isolates toward kanamycin, streptomycin or tetracycline. But in 2001, isolates resisting only chloramphenicol were absent. In two successive years, 2000 and 2001, complete absence of ARPs like AK, CK, CT, KS, KT, ST, AKT, CKT, CST, ACKS, AKST and CKST among MAR isolates were noted.

During December 2000, 447 bacteria were screened. Among them, 28.32% were doubly resistant, 27.29% were triply resistant, 23.93% were quadruply resistant and 20.35% were singly resistant. There was complete absence of the quintuply resistant population. The resistance to only chloramphenicol was dominant among the singly resistant group. Among the multi-resistance combinations, ACT (21.25%) and ACST (23.93%) were the most dominant ones. Among the 230 bacterial isolates that were screened during December 2001, 99.54% were MAR bacteria. Five different ARPs

were found among MAR isolates, of which AC (50.0%) followed by AT (41.30%) were the most frequent ones. During December 2000 and 2001, populations resisting only streptomycin or tetracycline were absent. Only ampicillin or chloramphenicol resisting singly resistant isolates were reported in December 2000 but not during December 2001. The isolates exhibiting resistance to only kanamycin were found in December 2001. Some of the ARPs like AK, AS, CK, CS, KS, KT, ST, ACK, ACS, AKT, CKT, CST ACKS, ACKT, AKST and CKST were not found in MAR isolates in water samples of December in two successive years, 2000 and 2001.

In March 2001, 307 bacteria were screened. Within the population, 52.11% were triply resistant, 28.66% were doubly resistant, 15.95% were quadruply resistant and 3.25% were quintuply resistant. The most dominant ARPs among MAR isolates were AT (28.66%), ACT (27.36%) and AST (20.19%). The singly resistant isolates were not present. Of the antibiotic resistant bacteria screened during June 2001, 99.5% were MAR. The MAR population consisted of 51.25% doubly resistant, 26.75% triply resistant, 17.0% quadruply resistant and 4.5% of the quintuply resistant population. AT (24.5%) and ACT (22.25%) were the most common ARPs found among the MAR isolates. A total of 391 antibiotic resistant bacteria were screened in September 2001. Among the MAR population, 32.45% were doubly resistant, 28.12% were triply resistant, 26.84% were quadruply and 9.97% were quintuply resistant. The most common ARP was ACT (24.55%). Only 2.54% of the populations were singly resistant.

The maximum occurrence of singly resistant bacteria toward ampicillin was noted in January 2001 and the lowest in October 2001. The occurrence of bacteria resistant to only chloramphenicol was highest in the month of January 2001 while in the same month isolates resisting only ampicillin was the least. Only 1.02% of the bacteria exhibited resistance singly to tetracycline in September 2001 and that was the highest for only tetracycline resisting population. Although kanamycin appeared in different multi-resistance combinations, isolates exhibiting only kanamycin resistance were too low and found only in the months of November 2001 and December 2001. Among the doubly resistant combinations, AC was the most frequent one. More than 50% of the screened populations, in the months of January 2000, October 2000, November 2001 and December 2001, exhibited this phenotype. The occurrence for AT combination was noted next to AC. Among the doubly resistant isolates, ARPs like CK and KS were absent. ACT was the most frequently occurring ARP among the triply resistant isolates. The highest occurrence of this pattern was found in October 2001. Maximum occurrence of quintuply resistant population was recorded in May 2000. During January 2000 to December 2000, maximum of eight different ARPs were observed among the MAR isolates. An increase in number of different ARPs was recorded from May 2001 to October 2001. Eleven to twelve different ARPs among MAR isolates were found during the said time frame.

1.3.6 Comparison of antibiotic resistant bacteria recovered from three sampling sites on river Torsa

The relative abundance of singly-resistant and multi-resistant (resistant to two or more antibiotics tested) bacteria in the pool of antibiotic-resistant-copiotrophs

(bacterial colonies picked up randomly from plates containing single antibiotic) from water samples collected in the same month of the two successive years, 2000 and 2001, from three different sampling stations (SS I, SS II, & SS III) on River Torsa have been presented in figures (Figure 1.5, 1.6, 1.7, 1.8, 1.9).

In the month of January 2000, in SS I singly resistant populations were dominant while in the next year triply resistant populations was most dominant. In January 2000, triply and quadruply resistant populations were completely absent while January 2001 was marked by the absence of quintuply resistant bacteria among antibiotic resistant bacteria. The water samples collected from SS II in month of January 2000 showed maximum incidence of triply resistant bacteria while in the next year doubly resistant populations was most dominant. In both the years, the SS II water samples were devoid of quintuply resistant bacteria among antibiotic resistant bacterial population. The water samples collected from SS III in month of January 2000 showed maximum incidence of doubly resistant bacteria while in 2001 singly resistant populations was most dominant. In both the years, the SS III water samples were devoid of quadruply as well as quintuply resistant bacteria among antibiotic resistant bacterial population. Though triply resistant bacteria could be isolated from the water samples of the same site in 2000 but it was absent in 2001.

In the month of May of both the years, 2000 & 2001, the water samples collected from SS I contained mostly triply resistant bacteria. The water samples collected from SS II in month of May 2000 showed

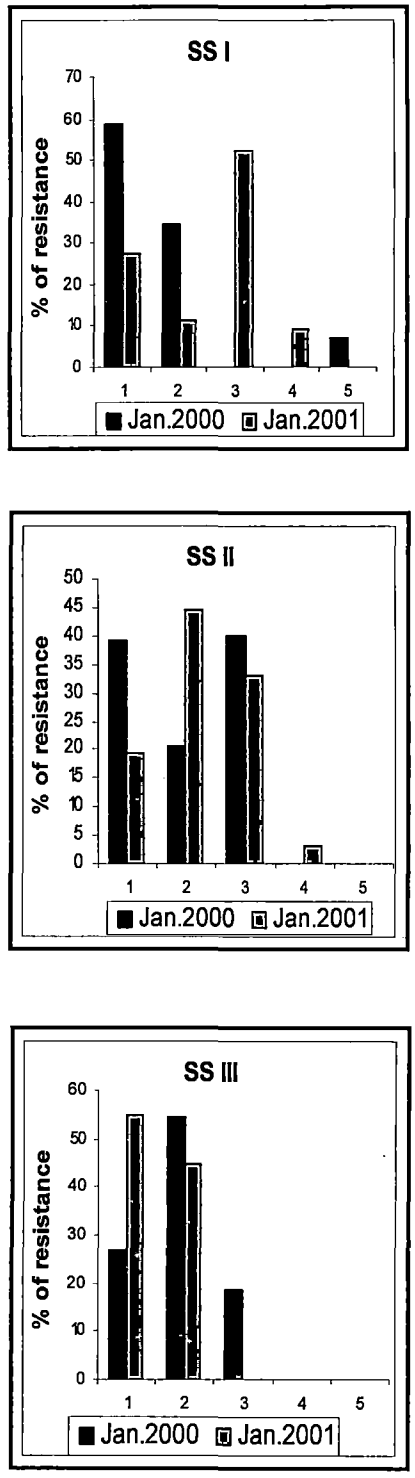


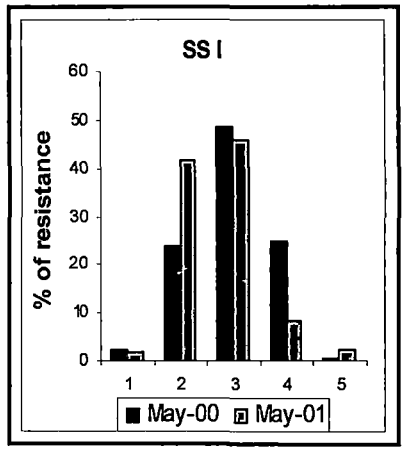
Figure 1.5. Relative abundance of singly resistant and multi-resistant bacteria in the pool of antibiotic- resistant copiotrophs from water samples collected in the month of January of the two successive years, 2000 and 2001, from three sampling stations [SS I, SSII and SS III] on river Torsa.
1, Singly resistant; 2, Doubly resistant; 3, Triply resistant; 4, quadruply resistant; 5, quintuply resistant.

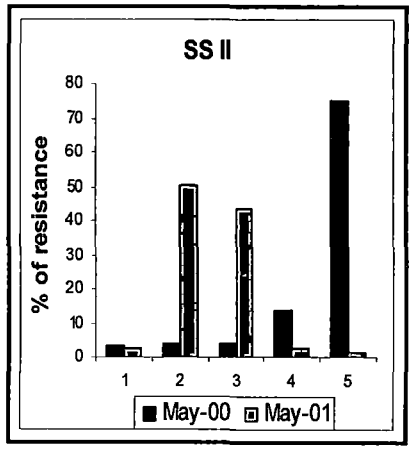
maximum incidence of quintuply resistant bacteria while in the next year doubly resistant populations was most dominant. The water samples collected from SS III in month of May 2000 showed maximum incidence of quadruply resistant bacteria while in 2001 doubly resistant populations was most dominant. In the year, 2000, the SS III water samples were devoid of singly resistant bacteria among antibiotic resistant bacterial population.

In the month of October 2000, in SS I triply resistant populations were dominant while in the next year quadruply resistant populations was most prevalent. The water samples, collected from SS II in month of October 2000, were marked by the absence of singly resistant and abundance of doubly resistant bacteria while in the next year triply resistant populations dominated. In the year 2000, the SS III water samples were devoid of quintuply resistant bacteria but dominated by doubly resistant among antibiotic resistant bacterial population. In the following year, 2001, triply resistant bacteria were most abundant.

In the month of November, triply and doubly resistant bacteria were most abundant in the year 2000 and 2001 respectively in the water samples collected from SS I. In both the years, doubly resistant bacteria were most dominant in water samples collected from both the sampling sites, SS II and SS III.

In the month of December, quadruply and doubly resistant bacteria were most abundant in the year 2000 and 2001 respectively in the water samples collected from SS I. The water samples of SS I in the month of December 2000 were devoid of singly and quintuply resistant bacterial





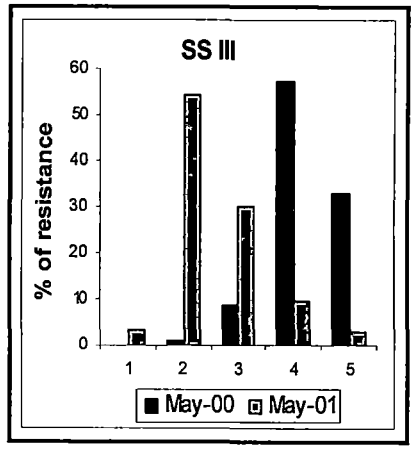


Figure 1.6. Relative abundance of singly resistant and multi-resistant bacteria in the pool of antibiotic- resistant copiotrophs from water samples collected in the month of May of the two successive years, 2000 and 2001, from three sampling stations [SS I, SSII and SS III] on river Torsa.
1, Singly resistant; 2, Doubly resistant; 3, Triply resistant; 4, quadruply resistant; 5, quintuply resistant

population The water samples, collected from SS II in month of December 2000, was marked by the sole presence of singly resistant and complete absence of bacteria resistant to two or more antibiotics, while emergence of multi-resistant bacteria was noted in the next year samples with dominance of singly resistant bacteria. In both the years, the SS III water samples contained maximum number of doubly resistant bacteria. Complete absences of quintuply and quadruply resistant bacteria were noted in the year 2000 and 2001 respectively, in water samples of SS III in the month of December.

1.3.7 Statistical analysis of data on singly and mutiply-antibiotic-resistant (resistance to two or more antibiotics tested) bacteria in the antibiotic-resistant population pooled from water samples collected from three different sampling stations, SS I, SS II, SS III, along the river Torsa

The data presented in the tables 1.5, 1.6 and 1.7 on percentage occurrence of singly, doubly, triply, quadruply and quintuply resistant groups were arcsine transformed before analysis and were rearranged as follows-

a. The percent occurrences of doubly, triply, quadruply and quintuply resistant groups were added together to form a group, tentatively termed as multi-resistant (treated as one variable) while percent occurrence of singly resistant was treated as another variable. Correlation coefficient (r) of the variable pair (singly and multi-resistant) revealed a strong negative correlation from the data of SS I (r= - 0.958) and SS III (r= -1.0). The correlation coefficient obtained for the SS III was - 0.458.

b. The existing data of singly, doubly, triply, quadruply and quintuply resistant

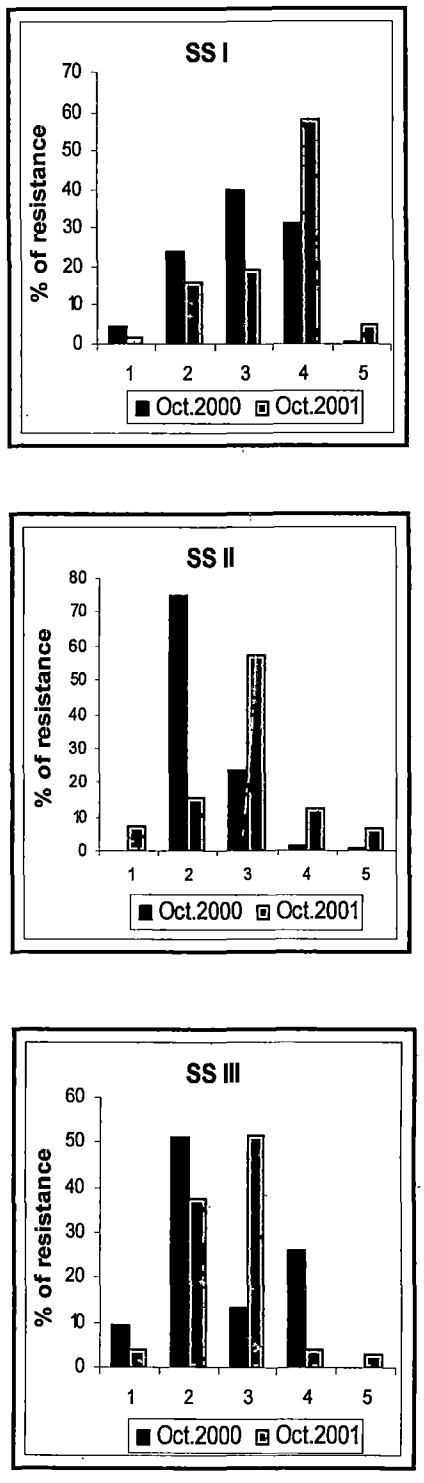
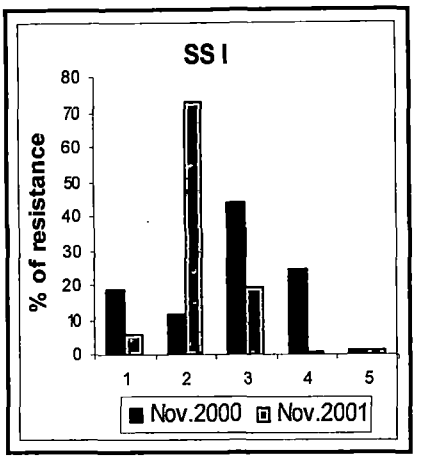


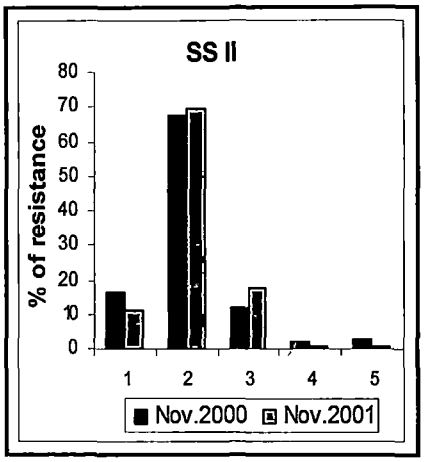
Figure 1.7. Relative abundance of singly resistant and multi-resistant bacteria in the pool of antibiotic- resistant copiotrophs from water samples collected in the month of October of the two successive years, 2000 and 2001, from three sampling stations [SS I, SSII and SS III] on river Torsa.
1, Singly resistant; 2, Doubly resistant; 3, Triply resistant; 4, quadruply resistant; 5, quintuply resistant

groups were treated as separate variables. Significant negative correlation coefficient(s) between occurrences of (i) Singly resistant with quadruply- resistant (r = -0.595), (ii) doubly-resistant and Quadruply-resistant (r = -0.627) proportions of antibiotic resistant bacteria were observed from the data of SS I.

In case of the data derived from water sample analysis of SS II, no significant correlation coefficient was obtained amongst the different proportions of antibiotic resistant bacteria screened for multiple-antibiotic-resistance. The data derived from water sample analyses of SS III revealed significant negative correlation coefficient(s) between the occurrence of doubly and quadruply-resistant (r = - 0.742), and singly and quintuply-resistant (r = - 0.693); while quadruply and quintuply-resistant bacterial proportions showed positive correlation coefficient (r = + 0.589).

The Wilcoxon matched pairs signed ranks test was used to compare the proportion and test the significance of the difference in abundance of the MAR groups. The significant difference in recovery of proportion of matched pairs were doubly and quintuply($p< 0.002$), triply and quintuply ($p< 0.003$), and quadruply and quintuply ($p< 0.007$) resistant bacteria in water samples of SS I. The occurrence of the matched pairs, singly and doubly, singly and triply, doubly and triply, and triply and quadruply resistant bacteria were significantly different with the probability < 0.003, < 0.002, < 0.002, and < 0.004 respectively, in water samples of SS II. There was significant difference in recovery of proportion of singly and doubly ($p< 0.002$), doubly and quintuply ($p< 0.004$), and triply and quintuply ($p< 0.006$)





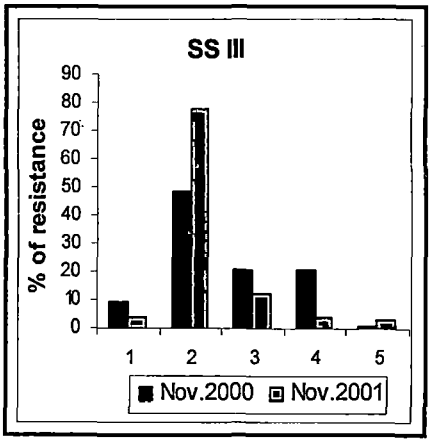


Figure 1.8. Relative abundance of singly resistant and multi-resistant bacteria in the pool of antibiotic- resistant copiotrophs from water samples collected in the month of November of the two successive years, 2000 and 2001, from three sampling stations [SS I, SSII and SS III] on river Torsa.
1, Singly resistant; 2, Doubly resistant; 3, Triply resistant; 4, quadruply resistant; 5, quintuply resistant

resistant bacteria in water samples of SS III.

1.4 Discussion

The reserves of water on the earth are immense, but this is mostly salt water which is unfit for drinking and irrigation purposes. Freshwater is the liquid of life, without which the planet would be a barren wasteland. 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. Globally between 12.5 and 14 billion cubic meters of water are available for human use on an annual basis. In 1989 this amount equaled about 9000 cubic meters per person per year and by 2000 had dropped to around 7800 cubic meters per person. In 2025 the amount of per capita expected to fall to 5100 cubic meters per person as the worlds population grows from 6 billion to over 8 billion (www.peopleandplanet.net). Even this amount would be enough to meet human needs with the even distribution of fresh water resources. But available fresh water supplies are not distributed evenly around the globe throughout the seasons or from year to year.

Rivers are the main sources of fresh water. There are no national waters in the rivers that travel through several countries. They are constantly moving, while the nations can divide land, they can never divide waters running above it. River waters do not mind political or national boundaries. 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

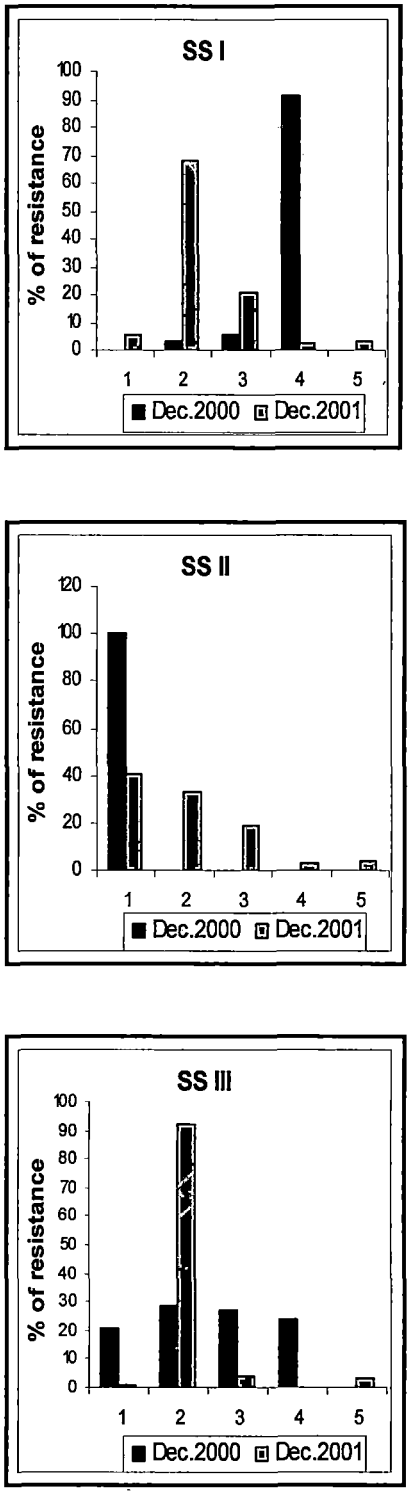


Figure 1.9. Relative abundance of singly resistant and multi-resistant bacteria in the pool of antibiotic- resistant copiotrophs from water samples collected in the month of December of the two successive years, 2000 and 2001, from three sampling stations [SS I, SSII and SS III] on river Torsa.
1, Singly resistant; 2, Doubly resistant; 3, Triply resistant; 4, quadruply resistant; 5, quintuply resistant

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 worlds total annual usable fresh water runoff (www.peopleandplanet.net).

The trends, in the contamination of river water world wide, 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. Most pharmaceuticals are found in natural waters in only very low concentrations. Antibiotics are of particular interest because we do not know currently whether their presence in natural waters contributes to the spread of antibiotic resistant organisms. Subsequent knowledge regarding the effect of sub-inhibitory concentrations of the antimicrobials on the survival of bacteria in environment is scarce and contradictory. But on the other side voluminous evidences are there which revealed the existence of antibiotic resistant bacteria in nature and horizontal transfer of antibiotic resistance determinants between them. Therefore there is a great dilemma between the choices of the major determinant for the stable maintenance of antibiotic resistant bacterial populations in different environmental compartments. Whatever the reasons for antibiotic resistance, such traits are apparently found in many bacteria and in different environments.

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 indigenous flora 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 (Aravanitidou *et al.* 2001). 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. To date, little work has been done to assess the prevalence of drug resistant bacteria in surface waters mainly rivers. Antibiotic resistance of the native bacterial population other than those of fecal origin must be considered for the precise assessment of the environmental pool of bacterial antibiotic resistance. Several groups have considered the whole bacterial populations, gram-negative bacteria, heterotrophic bacteria or viable bacteria and dealt with global antibiotic resistance (the frequency of cells able to grow on antibiotic supplemented media). In the present study, an intensive bacteriological investigation was made of the incidence and abundance of antibiotic resistant copiotrophic bacteria in water samples of the river Torsa.

In this study, water samples were collected from three sampling stations (shown in Figure 1.1). At the first sampling site (SS I) the river Torsa enters into Indian territory from Bhutan, and thereby analysis of water quality at this place throws light on different anthropogenic

activities influencing the water quality of Amo-Chu (synonym of Torsa at Bhutan) and also helps to understand the quality of water that flows within the Jaldapara Wild Life Sanctuary of North Bengal, which is the immediate destination of the river. At this sampling site the river is very turbulent, flows at high speed through pebbles and the river basin is around 700 meter wide. After SS I, the river is alienated into small channels and enters into Jaldapara Wildlife Sanctuary; serving as one of the source of drinking water for the wild life and forest tribes. After leaving the sanctuary, small channels of the river again fuse and form two large and wide branches, at village- Falakata of Jalpaiguri district, named Sil-torsa and Buri-torsa. Siltorsa is the major and wider (890 m) stream and second sampling station (SS II) was located at this point. At this site, the river is wide and less turbulent. The riverbank is made up of dry white sand. The entire hinterland of Torsa catchments between SS I and SS II sustains mainly the agricultural fields including tea gardens and small hamlets of human habitations. After leaving SS II, the tributaries, Siltorsa and Buritorsa, meet at Coochbehar Town; district Coochbehar, of the state of West Bengal, India. Here was the third sample collection site, SS III, where the depth of the river increases and flows slowly. Here the riverbank is made up of clay, sand and humus. Compared with the other sampling sites, the load of urban effluents on river water is quite high at this point as is evidenced from the results of the previous study. It was expected that frequency of resistance in the bacterial assemblages would be greater among the population isolated from the site with the putatively greatest human impact. In a previous study conducted on Yarra river of Australia, seven sites were used which has headwaters in the central highlands of Victoria and flows through forests, rural farmland and finally, through urban and inner city of Melbourne before discharging to the sea. It was anticipated that the incidence of antibiotic resistance in native bacteria isolated from polluted sites (such as river stretches in well developed urban areas, sewage treatment effluents) would be greater than the incidence in native bacteria isolated from pristine sites, such as drinking water catchments closed to public access and upstream reaches of rivers situated in forested uplands or flowing through areas of low intensity agriculture (Boon and Cattanach 1999).

Thirty-nine water samples were collected altogether and analyzed between January 2000 to December 2001 from the three sampling stations on the river Torsa. Standard plate count was made to assess the general copiotrophic bacterial content of the water samples collected from the three sampling stations. For the cultivation of bacteria, Luria Bertani agar plates were used. The recovered bacteria did not represent all the bacterium present in water but only those able to grow copiously and produce visible colonies on the nutrient rich media used and under prescribed condition of temperature and incubation. The cultivable bacteria represent only a small portion of the vast number of bacteria present in the environment and that the number that can be enumerated is higher than can be cultivated. It has been observed that the known and cultivable environmental bacteria add up to only 5-10% of the total number assumed to be present in waste water and waste water treatment plants. Use of classical microbiological methods can lead to the cultivation and Identification of only 1% of the soil

bacteria (Kummerer 2004).

The maximum recovery of the culturable copiotrophic bacterial populations took place mainly in the month of May in both the years from all three sampling sites. The minimum occurrence of the population was recorded during the months of January and March, in all the three sampling stations. The general population of bacteria in surface water may include some genera that could, under special circumstances, contribute a health risk. Although copiotrophic bacterial counts is not a true indicator of potential pathogens that might be present. It seems reasonable to assume, however, that chance occurrences are proportionately greater as the copiotrophic bacterial population increases. A wide range of variation in the occurrence of recovered bacterial population was observed. It is likely that tremendous fluctuations occur within the river water populations as related to season, temperature, turbidity, total organic carbon and chemical content and this probability accounts for much of the variability that have been observed in regard to the recovery of the culturable copiotrophs obtained from three different sampling sites. We found that the culturable antibiotic resistant bacteria were widespread in water samples from three different sampling sites located on river Torsa.

In the second step, the isolates were classified according to their susceptibility to a certain antibiotic. Five different antibiotics, namely ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline were used singly in nutrient rich solid medium to recover the fraction of the copiotrophs that could withstand the vulnerability of the specific antibiotic and could be termed as resistant to that particular antibiotic. The fractions resisting ampicillin and chloramphenicol were recovered maximally from the water samples of all the three sites. The percent occurrences of kanamycin resisting populations were recorded to be the lowest among all other antibiotic resisting populations. It was observed that in all the water samples analyzed, in the months where maximum recoveries of the ampicillin or chloramphenicol resisting populations were recorded, the occurrences of other four antibiotic resisting populations were quite low.

The data on percent occurrence of the different antibiotic resistant bacterial populations were arcsine transformed before performing different statistical analyses. The arcsine transformation, also known as angular transformation, is especially appropriate to percentages and proportions. The arcsine transformation finds $\theta=\arcsin\sqrt{p}$, where p is a proportion. The term arcsin is synonymous with inverse sin or $\sin^{-1}$, which stands for the angle whose sine is the given quantity. The arcsine transformation stretches out both tails of distribution of percentages or proportions and compresses the middle. When the percentages in the original data fall between 30 and 70%, it is generally not necessary to apply the arcsine transformation. In the present study data fell between 0.03 and 89.28%, and therefore arcsine transformation was applied to stabilize the data set.

Trend is the smooth, regular and long term movement of time series exhibiting the basic tendency of growth, decline or stagnation over a period of time. It was observed that fraction of population exhibiting resistance to ampicillin or

kanamycin or tetracycline have shown similar trends of increase in the sampling sites, SS I and SS III. The water samples analyzed from all the three sampling sites, SS I, II, and III, did not have any significant difference in occurrence of the proportion of ampicillin and chloramphenicol resistant fraction of the culturable copiotrophs of River Torsa. Analyses of Wilcoxon matched pairs signed ranks test revealed significant difference(s) in occurrence, irrespective of the sampling sites, for following pairs: Kanamycin and ampicillin, streptomycin and ampicillin, tetracycline and ampicillin, kanamycin and chloramphenicol, streptomycin and chloramphenicol, and tetracycline and chloramphenicol, antibiotic resistant fractions of copiotrophic bacteria.

Analysis of variance (ANOVA) was conducted to test – 1) relation between sampling site and fraction of copiotrophs resistant to different antibiotics, 2) to test whether seasonal variations in percent occurrences for antibiotic resistant populations were evident and 3) to observe any significant differences in occurrence of particular antibiotic resistant population in three different seasons or sites. Significant differences ($p < 0.05$) between the sampling sites SS I and SS II were noted in the months of May 2000, December 2000 and March 2001. In the other sampling months, no significant differences were scored between the sampling sites. Antibiotic resistant populations exhibited significant differences in occurrence in various sampling months. It was observed from this analysis that percent occurrence of ampicillin and chloramphenicol differed significantly from other antibiotic resistant populations.

This investigation also documents the occurrence of multiple antibiotic resistant (MAR) copiotrophic bacterial isolates in river water of Torsa. Multiple-antibiotic-resistance (MAR) profile of the recovered copiotrophic bacterial population on plates containing single antibiotic was obtained by the replica plating technique against five antibiotics. It was found that more than 90% of the antibiotic resistant bacteria obtained from each sampling station were MAR. The percent occurrence of population exhibiting resistance to only one antibiotic was in the range of 5.04-9.73%. Lin *et al.* (2004) studied the antibiotic resistance profiles of the enteric bacteria isolated from the Mhlathuze catchment and found that 94.7% of these isolates were resistant to at least one class of antibiotic while 75.2% were multi resistant. However, Park *et al.* (2003) showed that 53.6% of coliform isolates of an aquatic environment were resistant to one or more antibiotics tested. Ampicillin as one of the constituent in the ARPs happened to occur in 90.10%, 84.84% and 88.91% of the total MAR bacteria analyzed from SS I, SS II, and SS III respectively. Similarly, occurrence of chloramphenicol in the ARPs of MAR population were 72.98% in SS I, 80.04% in SS II, and 71.22% in SS III water samples. The maximum occurrence of ampicillin and chloramphenicol as a constituent in the ARPs of the MAR bacteria actually supported the insignificant difference in occurrence of ampicillin and chloramphenicol resistant fractions in Wilcoxon matched pairs signed ranks test. Presence of high percentage of ampicillin resistant bacterial populations indicated that beta-lactamase gene might be widely present in the gene pool of microbes in the environment. The analysis of antibiograms revealed that the MAR bacteria were in a

dynamic state of fluctuation within the aquatic environment. An examination of MAR bacteria isolated from three different sampling sites revealed striking differences in types and frequencies. Such observation is comparable to the results of an earlier study (Armstrong *et al.* 1981) where striking differences were observed for the types and frequencies of MAR bacteria isolated from two nearby sites. But the observation was quite different from the previous work conducted by Lin *et al.* (2004) on Mhlathuze river. They found that antibiotic resistance profiles of *E.coli* and non-*E. coli* isolates were similar regardless of the site. There was no clear pattern of antibiotic resistance in the antibiotic resistant bacterial population isolated from water samples of the river Torsa during January 2000 to December 2001. Population fluctuation was also observed at a single site sampled on a month-to-month basis. Variations were observed in the percentage of MAR organisms recovered and the resistance markers selected. Similar observation was made by earlier authors (Boon and Cattanach 1999) who found no distinct pattern to antibiotic resistance in the native bacteria isolated from the various river sites of Yarra river in Australia. It was observed that maximum percent of multiple antibiotic resistant bacteria were isolated from downstream (SS III) which is mainly urban than those from the upstream point (SS I and SS II) that is predominantly rural. The results suggest that urban effluents may have impact on the level of antibiotic resistance in the environment.

Correlation analysis between the singly and multi-resistant groups revealed whether any systematic or nonsystematic variations exist between the different MAR groups. A high correlation indicates systematic variation while a low correlation indicates nonsystematic variations between antibiotic resistance patterns of the bacterial population. Significant negative correlation was scored between the singly and quadruply resistant and doubly and quadruply resistant MAR population of SS I. It means that increase in occurrence of singly or doubly resistant groups will lead to the decrease in occurrence of the quadruply resistant population. Very low correlation was scored between the other antibiotic resistant populations from the same site. No significant correlation was scored between the antibiotic resistant populations recovered from water samples of SS II, which led to conclude that nonsystematic variation took place between the different antibiotic resistant groups. Significant negative correlation between doubly and triply resistant and singly and quadruply resistant groups was scored among the recovered antibiotic resistant population of the SS III. A positive correlation was observed among the quadruply and quintuply resistant groups, meaning that increase in occurrence of one group would be leading to the simultaneous increase of the other group. The values obtained from the correlation matrix revealed that antibiotic resistances of the culturable copiotrophs were random rather systematic in their occurrence. Such results were quite different from the results of the earlier findings where systematic variations were scored among the antibiotic resistances for the bacterial isolates recovered from different environmental sources (Kelch and Lee 1978).

The comparison of the percentage of resistant strains with published work from

other times and places are complicated because different groups have used different numbers and kinds of antibiotics in their studies. It is difficult to provide a single consistent picture and to make a comparison between the results obtained in this study with the results of previous works. Because the factors, that affect the survival and proliferation of antibiotic resistant populations, change with respect to land and water use pattern in different regions of the same country as well as between different countries. If the incidence of resistance to antibiotics and synthetic antimicrobial drugs is to be compared in different areas and at different times, it is necessary to identify the bacteria and to use the same set of antimicrobial drugs in the tests.

1.5 Conclusion

The problem of antibiotic resistance is a global one. Global surveillance are needed to look after antibiotic use and the residence of resistant strains, since genetic fluidity of bacteria allow them to cross national and international boundaries. It is essential to identify the ecological nature of the problem and to concentrate on the kinds of resistances in the so called reservoirs – both commensal and the non-clinical bacteria. Because the data going to generate from this background will forecast about the commencement of the next resistance story and will also identify the site where antibiotic selection pressure for resistance is high. Studies have found that rivers have become major reservoirs of antibiotic resistance genes (Park *et al.* 2003 and Biyela *et al.* 2004). Finding antibiotic resistant bacteria in rivers is hardly novel. What has not been appreciated is the extent of contamination. A noteworthy paper by Chee *et al.* (2001) addressed the critical area of antibiotic resistance and dissemination of resistance genes in the environment. The ground water was shown to be contaminated with genes that were identical to those present in swine farm waste lagoons; therefore, the authors concluded that the contaminants seat into the ground water from lagoons. The potential risk to human health from antibiotic resistance contamination of ground water is several folds. Genes may be mobilized into soil microorganisms and hence into food chain. Ingestion of microbes, whether or not they are pathogenic, can enable acquisition of antibiotic resistance genes by gutflora of humans and animals. Subsequent discharge in fecal material continues the cycle of antibiotic resistance. Moreover, ground water discharged to 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. In present days perspective, with alarming rise in antibiotic resistance, reemergence of once fatal illnesses and with the changing nature of the drug resistant pathogens, not only the increased numbers of surveillance programmes are needed but also resistance surveillance programmes are needed to be coordinated and the results made available.

1.6 Summary of chapter 1

A total of thirty-nine water samples, thirteen from each sampling site on river Torsa, were collected and studied on monthly basis from January 2000 to December 2001. Maximum occurrence of culturable copiotrophs was recorded in the month of May in water samples irrespective of the sampling sites. Copiotrophic bacterial counts (CBCs) recorded in different months per sampling site exhibited very wide variations with large dispersion values. The geometric mean of CBCs in water samples ranged from 2.64×10^4 to 4.80×10^4. Five different antibiotics, ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline, were used singly in Luria-Bertani agar plates to enumerate the recovery of resistant bacteria against each antibiotic. Percent occurrence of ampicillin and chloramphenicol resistant population were much higher than other antibiotic resistant bacteria. The lowest occurrence was recorded for kanamycin resistant population. In the months where recovery of either ampicillin or chloramphenicol resistant population was exceedingly high, the occurrences of other antibiotic resistant populations were quite low. In spite of the least occurrence of kanamycin resistant populations among antibiotic resistant bacteria, an upward trend of occurrence of the said population was recorded in SS I and SS III with passage of time. Wilcoxon matched pairs signed ranks test was used to compare the proportion and test the significance of the difference in abundance of the five antibiotic resistant groups. No significant difference in occurrence of the proportion of ampicillin and chloramphenicol resistant fraction of copiotrophs was found among water samples analyzed. Analysis of variance (ANOVA) (two way classified data with and without replication) was performed to test- 1) relation between sampling site and fraction of copiotrophs resistant to different antibiotics, 2) the effect of seasonal variations on percent occurrences of antibiotic resistant populations and 3) any significant differences in occurrence of particular antibiotic resistant population in three different seasons or sites. Antibiotic resistant populations exhibited significant differences in occurrence in various sampling months. The percent occurrence of ampicillin and chloramphenicol differed significantly from other antibiotic resistant populations. Antibiotic-resistant-patterns (ARPs) of a total of 14,395 antibiotic- resistant isolates were determined. Analyses of ARPs revealed that more than 90% were resistant to two or more antibiotics tested. The occurrence of ampicillin and chloramphenicol as one of the constituent of the ARP combinations of MAR isolates was most frequent. Some ARP combinations like AC, AT, ACT were most dominant ARPs among MAR bacteria. It was found that when singly resistant bacteria dominated in water samples, MAR bacteria were only a few and vice-versa. This observation was supported by negative correlation coefficient value. Among MAR groups, significant negative correlation was observed between the occurrence of doubly and quadruply resistant ($r = -0.742$) and singly and quintuply resistant ($r = -0.693$) bacteria isolated from water samples of SS III. To compare the proportion and test the significance of the difference in abundance of MAR groups, Wilcoxon matched pairs signed ranks test was used. Significant differences in occurrence found among matched pairs were: doubly and quintuply ($p < 0.002$); triply and quintuply ($p < 0.003$); and quadruply and quintuply ($p < 0.007$) resistant bacteria isolated from SS I. Similar significant differences in recovery of proportion of singly and doubly ($p < 0.003$); singly and triply ($p < 0.002$); doubly and triply ($p < 0.002$); triply and quadruply ($p < 0.004$) resistant bacteria were noted in samples from SS II. The occurrences of singly and doubly ($p < 0.002$); doubly and quintuply ($p < 0.004$) and triply and quintuply ($p < 0.006$) resistant bacteria were also significantly different in water samples collected from SS III.

1.7 References

Alcaide, E., M-D. Blasco, and C. Esteve. 2005. Occurrence of drug-resistant bacteria in two European eel farms. Appl. Environ. Microbiol. **71** : 3348-3350.

Al-Jebouri, M.M. 1985. A note on antibiotic resistance in the bacterial flora of raw sewage and sewage polluted River Tigris in Mosul, Iraq. J. Appl. Bacteriol. **58** : 401-406.

Aravanitidou, M., V. Katsouyannopoulos, and A. Tsakris. 2001. Antibiotic resistance patterns of enterococci isolated from coastal bathing water. J. Med. Microbiol. **50** : 1001-1005.

Armstrong, J., D. S. Shigeno., J. J. Calomiris, and R. J. Seidler. 1981. Antibiotic-resistant bacteria in drinking water. Appl. Environ. Microbiol. **42**: 277-83.

Armstrong, J. L., J. J. Calomiris, and R. J. Seidler. 1982. Selection of antibiotic-resistant standard plate count bacteria during water treatment. Appl. Environ. Microbiol. **44**: 308-316.

Ash, R. J., B. Mauck, and M. Morgan. 2002. Antibiotic resistance of gram-negative bacteria in rivers, United States. Emerg. Infect. Dis. **8** : 713-716.

Barkay, T., N. Kroer., L. D. Rasmussen, and S. J. Sørensen. 1995.Conjugal transfer at natural population densities in a microcosm simulating an estuarine environment. FEMS. Microbiol. Ecol. **16** : 43-53.

Bhadra, B., S. Mukherjee., R. Chakraborty, and A. K. Nanda. 2003. Physicochemical and bacteriological investigation on the river Torsa of North Bengal. J. Environ. Biol. **24** : 125-133.

Biyela, P.T., J. Lin, and C.C. Bezuidenhout. 2004. The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. Water. Sci. Technol. **50** : 45–50.

Boon, P. I, and M. Cattanach. 1999. Antibiotic resistance of native and faecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, south-eastern Australia. Lett. Appl. Microbiol. **28**: 164-168.

Chee-Sanford, J. C., R. I. Aminov., I. J. Krapac., N. Garrigues-Jeanjean, and R. I. Mackie. 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. Appl. Environ. Microbiol. **67**: 1494-1502.

Cohen, E, and R. Colwell. 1986. Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. Appl. Environ. Microbiol. **51** : 1285-1292.

Davison, J. 1999. Genetic exchange between bacteria in the environment. Plasmid. 42 : 73-91.

Diab, A. M., M. H. Abdel Aziz., and S. A. Salim. 2000. Antibiotic-resistant gram-negative bacteria isolated from drinking water in Ismalia city. The Tenth Conference on Microbiology. Cairo, Egypt. November 12-14. 2000. pp. 1339-153.

Goni-Urizza, M., M. Capdepuy., C. Arpin., N. Raymond., P. Caumette, and C. Quentin. 2000. Impact of an urban effluent on antibiotic resistance of riverine *Enterobacteriaceae* and *Aeromonas* spp. Appl. Environ. Microbiol. **66**: 125-132.

Grabow, W. O. K., O. W. Prozesky, and L. S. Smith. 1974. Drug resistant coliforms call for review of water quality standards. Water. Res. **8** : 19.

Harwood, V. J., J. Whitlock, and V. Withington. 2000. Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in predicting the source of fecal contamination in subtropical waters. Appl. Environ. Microbiol. **66** : 3698-3704.

Heuer, H., E. Krögerrecklenfort., E. M. H. Wellington., S. Egan., J. D. van Elsas., L. van Overbeek., J. -M. Collard., G. Guillaume., A. D. Karagouni., T. L. Nikolakopoulou, and K. Smalla. 2002. Gentamicin resistance genes in environmental bacteria: prevalence and transfer. FEMS. Microbiol. Ecol. **42** : 289-302.

Hirsch, R., T. Ternes., K. Harber, and K –L. Kratz. 1999. Occurrence of antibiotics in the environment. Sci. Total. Environ. **225** : 109-118.

Jones, J.G. 1986. Antibiotic resistance in aquatic bacteria. J. Antimicrob. Chemother. **18**: 149-154.

Kaspar, C. W., J. L. Burgess., I. T. Knight., and R. R. Colwell. 1990. Antibiotic resistance indexing of *Escherichia coli* to identify sources of fecal contamination in water. Can. J. Microbiol. **36**: 891-894.

Kelch, W. J, and J. S. Lee. 1978. Antibiotic resistance patterns of gram negative bacteria isolated from environmental sources. Appl. Environ. Microbiol. **36** : 450-456.

Kolpin, D. W., E. T. Furlong., M. T. Meyer., E. M. Thurman., S. D. Zaugg., L. B. Barber, and H. T. Buxton. 2002. Pharmaceuticals, hormones and other organic waste water contaminants in U. S. streams, 1999-2000: A national

reconnaissance. Environ. Sci. Technol. **36**: 1202-1211.

Kolwzan, B., T. Traczewska, and M. Pawlaczyk-Szipilowa. 1991. Examination of resistance bacteria isolated from drinking water to antibacterial agents. Environ. Prot. Eng. **17** : 53-60.

Kummerer, K. 2004. Resistance in the environment. J. Antimicrob. Chemother. **54** : 311-320.

Leff, L.G., J.V. McArthur, and L.J. Shimkets. 1993. Spatial and temporal variability of antibiotic resistance in freshwater bacterial assemblages. FEMS. Microbiol. Ecol. **13** : 135-144.

Lin. J., P. T. Biyela, and T. Puckree. 2004. Antibiotic resistance profiles of environmental isolates from Mhlathuze River, KwaZulu- Natal (RSA). Water SA. **30** : 23-28.

Magee, A.M, and J.P. Quinn. 1991. Antibiotic resistance in the bacteria of a remote upland river catchment. Lett. Appl. Microbiol. **13** : 145-149.

McArthur, J. V, and R. C. Tuckfield. 2000. Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. Appl. Environ. Microbiol. **66** : 3722-3726.

McKeon, D. M., J. P. Calabrese, and G. K. Bissonnette. 1995. Antibiotic resistant gram-negative bacteria in rural ground water supplies. Water. Res. **29** : 1902-1908.

Mukherjee, S., B. Bhadra., R. Chakraborty., A. Gurung., S. Some, and R. Chakraborty. 2005. Unregulated use of antibiotics in Siliguri city vis-à-vis occurrence of MAR bacteria in community waste water and river Mahananda, and their potential for resistance gene transfer. J. Environ. Biol. **26** : 229-238.

Niemi, M., M. Sibakov, and S. Niemala. 1983. Antibiotic resistance among different species of fecal coliforms isolated from water samples. Appl. Environ. Microbiol. **45** : 79-83.

Ogan, M.T, and D. E. Nwiika. 1993. Studies on the ecology of aquatic bacteria of the Lower Niger Delta: multiple antibiotic resistance among the standard plate count organisms. J. Appl. Bacteriol. **74** : 595-602.

Park, J. C., J. C. Lee., J. Y. Oh., Y. W. Jeong., J. W. Cho., H. S. Joo., W. K. Lee, and W. B. Lee. 2003. Antibiotic selective pressure for the maintenance of antibiotic resistant genes in coliform bacteria isolated from the aquatic environment. Water. Sci. Technol. **47**: 249-253.

Parveen, S., R. L. Murphree., L. Edmiston., C. W. Kasper., K. M. Portier, and M. L. Tamplin. 1997. Association of multiple-antibiotic-resistance profiles with point and nonpoint sources of *Escherichia coli* in Apalachicola Bay. Appl. Environ. Microbiol. **63** : 2607-2612.

Pillai, S. D., K. W. Widmer., K. G. Maciorowski, and S. C. Ricke. 1997. Antibiotic resistance profiles of *Escherichia coli* isolated from rural and urban environments. J. Environ. Sci. Health. **A32** : 1665-1675.

Sacher, F., F. T. Lange., H. -J. Brauch, and I. Blankenhorn. 2001. Pharmaceuticals in groundwaters: Analytical methods and results of a monitoring program in Baden-Wuettemberg, Germany. J. Chromatogr. A. **938** : 199-210.

Schmidt, A. S., M. S. Bruun., I. Dalsgaard., K. Pedersen, and J. L. Larsen. 2000. Occurrence of antimicrobial resistance in fish pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Appl. Environ. Microbiol. **66** : 4908-4915.

Schwartz, T., W. Kohnen., B. Jansen, and U. Obst. 2003. Detection of antibiotic-resistant bacteria and their resistance genes in waste water, surface water and drinking water biofilms. FEMS Microbiol. Ecol. **43** : 325-35.

Sokari, T. G., D. D. Ibiebele, and R.M. Ottih. 1988. Antibiotic resistance among coliforms and *Pseudomonas* spp. from bodies of water around Port Harcourt, Nigeria. J. Appl. Bacteriol. **64** : 355- 359.

Swartz, M. N. 2000. Impact of antimicrobial agents and chemotherapy from 1972 to 1998. Antimicrob. Agents. Chemother. **44**: 2009-2016.

Wiggins, B. 1996. Discriminant analysis of antibiotic resistance patterns in fecal streptococci, a method to differentiate human and animal sources of fecal pollution in natural waters. Appl. Environ. Microbiol. **62** : 3997-4002.

Wiggins, B. A., R. W. Andrews., R. A. Conway., C. L. Corr., E. J. Dobratz., D. P. Dougherty., J. R. Eppard., S. R. Knupp., M. C. Limjoco., J. M. Mettenburg., J. M. Rinehardt., J. Sonsino., R. L. Torrijos, and M. E. Zimmerman. 1999. Use of antibiotic resistance analysis to identify nonpoint sources of fecal pollution. Appl. Environ. Microbiol. **65** : 3483-3486.

Chapter 2

Transferable plasmid mediated resistance to multiple antimicrobial agents in copiotrophic bacterial isolates in river Torsa

2.1 Introduction

Bacterial 'antimicrobial resistance' has become a matter of vital importance in the present day scenario with economic and social implications throughout the World. The last five decades have seen an outstanding ability of bacterial populations to develop and share resistance to every antibiotics that has been developed, often by quite startling mechanisms and much more readily than was formerly predicted. Over millions of years, bacteria have evolved a number of strategies to coexist peacefully, including the capacity to produce antibiotics to fight off competitors. Many genes determining resistance had been present in nature and predate the clinical use of antimicrobial drugs. Antibiotic resistant bacteria, estimated at over 2000 year old have been isolated from deep within glaciers in Canada's high arctic regions (Dancer *et al.* 1997). Resistant microorganisms have also been found among historic bacterial cultures collected before the beginning of antimicrobial era (Smith 1967). However, before the widespread use of antibiotics, resistant strains constituted a small fraction of the microbial ecosystem.

The successive development of antibiotic resistant bacterial populations follows the Darwinian principle of 'Survival of the fittest' (White and McDermott 2001). The selective pressures, exerted following extensive use of antimicrobials in different arenas, such as human and veterinary medicine and agriculture, and as food animal growth-promoting agents brought about serious problems- the overgrowth of resistant bacterial strains. With increasing rate of antibiotic use, resistant strains of both harmful and harmless bacteria are replacing antibiotic susceptible bacteria. In addition, resistant bacteria from one environment can be carried far away by means of several vectors. All of these factors are playing together to change the balance between antibiotic susceptible and the antibiotic resistant bacteria in our ecosystem, locally and globally.

The problems related to the growing incidence of bacterial antibiotic resistance are especially evident within hospital environment. In the late 1940s, just a few years after the penicillin was put on the market, unresponsive strains of the bacterium *Staphylococcus aureus*, the leading cause of hospital acquired infections, were detected in English hospitals (Cohen 1992). Resistant strains of gonorrhoea, dysentery-causing *Shigella* and *Salmonella* rapidly followed. About a decade later the first report on resistance to the second generation of penicillins arrived; it came from a Boston hospital, where methicillin resistant strains of *Staphylococcus aureus* (MRSA) had been identified (Barret *et al.* 1968). Since the 1980s the frequency of isolates of MRSA among *Staphylococcus aureus* has increased from close to zero to nearly 70% in Japan and the Republic of Korea, 30% in Belgium and around 40% in the United kingdom and the United States. The problem soon became serious for other pathogens as well. Infections caused by multi-resistant bacterial strains such as *Acinetobacter* and *Stenotrophomonas* can in some cases no longer be treated with modern antibiotics and the only available treatment is an old antibiotic, colistin. Globally, escalating levels of the multiresistant intestinal pathogens *Salmonella* and *Shigella* cause severe infections that are difficult to treat

especially in children. Resistance to remaining effective therapy, such as fluoroquinolones, is already increasing, and the industry pipeline for antibiotics against important intestinal pathogens is running dry. Increasing reliance on vancomycin has led to the emergence of vancomycin-resistant enterococci (VRE) infections. Prior to 1989, no U.S. hospital had reported any vancomycin resistant enterococci, but over the next decade, such microbes have become common in U.S. hospitals, according to CDC (Centers for disease control and prevention). Multi-drug resistant tuberculosis is no longer confined to any one country or to those co-infected with HIV, but has appeared in locations as diverse as Africa, Asia and Western Europe, among health care workers and in the general population. Penicillin resistant pneumococci are likewise spreading rapidly, while resistant malaria is on the rise. Now formerly effective drugs are largely useless in the battle to control cholera epidemics. Drug resistant microbes almost always cause nosocomial infections, which account for 40,000 deaths a year in the United States alone. Food borne infections are also on the increase- promoting growing concern about drug resistance in pathogens such as *Salmonella* and *Campylobacter* (WHO 2000) (www.who.int). A resistant strain of *Streptococcus pneumoniae*, first identified in Spain, was soon afterwards found in Argentina, Brazil, Chile, Taiwan, Malaysia, the USA, Mexico, the Philippines, the Republic of Korea, South Africa and Uruguay (Smith and Coast 2002). The first *S. aureus* infections resistant to vancomycin emerged in the United States in 2002 (CDC). In 2003, epidemiologists reported in The *New England Journal of Medicine* that 5 to 10 percent of patients admitted to hospitals acquire an infection during their stay, and that the risk for a hospital-acquired infection has risen steadily in recent decades. A 2003 study in The *New England Journal of Medicine* found that the incidence of blood and tissue infections known as sepsis almost tripled from 1979 to 2000. In *Shigella* strains from Indonesia, Thailand and India 80-90% resistance is seen for two or more antibiotics (Okumara *et al.* 2004).

It is not possible for a single country to protect itself from the threat related to the MDR (multi-drug resistant) bacterial pathogens as they are rapidly thinning out the international boundaries. Apart from environment with profound antibiotic selection pressure (eg. hospital environment), conspicuous incidences of such bacterial populations have been noted in different ecological niches, be it soil (Tolls 2001, Golet *et al.* 2002), sediment (Andersen *et al.* 1994) or river water (Ash *et al.* 2002, Biyela *et al.* 2004, Mukherjee *et al.* 2005). Antibiotics enter into municipal sewage and sewage treatment plants (STP) following disposals in household drains, subsequently they find their way into surface water or sludge. Goni-Urizza *et al.* (2000), made a study to evaluate the effect of urban effluent on antibiotic resistance of fresh water bacterial populations. They found that the urban discharge resulted in the increase of resistant strains of riverine autochthonous and allochthonous bacteria.

2.1.1 Origin and development of antibacterial resistance in bacteria

The interplay of many mechanistic and epidemiological factors brings about the evolution and spread of antibiotic resistance. Mechanistically, antibiotic resistance can be achieved via three routes: modification of the target action,

reduction in the concentration of the drug that reaches the target, and drug inactivation which can occur by both chromosomal mutation and acquisition of new genetic elements. The origin of antibiotic resistance genes in pathogenic bacteria is unclear. The period from the beginning of antibiotic treatment (50 to 60 years ago) to the emergence of bacteria expressing effective resistance mechanisms is too short to explain the development of resistance factors from other proteins by spontaneous mutation. In particular, if a resistance mechanism requires the cooperative action of several proteins (e.g., vancomycin resistance) the de novo generation of such a resistance mechanism in the pathogen is very unlikely (Grohmann *et al.* 2003). Most of the antimicrobial drugs currently in use are derived from metabolites of soil organisms, mainly fungi and actinomycetes. All resistance mechanisms that have been identified in pathogenic bacteria, including RNA methylases, ATP-binding cassette transporters, aminoglycoside phosphotransferases, and ß-lactamases, already exist in the respective antibiotic producers. In *Streptomyces coelicolor* (http://www.sanger.ac.uk/Projects/S_coelicolor/), as well as in the glycopeptide producers of the genus *Amycolatopsis*, even the vancomycin resistance determinants *vanH* (D-Ala dehydrogenase), *vanA* (D-Ala-D-Lac ligase), and *vanX* (D,D-dipeptidase) are present in the very same gene organization as found in the enterococcal conjugative transposon Tn*1549* (Garnier *et al.* 2000).

A single genetic mutation may cause resistance without changing the pathogenecity or viability of a bacterial strain (Gold and Moellerring, 1996). The examples of this type of change include resistance development to antituberculous drugs such as streptomycin (Snider *et al.* 1991). Further examples include the development of fluoroquinolone resistance in Staphylococci, *Pseudomonas aeruginosa*, and other pathogens through alterations in DNA topoisomerase (Hooper and Wolfson 1993). Resistance usually has a biological cost for the microorganisms but compensatory mutations accumulate rapidly that abolish the fitness cost explaining why many types of resistance may never disappear in bacterial population (Normark and Normark 2002), altering existing mechanisms of resistance to make them more active or give them a broader spectrum of activity (Gold and Moellering 1996).

Recent attention has also brought to the light the importance of different physiological states for the survival of bacteria in the presence of antibiotics. Baquero *et al* (1998) found that selective effects occur in selective compartments, where particular antibiotic concentrations result in a different growth rate of resistant bacterial variants. The consecutive development of drug resistance in a population occurs stepwise and organisms with low-level resistance may form the genetic platform for the development of higher resistance levels (Normark and Normark 2002). Once resistance is developed, resistant bacteria appear to acquire a 'life of their own' (Barbosa and Levy 2000). The proliferation and maintenance of these resistant strains in nature are independent of antibiotic selection. Extensive use of vancomycin and cephalosporins has promoted the rise of resistant strains of *Enterococcus faecalis* (Levy 1998). It has been observed that in presence of more than one antibiotics, selection of bacterial variants depends on

the multiple and fluctuating pressure produces or optimization of a single resistance mechanism is essential for survival under the variable environmental conditions. A clear understanding of the biology of antibiotic resistance depends on the detailed analysis of selective environment and related-antibiotic-host-bacteria interactions (Baquero *et al.* 1998). The results of a study revealed that directed evolution could be a powerful tool to predict antibiotic resistance. Directed evolution coupled with structural analysis could be used to predict future mutations that lead to antibiotic resistance (Orencia *et al.* 2001). The relationship between the epidemiological patterns in resistance and major external events those are responsible for the development of resistance has been identified by Philips *et al.* (1998). The findings of this study take into account the antibiotic use and the creation of opportunities for the spread of resistance determinants and resistant organisms as the major contributors for starting point of drug resistance. However, acquisitions of resistance genes by horizontal transfer lead to the development of multi-resistance (resistance to two or more antibiotics).

2.1.2 Horizontal gene transfer - as a means for spread of resistance genes and resistance development

Horizontal gene transfer (HGT) entails the incorporation of genetic elements transferred from another bacterium – perhaps in an early generation – directly into the genome, where they can form genomic islands, i.e blocks of DNA with signatures of mobile genetic elements (Hacker and Carniel 2001). It differs from vertical transfer of genes that describes the inheritance of a gene from a progenitor. In nature, gene dissemination through horizontal gene transfer involves many different players (plasmids, phages, transposons, conjugative transposons, integrons, genomic islets<10 kb and genomic islands>10kb) and mechanisms (homologous and site specific recombination, transposition, conjugation, transformation and transduction) (Lawrence 1999). Gene transfer events that involve members of different species and different genera are called broad host range transfers. Those that occur between very closely related bacteria, such as members of the species, are called narrow host range transfers. Broad and narrow host range transfers are mediated by same mechanism of DNA transfer. Three mechanisms of horizontal gene transfer have been studied extensively-transformation, transduction and conjugation. Conjugative transfer of bacterial plasmids is the most efficient way of horizontal gene spread, and it is therefore considered one of the major reasons for the increase in the number of bacteria exhibiting multiple-antibiotic resistance. This was the first mechanism of gene transfer studied extensively as a way bacteria might disseminate genetic material in non laboratory areas, provides an important means for the horizontal transfer of genetic traits among bacterial populations as well as transphylic transfer of genes from *Agrobacterium tumefaciens* to plants.

2.1.2.1 Conjugation

The traditional view on the bacterial conjugative gene exchange is a gene flow from the plasmid containing donor strain into the plasmid free recipient strain. It encompasses a wide variety of DNA transmission systems operative among gram positive as well as gram-negative bacterial species (Grohmann *et al.* 2003).

In 1952, J. Lederberg, L. L. Cavalli and E. M. Lederberg and in 1953 Hayes reported the presence of an infectious particle, F (fertility factor), that enables a bacterium to function as donor during conjugation. Basic functions exhibited by F remain typical for gram-negative conjugation systems. Particularly useful traits of the F transfer system include constitutive transfer gene expression and an ability to effect efficient gene transfer among cells in liquid culture (Minkley 1986). These self-transmissible plasmids encode functions necessary for both mating pair formation and the transfer of DNA (Willetts and Skurray 1987). There are some self-transmissible plasmids that can effectively cross at least the barrier between bacteria belonging to two different branches of the proteobacteria (Szpirer *et al.* 2001). Many other plasmids unable to promote mating pair formation nevertheless specify cis and trans acting functions that allow them to be effectively mobilized by self-transmissible plasmids (Lanka and Wilkins 1995). These are nonconjugative but mobilizable plasmids. However, even without specialized functions, virtually any DNA segment either from the bacterial chromosome or a plasmid can be originally transferred by recombining with a self-transmissible or mobilizable plasmids (Reiman and Haas 1993).

When mobilization of a self-transmissible plasmid is described, it is either a bi parental mating with a donor containing both conjugative and non conjugative but mobilisable plamids and a plasmid free recipient, or a triparental mating with a donor, containing the mob plasmid, a helper strain harboring a conjugative helper plasmid and again a plasmid free recipient strain. In these scenarios, mobilization was always considered as a gene flow from the original donor to the recipient strain. However, it has been observed that a donor strain can sometimes acquire DNA from a recipient. Both plasmid and chromosomal DNA segments can be transferred from the recipient to donor. This mode of transfer was described as retrotransfer, shuttle transfer, plasmid mediated gene capture or even a kind of bacterial hermaphroditism. The first report of gene flow at high frequencies in two directions was published by Mergeay *et al.* in 1987.

The importance of conjugation to plasmids is underlined by the large percentage of them that encode conjugation systems, by the likelihood that even small non-conjugative plasmids frequently carry an OriT and perhaps mobilization genes and relatively large proportion of the plasmid DNA dedicated to this function. From an evolutionary point of view this importance is not surprising, since as a result plasmid genes are better able to survive. Firstly, conjugation allows plasmids repeatedly to express their phenotype genes (which, as in the case of antibiotic resistance, typically confer only a transient advantage) in different hosts in different environments. Secondly, conjugation is essentially a replication process and allows plasmid genes to replicate faster than the host chromosomal genes. This can give rise, for example, to infectious spread of a conjugative plasmid through a bacterial population. Transmissible non-conjugative plasmids may have the additional advantages of exploiting several types of conjugation systems (with the corollary of a wider host range) and of their small size being compatible with a high copy number.

During conjugation, transfer begins at a defined point in DNA sequence, usually

called the origin of transfer (OriT). One of the most studied conjugative systems is the F plasmid of *E.coli* K12, which has been the paradigm since its discovery (Klimke and Frost 1998). It has also provided the first evidence for the existence of a plasmid replicon and of a plasmid specified conjugation system. Since this time, a variety of bacterial plasmids and plasmid conjugation systems in a variety of enterobacterial host cells have been described (Minkley 1986). Although all these plasmid determined systems are phylogenetically and biochemically similar but they are genetically dissimilar. These dissimilarities depend on the fact that sex pili differ in their morphology and serology, in the particular varieties of male specific bacteriophages that they absorb and in their abilities to allow conjugation in liquid, as opposed to solid medium (Bradley 1980, Bradley *et al.* 1980). Again the differences depend on the nature of the incompatibility group (as for IncN, P, W or X plasmids) or to one of a small collection of incompatibility groups (as for the IncF or IncI complexes). Furthermore, the lack of complementation between different conjugation systems implies that recognition of a particular OriT sequence by a Dtr system and of a particular Dtr system by an Mpf system, is both highly specific processes. Because of the large proportion of the plasmid DNA devoted to conjugation, plasmids with similar conjugation systems share a large proportion (40-80%) of DNA homology, while plasmids with different systems do not (<10 %) (Falkow *et al.* 1974). The absence of DNA homology itself provides an indication that the conjugation systems determined by a particular pair of plasmids are likely to be dissimilar.

F is a representative of a numerous class of F like plasmids that are related in the type of conjugative pilus that they specify (the distinguishing characteristics include morphology, serology and pilus specific phages) complementation between (the majority of) their conjugation genes and extensive homology between the DNA of these *tra* genes. The F transfer region (over 33kb) contains about 40 genes (*tra*) arranged contiguously. Five different classes of genes have been identified so far: those for (1) pilus bisynthesis and assembly, (2) surface exclusion, (3) mating pair stabilization, (4) regulation and (5) origin of transfer nicking and mobilization. About 20 genes encoded in the *tra* region are absolutely required for conjugation to occur since mutations in these genes completely abrogate transfer. In other cases, mutations within the tra genes cause transfer levels to decrease by several orders of magnitude (Klimke and Frost 1998).

Gene transfer between bacterial taxa in diverse niches could be mediated by conjugative plasmids. The transfer of F type conjugative plasmid R1 from the donor to the recipients varies to a great extent among enteric bacteria because of the interaction of the system that represses sex-pili formations (products of finOP) of plasmids already harbored by a bacterial strain with those of the R1 plasmid. The transfer of plasmids could be accelerated and disseminations could be increased several folds in the presence of efficient donors in heterogeneous bacterial populations. Innumerable numbers of other bacteria were found to acquire the plasmid from such donors in few days while in absence of such strains; the dissemination of plasmids would take several years. This "amplification effect"

could have an impact on the evolution of bacterial pathogens that exist in heterogeneous bacterial communities because conjugative plasmids can carry virulence or antibiotic-resistance genes (Dionsio *et al.* 2002).

2.1.3 Plasmids - as a vehicle for carrying antibiotic resistance genes: Their role in the spread antibiotic resistance among bacteria

The existence of plasmids was initially revealed as the F factor in *Escherichia coli*. One of the features that keep plasmids at the forefront of microbiology is their ability to carry and transmit genes encoding resistance to antimicrobial compounds. These plasmids are known as R plasmids. They represent the most common genetic instrument for resistance among bacteria and are often self-transmissible. Due to their transmissibility, they are abundant in environments with the greatest potential for significant contamination by antimicrobial agents eg. hospitals and hospital sewage effluent, commercial fisheries and abattoirs. However, bacteria carrying R factors have also been isolated from apparently nonselective environments, including plants, estuaries, deep ocean water, sediment and drinking water. Isolation of R plasmid bearing bacteria from natural environmental sources has led to such speculation about the possibility of in situ resistance transfer, the frequency with which it occurs and its effect on public health. Acquisition of these plasmids occurs via all three types of recombination (transformation, transduction and conjugation), although conjugation appears to be the most common method for invivo transfer. Numerous authors have demonstrated the transfer capacity of plasmids through conjugation both in laboratory experiments and in situ experiments using diverse microcosms. It has been predicted that transfer as well as emergence of new combinations of resistance genes will occur most often where bacterial density is high, i.e, biofilms (Murray 1997).

The discovery by Japanese workers in 1959 that the multiple drug resistance of Shigellae could be transferred to other members of the Enterobactericeae by conjugation was an important discovery in the study of bacterial antibiotic resistance. From then on resistance factors (R factors) have been found in a host of different environments and in a variety of different organisms-both gram negative and gram positive. Plasmids have been shown to play an important role in the pathogenecity of most enterobacteria. Their involvement in various enterobacteria was discovered at different times and from different regions of the world.

Richmond *et al.*(1975) reported an abrupt increase in gentamicin-resistant isolates in the Manhattan Veterans Administration Hospital in 1973 and 1974. The presence of conjugative R plasmids in bacterial strains suggested in vivo interbacterial spread of the R factor. Another study reported the resistance pattern and the transferability of the *Salmonella wien* strains isolated in Sicily. The results of the said study suggested that the various outbreaks occurred in Italy might have the same origin (Marranzano *et al.* 1976). There was a profound increase in the frequency of *Shigella* strains carrying R plasmids from 28% in 1969-1970 to 72.6% in 1977 in different regions of the USSR (Solodovnikov *et al.* 1979). The results of a study revealed that gentamicin

resistance in strains of *P. aeruginosa* isolated in Auckland was mediated by R plasmids. The said study included 422 clinical strains of *Pseudomonas aeruginosa*, of which 23 (5.5%) were resistant to gentamicin, 19 were resistant to both tobramycin and sisomycin and one was resistant to tobramycin, sisomyucin and amikacin. Sixteen strains with a high level of resistance to gentamicin transferred all their resistance determinants to recipient strains of *P. aeruginosa* and four transferred some resistance to *P. aeruginosa* (Bremner 1979). During 1977–78, *Escherichia coli* strains, isolated from patients and carriers as well as from the environment, were analyzed for their drug resistance. The antibiotics used for the purpose included tetracycline, streptomycin, chloramphenicol, ampicillin, neomycin, kanamycin, nalidixic acid and sulfonamide. 60% of the isolates exhibited resistance to two or more antibiotics and 85% of the MAR population carried conjugative R plasmids. It was found that the frequency of transmission of R plasmids in the strains isolated from the humans was higher than that in the strains isolated from the environment (Rudneva *et al.* 1980). An American hospital (Womens hospital, Boston) had witnessed a gradual increase in the percentage of trimethoprim resistant clinical isolates of several species of Enterobacteriaceae, particularly *E. coli* and *Klebsiella pneumoniae*. Resistance to trimethoprim, beta lactams and sulfamethoxazole was subjected to conjugal transfer. Trimethoprim resistance in multiple species of Enterobactericeae was found to be spread in one hospital by a single, stable conjugative plasmid that exhibited a wide host range and carried the type II DHFR gene (Mayer *et al.* 1985).

The molecular basis of antibiotic resistance was studied in 32 epidemiologically unrelated Danish clinical isolates of *Haemophilus influenzae*. Plasmid DNA was found in eleven strains, all of which were antibiotic resistant. Antibiotic resistance was transferred to an Rd *Haemophilus influenzae* recipient from 5 to 6 prospective donors. The clonal spread and horizontal transmission of related drug resistance plasmids in *Haemophilus influenzae* was evidenced (Jorgensen *et al.*1989). In a farm environment, the spread of wild type *Escherichia coli* bearing a transferable plasmid was studied. The results revealed that *E. coli* of animal origin could spread rapidly and colonize the intestinal tract of humans and of other animals in the absence of antibiotic selection (Marshall *et al.* 1990). Johnson *et al.* (1994) reported that resistance to gentamicin and apramycin in clinical isolates of *E. coli* resulted from the spread of resistant organisms from animals to man, with subsequent interstrain or interspecies spread, or both, of resistance genes on transferable plasmids. Boyd *et al.* (1996) demonstrated a relatively high incidence of F related plasmids among natural isolates of *E. coli*. They found that 15% of the isolates of the ECOR collection possessed F related plasmids. Sequence analysis of chromosomal genes and MLEE revealed frequent plasmid transfer among strains within and between the major ECOR groups. In order to determine the evolutionary significance of F plasmid transfer between *E. coli* and *S. enterica* they also examined strains of subspecies I for F plasmid occurrence, distribution and genetic diversity. F like plasmids was isolated from *S.enterica* that showed a common ancestry with the *E. coli* F plasmids. The important inference of this finding was that there was relatively

frequent conjugational transfer between those species, despite their different niche specializations. The importance of F plasmids as a mechanism for interspecies recombination between these genetically divergent taxa could be speculated from the demonstration of overlapping plasmid pools. In another study, seventy-one natural isolates from a *Salmonella* reference collection were examined for the presence of plasmids closely related to the *E. coli* F plasmid. The collection consisted of several serovars of the *S. enterica* Typhimurium complex, subspecies 1, to which belonged 99% of the pathogenic *Salmonella* strains. The unexpected finding of a shared pool of F like plasmids between *S. enterica* and *E. coli* demonstrated the significant role of conjugation in the histories of these important bacterial species (Boyd *et al.* 1997). An association of transferable antibiotic resistance traits in *E. coli* of animal origin with the presence of conjugative R plasmids, in Sarawak, East Malaysia, was reported by Son *et al.* (1997). Mobile pathogenecity islands or multidrug resistance plasmids provided vivid examples of adaptive transfer of alien genes between bacteria that may be quite unrelated. As much as 17% of the genome of different *E.coli* strains have been identified by anomalous nucleotide composition as transferred sequence (Medigue *et al.* 1991, Lawrence and Ochman 1998). In 19 different species of bacteria the mean genome fraction identified as transferred sequences is 6% (Kurland 2000, Ochman *et al.* 2000). Multi-drug resistant coliform bacteria were isolated from feces of cattle exposed to antimicrobial agents and humans associated with the animals. Isolates from both cattle and humans harbored an R plasmid of 65 kb (pTMS1). Horizontal transfer of the said plasmid took place between those diverse hosts due to selective antibiotic pressure in the farm environment (Oppegaard *et al.* 2001). Ten different *Klebsiella pneumoniae* strains according to distinct bacteriocin typing and REP-PCR were examined for their plasmid content, their ability to transfer their resistance to aminoglycosides and third generation cephalosporins and their production of aminoglycoside modifying enzymes and beta-lactamases. Transfer of resistance to the said antibiotics as well as to cotrimoxazole and tetracycline to *E. coli* strain R85 was achieved for all strains by conjugation. Similar strains harbor a self-transmissible multi-resistance plasmid (80 kb) with similar *Eco*RI and *Hind* III restrction patterns. The plasmid was found to encode an extended spectrum beta-lactamase that conferred high level of resistance to third generation cephalosporins and aztreonam. Aminoglycoside resistance was also cotransferred (Galani *et al.* 2002).

The conjugative genetic exchange between *Escherichia coli* and *Yersinia pestis* in the flea midgut was studied in detail (Hinnebusch *et al.* 2002). *Yersinia pestis*, the plague bacillus, infects normally sterile sites in mammalian host, but forms dense aggregates in the nonsterile digestive tract of its flea vector to produce a transmissible infection. The study has shown that unrelated coinfecting bacteria in the flea midgut were readily incorporated into these aggregates and the close physical contact led to high frequency conjugal transfer of an antibiotic resistance plasmid from an *E. coli* donor to *Yersinia pestis* at a frequency of 10^{-3}. It was thought that horizontal gene transfer in the flea might have been the source of antibiotic resistant *Y. pestis* strains isolated from plague patients in Madagascar.

In order to assess the role of antibiotics in the environment on the spread of resistance factors, the impact of subinhibitory concentrations of antibiotics in sewage on gene transfer was investigated using conjugative gentamicin resistance plasmids of *S. aureus*. Resistance plasmid transfer occurred both on solidified sewage and in liquid sewage in a bioreactor with a frequency of 1.1×10^{-5} to 5.0×10^{-8}. It was observed that low antibiotic concentrations in sewage could not increase plasmid transfer frequencies of gentamicin resistance plasmids of staphylococci (Ohlsen *et al.* 2003).

2.1.4 Conjugal transfer in aquatic environment

In natural bacterial communities the microbial structure and functions are subjected to dynamic environmental and genetic adaptation. Plasmid mediated horizontal gene transfer has a major impact on the adaptability of bacteria, exemplified by the interspecies and intergeneric transfer of antibiotic resistance genes in a variety of aquatic bacteria. The high incidence of resistant bacteria has been documented for freshwaters, marine waters and chronically polluted waters.

In a wastewater treatment plant, enteric bacteria had been examined for their ability to transfer antibiotic resistance. Drug resistant strains of *Salmonella enteritidis, Proteus mirabilis* and *Escherichia coli* were found to transfer their R plasmids when mated with susceptible *Escherichia coli* and *Shigella sonnei*. Mean transfer frequencies for laboratory matings were 2.1×10^{-3} while in situ matings resulted in frequencies of 4.9×10^{-5} and 7.5×10^{-5} respectively. The findings suggested that a significant level of resistance transfer occurred in wastewater treatment plants in the absence of antibiotics as selective agents (Mach and Grimes 1982).

The contribution of water as a means of spreading R factor containing bacteria became evident from the work of Bell *et al.* (1980). Coliforms, fecal coliforms and *Salmonella* isolated from the Red river, Manitoba, Canada were identified and antibiograms of these organisms was determined against 12 different antibiotics. A total of 52.9% of the fecal coliforms resistant to three or more antibiotics were able to transfer single or multiple resistance determinants (R) to the *Salmonella* recipient and 40.7% could transfer R determinants to the *Escherichia coli* recipient. Of the resistant *Salmonella*, 57% transferred one or two determinants to the *Salmonella* recipient and 39% transferred one or two determinants to the *E. coli* recipient. It was calculated that populations of fecal coliforms containing R factors were as high as 1400 per 100 ml and an accidental intake of a few milliliters of water could lead to transient or permanent colonization of the digestive tract. *Escherichia coli* isolates, selected at random from the Tisza river in Csongrad County, were tested for resistance to five antibiotics and R plasmid carriership. It was found that R plasmids were carried by 43% of the resistant isolates tested, most of which were multi resistant (Lantos *et al.* 1982). Aerobic, heterotrophic bacteria from the sediment of polluted and unpolluted sites in a fast flowing Southwales river, were screened for the presence of plasmid DNA. The majority of the plasmids detected were large enough to carry genes for conjugal transfer, suggesting the possibility of such transfer in the environment (Burton *et al.* 1982).

The fecal coliform populations found in the raw sewages and final sewage effluents of mechanical treatment plants, a long-term retention lagoon, shorter-term retention lagoons, a remote northern Canada river, and a heavily urbanized prairie river were examined for antibiotic resistance and the possession of R factors. A striking contrast was found between the populations of the remote northern Slave River and those of the urbanized Red River. Of the fecal coliforms in the Slave River, 7.1% were multiresistant, and only 0.79% possessed transmissible R factors. The Red River fecal coliform populations were 52.9% multiresistant, and 18.77% of the total population possessed transmissible R factors. The influence of urbanization and the type of sewage treatment had been shown to affect the selection and survival of multiresistant fecal coliforms and R plasmid containing fecal coliforms (Bell *et al.* 1983). In another study it had been shown that water in many streams might function as the reservoirs of strains bearing the determinants of transferable resistance. Such strains may play an important role not only in the ecology and epidemiology of R plasmids but also in the accidental spread of the so called DNA recombinants that might escape during gene manipulations (Kralikova *et al.* 1984). In 1984, a total of 600 isolates of *Escherichia coli* were isolated from healthy human adults, raw sewage and the sewage-polluted River Tigris in Nineva. Resistance of these organisms to 11 antimicrobial drugs was assessed. Over 40% were antibiotic-resistant and of these 77.1% were resistant to more than one antibiotic. The high incidence of antibiotic-resistant *E. coli* in this locality and the possible implications to human health were also take into account (Al-Jebouri *et al.* 1985). During 1977-1982, two hundred fifty five *V. cholerae* strains, unrelated to vibrio O group I (NAG vibrios), were isolated from water bodies in the region of the Volga delta. R factor was detected among 37.7% of those strains (Trichomirov *et al.* 1985). In another study, it was found that about 0.006 to 0.3% of total coliforms, isolated from river water samples, were gentamicin resistant. 86.5% of the gentamicin resistant coliforms were identified as *Klebsiella*. All gentamicin resistant wild strains transferred their resistance to *E. coli* K-12 (Stelzer and Ziegert 1986). 262 samples of river water were analyzed for the occurrence of antibiotic resistant *Aeromonas* and *Vibrio* strains by Kontny *et al.* 1988. The bacteria were cultured on selective media containing the antimicrobial chemotherapeutic agents oxytetracycline, chloramphenicol, streptomycin, ampicillin, kanamycin, gentamicin or trimethoprim. R plasmids were found by means of conjugation in 21.2% of the 826 tested *Aeromonas hydrophila* strains. In eleven *Aeromonas hydrophila* strains with transferable and in 3 from 4 strains with non-transferable multiple antibiotic resistance plasmid DNA with 100 megadalton were found.

The occurrence of drug resistance and its plasmid-mediated transferability was investigated in 140 environmental strains of *Vibrio cholerae* non-O1 and 6 strains of *Vibrio cholerae*, both O1 and non-O1, of clinical origin. Of the 146 strains tested, 93% were resistant to at least one drug and 74% were resistant to two or more antibiotics. A total of 26 of 28 selected resistant wild strains carried R plasmids that were transferable by intraspecific and intergeneric matings. The most common transmissible R factor determined resistance to ampicillin, amoxicillin, and

sulfanilamide (30%), followed by resistance to ampicillin and amoxicillin (13%) and resistance to ampicillin, amoxicillin, phosphomycin, and sulfanilamide (9%). Most strains harbored more than one plasmid, and the molecular sizes ranged from 1.1 to 74.8 megadaltons. The plasmids of high molecular size (around 74 megadaltons) were responsible for the resistance pattern transferred and were maintained with high stability in the hosts (Amaro *et al.* 1988).

Multiple antibiotic resistant enterobacterial isolates were also recovered from the Monocacy river in Frederick County, Maryland (Rinker *et al.* 1988). The presence of R plasmids in coliform bacteria isolated from sewage and the water of surface reservoirs led to some important conclusions. Firstly, antibiotic-resistant coliform bacteria could be pathogenic and play the role of a source of R plasmids for other pathogenic bacteria and secondly, they could be considered as suppliers of the signal information on the distribution of antibiotic resistance which is useful for defining the strategy and tactics of antibiotic therapy (Moissenko 1994).

Adhikari *et al.* (2000) reported the presence of multi drug resistant strains of *Escherichia coli* with transferable traits from four different community ponds of Kathmandu valley. Combined resistance to ampicillin, tetracycline and trimethoprim was most common and ressitance to these three antibiotics was transferred in conjugation experiments. As the water of these ponds is used for recreational purposes, there was every possibility of transmission of such bacteria to humans. The study pointed to the fact that most community ponds of the developing countries might function as reservoir of drug-resistant bacteria. Bacterial resistance to antimicrobial agents is a major public health problem in many tropical countries (Amyes *et al.* 1992). In the countries of South Asia, this problem has been particularly conspicuous with enteric pathogens with both India and Bangladesh reporting outbreaks of *Shigella* with multiple antibiotic resistances (Shears *et al.* 1995).

The transfer of diverse plasmids from MAR *E. coli* strains isolated from river water was studied (Cernat *et al.* 2002). All the strains transferred two or more their resistance markers to potential recipients. The phenotypic data revealed the frequency and dynamic flow of multiple antibiotic resitant *E. coli* strains in aquatic media. Antibiotic resistant bacteria were isolated from fresh water samples from 16 U.S rivers at 22 sites and prevalence of organisms resistant to β-lactam and non β-lactam antibiotics were measured (Ash *et al.* 2002). Over 40% of the US river bacteria resistant to more than one antibiotic and all these antibiotic resistant population contained at least one plasmid. Ampicillin resistance genes, as well as other resistant traits, were identified in 70% of the plasmids.

2.1.5 Concept of horizontal gene transfer in the post-genomic era

The process of horizontal gene transfer provides an organism with access to genes along with inherited ones (Spratt *et al.* 1992, Hilario and Gogarten 1993, Syvanen 1994, Rivera *et al.* 1998, Doolittle 1999, Martin 1999, Ochman *et al.* 2000). It is a rapid process, occurring extensively among prokaryotes especially in response to the changing environment and can be considered as one of the key processes leading to rapid and dramatic change in

bacterial genome composition, as it leads to "evolution in quantum leaps" (Jain *et al.* 2003).

The contributions as well as consequences following horizontal gene transfer process remained highly debated for a long time (Snel *et al.* 2002, Mirkin *et al.* 2003). In general, HGT participates in shaping of prokaryotic genome structure (Doolittle *et al.* 2003, Eisen 2000, Jain *et al.* 2003). It was believed earlier that bacteria are unlikely to transfer genes for more fundamental processes, such as cell division. That view has changed in recent years. The very existence of unexchangeable genes, be it fundamental or not, has been put into question. Two hypotheses have been set forth by Jain *et al.* to describe temporal flow of horizontal transfer (1999). According to the continual horizontal transfer hypothesis, horizontal transfer of operational genes is a far more important factor in prokaryotic evolution than previously thought. The second, massive horizontal transfer hypothesis proposes that one or a few, massive ancient exchanges of (operational) genes occurred early in prokaryotic evolution before the diversification of modern prokaryotes. It supports the idea that massive horizontal exchange could have created modern prokaryotes. The findings of this work led them to propose that a major factor in the more frequent horizontal transfer of operational genes is that informational genes are typically members of large, complex system, whereas operational genes are not, thereby making horizontal transfer of informational gene products less possible (complexity hypothesis) (Jain *et al.* 1999).

Analysis of prokaryotic gene and genome sequences reveal that the exchange of genetic information through both homology dependent recombination and horizontal gene transfer is far more important in quantity and quality than thus far imagined. During bacterial evolution, the ability of bacteria to exploit new environments and to respond to new selective pressures can often be more readily explained by the acquisition of new genes by horizontal transfer rather than by sequential modification of gene function by the accumulation of point mutations (Syvanen 1994). Gene loss and other chromosomal alterations along with the reacquisition of genes through horizontal transfer act together to provide a rich explanatory paradigm (Gogarten *et al.* 2002). This concept was supported by the history of B12 metabolism in enteric bacteria, which includes loss of multiple functions and reacquisition of genes from a foreign source. Many bacterial genes are located in co transcribed clusters or operons; together, the genes in an operon generally provide a single function or selectable phenotype. Conditionally dispensable functions are usually encoded by operons; essentially genes are less likely to be clustered. Operon formation may be driven by gene loss (by mutation during periods of dispensability) and reacquisition (by horizontal acquisition of small chromosome fragments followed by selection). Clustered genes can be cotransfered horizontally and therefore can spread faster than identical unclustered alleles; thus clustered alleles have higher fitness. Gene clustering may provide no immediate fitness benefit to the host organism and can be considered a selfish property of genes (Lawrence and Roth 1998). Transferred genes can confer novel metabolic phenotypes to their new hosts and allow rapid, effective exploitation of new environmental niches (Lawrence

1997). The selfish operon model postulates that the organization of bacterial genes into operons is beneficial to the constituent genes in that proximity allows horizontal cotransfer of all genes required for a selectable phenotype. Horizontal transfer of selfish operons likely promotes bacterial diversification facilitating bacterial speciation (Lawrence 1999).

Some recent gene analysis has hinted on the magnitude of horizontal gene transfer. The availability of complete sequence of *E.coli* MG1655 provided the first opportunity to asses the overall impact of horizontal genetic transfer on the evolution of bacterial genomes. It has been estimated that phylogenetic lineages leading to *E. coli* and *Salmonella enterica* separated about 100 million years ago. Since that time horizontal transfer of genetic material has enabled these lineages to acquire several new genetic systems. For example, the phenotypic characteristics differentiating *E. coli* and *S.enterica* are all due to chromosomal genes (lactose utilization, citrate utilization, propanediol utilization, indole production, pathogenecity) that have been acquired since the development of these lineages from a common ancestor. An analysis based on the differences in base composition and codon utilization patterns, has permitted an estimation of the extent of this horizontal transfer (Lawrence and Roth 1998). Lawrence and Ochman (1998) found that 755 of 4288 ORFs (547.8 kb) have been introduced into the *E. coli* genome in at least 234 lateral transfer events. The average age of introduced genes was 14.4 Myr, yielding a rate of transfer 16kb per Myr per lineage since divergence. Although most of the acquired genes were subsequently deleted, the sequences that have persisted (~18 % of the current chromosome) have conferred properties permitting *E. coli* to explore otherwise unreachable ecological niches. In case of *E. coli* they suggest the introduction of lac operon probably permitted the bacterium to establish its current home in the colons of mammals (Lawrence and Ochman 1998).

Some transformable bacteria have acquired target mediated antibiotic resistance by horizontal genetic exchange of fragments of chromosomal genes. Examples include penicillin resistant penicillin binding proteins (PBPs) in *Streptococcus pneumoniae* and the pathogenic *Neisseria meningitides*. HGT permits movements of alleles among bacterial lineages, increasing the opportunities for the spread of antibiotic resistance (Maiden 1998). The spread of antibiotic resistance genes and xenobiotic degradation genes is due to the horizontal gene transfer coupled to the selective pressures caused by the presence of increasing amounts of these substances in the environments (Hacker and Carniel 2001).

HGTs among bacteria have immediate and practical effects on human health. Not only HGT is associated with the rapid spread of antibiotic resistance genes but also thought to have contributed to the evolution of disease causing bacteria. An example of latter phenomenon could be exemplified by the phenomenon of creation of a new toxin producing killer *E. coli* strain, *E. coli* O157: H7, through HGT to a less virulent strain *E. coli*.

The review made so far revealed the general role played by horizontal gene

transfer with emphasis on the antimicrobial resistance phenotype. The quanta of existing knowledge are insufficient to answer conclusively the questions like how resistance strains and resistance genes spread in nature and the significance of this for man in conjunction to his ecological ties. More data are needed with respect to the input of already resistant bacteria into the environment because on the basis of our present knowledge, an increased direct impact of antibiotic on bacteria in the aquatic environment and in soils is questionable. The average concentrations of antibiotics in rivers and lakes are so low (in the range of ppm.) that they could hardly be able to bring about changes in bacterial populations. The input of bacteria already resistant following the use of antibiotics in human and veterinary medicine seems to be more important source of resistance genes in the environment (Kummerrer 2004). It was also evident that 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. River waters are the main receptacle for antibiotic resistant bacteria, since they receive the sewage of urban effluents. Because rivers are the major sources of water, directly or indirectly for human and animal consumption, this pollution may contribute to the maintenance and even the dissemination of antibiotic resistance genes (Goni-Urizza *et al.* 2000). In the context of India, environmental studies on rivers were largely restricted to routine quantification of total and fecal coliforms in addition to the monitoring of physico-chemical parameters (Pathak *et al.* 1992, Chaurasia 1994, Bhadra *et al.* 2003). However, the information regarding antimicrobial resistance patterns of the development and evolution of the native Indian river water bacteria and assessment of transferability of antibiotic resistance genes is virtually absent or remain untraceable. A previous study conducted by the present author on the Mahananda river of Northern West Bengal, India, has shown the occurrence of antibiotic resistant bacteria and their potential for resistance gene transfer (Mukherjee *et al.* 2005).

The first chapter of the thesis dealt with the incidence and abundance of antibiotic resistant copiotrophic bacterial populations from the water samples collected from three sampling sites along the river Torsa. The second chapter was therefore naturally dictated towards revelation of the molecular mechanisms involved in the development of multiple antibiotic resistance (MAR) phenotypes of the culturable copiotrophs of river Torsa. It was thus imperative to strike a relation between plasmid carriage and antibiotic resistance. Studying the transfer of antibiotic resistance genes via conjugal route for identifying the major driving force behind the spread of resistance genes in the environment was an important aspect of this chapter.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

All the chemicals used in the experiment were purchased from SRL Fine Chemicals (SRL India Ltd.). De-ionized double distilled water was used for the preparation of reagents. The reagents were either filter-sterilized or autoclaved, wherever required, before use and preserved in glass containers.

2.2.2 Bacterial isolates

Bacterial isolates, used for studying the

distribution of plasmids and transferability of those plasmids, were recovered from the water samples obtained from the sampling site III. A total of 4254 antibiotic resistant culturable copiotrophs were recovered from the water samples of SS III (Coochbehar). 92.85% of that population (i.e 3950 isolates) exhibited the MAR phenotype (resisted at least two antibiotics). A total of 3786 isolates, representing 88.99% of the MAR population, exhibited ampicillin resistance. One hundred, ampicillin resistant, MAR isolates, were selected for the following analyses.

2.2.3 Antibiotic resistance determination

Antibiotic resistance was determined by the method described earlier in section 1.2.4 of chapter one. The antibiotics and the concentrations used were as follows: amikacin (25μg μl^{-1}), ampicillin (100μg μl^{-1}), cefotaxim (25μg μl^{-1}), cephalexin (25μg μl^{-1}), chloramphenicol (100μg μl^{-1}), gentamicin (25μg μl^{-1}), kanamycin (50μg μl^{-1}), netilmicin (25μg μl^{-1}), nitrofurantoin (25μg μl^{-1}), streptomycin (100μg μl^{-1}), tetracycline (20μg μl^{-1}), and tobramycin (25μg μl^{-1}). The isolates were considered multiple-antibiotic-resistant (MAR) if growth on at least two different antibiotic containing plates was at least equal to that on the growth control without antibiotics.

2.2.4 MAR indexing

The MAR index of each individual isolate was scored by dividing the number of antibiotics (a) to which the isolate was resistant by the total number of antibiotics (b) to which the isolate was exposed, i.e, a/b (Krumperman 1983). For example if the isolate was exposed to 12 different antibiotics and were resistant to 6, the index for the isolate would be 6/12 or 0.50.

2.2.5 Isolation of plasmid DNA (Mini Prep)

Copiotrophic bacterial isolates were screened for plasmid DNA by the procedure of Birnboim and Doly (1979).

I. Harvesting

A single bacterial colony was transferred into 3ml of LB medium containing appropriate antibiotic in a test tube. The culture was incubated overnight at 37° C with vigorous shaking. 1.5ml of the culture was poured into a microfuge tube and centrifuged at 10,000 r.p.m for 5 minutes at room temperature. The medium was removed by aspiration, leaving the bacterial pellet as dry as possible.

II. Lysis by alkali

The bacterial pellet was suspended in 100μl of ice cold solution I by vigorous vortexing, followed by addition of 200μl of freshly prepared solution II. The contents were mixed by inverting the tube several times and stored on ice for 6-8 minutes. 150μl solution III was then added and mixed by inverting the tubes several times followed by storing on ice for 6-8 minutes. The tube was centrifuged at 10,000 rpm for ten minutes at room temperature. After centrifugation the supernatant was transferred to a fresh tube (about 450μl). To the supernatant 100μl sterile distilled water was added (volume becomes 550μl). Then equal volume of phenol (550μl) was added to that supernatant and was mixed thoroughly for 8-10 times followed by centrifugation at 10,000 rpm for 10 minutes. After that the supernatant was transferred to a fresh micro centrifuge tube (without disturbing the interphase). An equal volume of phenol: chloroform

was added to the supernatant, mixed thoroughly and was centrifuged at 10,000 rpm for 5 minutes. Again the upper aqueous phase was transferred to a fresh micro centrifuge tube. 0.3M (final concentration) sodium acetate from a 3M stock of sodium acetate was added to the supernatant and mixed by inverting the tube.

III. Recovery of DNA

DNA was precipitated with two volumes of ethanol at room temperature. The mixture was allowed to stand for 10 minutes at room temperature. It was then centrifuged at 12,000 rpm for 15 minutes at 4°C in a micro centrifuge tube. The supernatant was removed by gentle aspiration and the tube was placed on a paper towel in an inverted position to drain off the fluid adhering to the walls of the tube. The pellet was air dried by keeping it in a dessicator for overnight and dissolved in 20µl TE (10:1) buffer.

2.2.6 Preparation of plasmid DNA by alkaline lysis with SDS: Midi preparation

30ml of rich medium (LB medium) containing the appropriate antibiotic was inoculated with a single bacterial colony carrying the plasmid of interest. The culture was incubated overnight at 37°C with vigorous shaking.

I. Harvesting

Overnight culture was transferred to a 50ml centrifuge tube and bacterial cells were harvested by centrifugation at 500 rpm for 15 minutes at 4°C. Supernatant was discarded. Left medium was discarded by gentle aspiration leaving the bacterial pellet as dry as possible.

II. Lysis of cells by alkali

The bacterial pellet was suspended in 1.5ml of solution I by vigorous vortexing. 3ml of freshly prepared alkaline lysis solution II was then added to the mixture. The tubes were closed tightly and inverting the tubes for 5-6 times mixed the contents and the tubes were stored on ice for 10 minutes. Then 2.25ml of ice cold solution III was added; again the contents were mixed by only inverting the tubes for several times and then kept on ice for 10 minutes. The bacterial lysate was centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was transferred to a fresh centrifuge tube. Then equal volumes of phenol: chloroform (1:1, v/v) was added. Organic and aqueous phases were mixed by vortexing and then the emulsion was centrifuged at 12,000 rpm for 10 minutes at room temperature. Upper aqueous layer was transferred to a fresh tube. Then equal volume of chloroform : isoamyl alcohol was added.

III. Recovery of plasmid DNA

Nucleic acids were precipitated from the supernatant by adding sodium acetate and double volume of ethanol at room temperature. The solution was mixed by inverting the tube and was allowed to stand for10 minutes at room temperature. The precipitated nucleic acids were collected by centrifugation at 12,000 rpm for 15 minutes at 4°C. Then the supernatant was decanted as completely as possible. The tube was allowed to stand in an inverted position on a paper towel to allow all of the fluid to drain away. All the drops of the fluid adhering to the walls of the tube was removed ultimately by gentle aspiration. Then the tube was left open at room temperature to allow the evaporation of ethanol from the tube. Finally, the precipitated nucleic acid was dissolved in

100µl of TE (pH 8.0) and the DNA solution was stored at -20°C.

2.2.7 Agarose gel electrophoresis

For separation of large and medium sized DNA fragments, 0.8% agarose gel electrophoresis was done. Electrophoresis was performed in horizontal electrophoresis tank using 1X TAE buffer containing 1µg ml^{-1} ethidium bromide. DNA digested with *Hind*III was used as the size marker.

2.2.8 Selection of bacterial isolates for conjugation study

One hundred isolates, with MAR index of 0.41 and above, were primarily differentiated into Pseudomonads and enteric bacteria by oxidase and glucose fermentation tests. 19 out of 100 isolates were oxidase positive and could not ferment glucose. They were grouped as Pseudomonads and were excluded from further tests. Gram negativity of the isolates were also checked. The multiple antibiotic resistant isolates that were sensitive to rifampicin were then sorted out from the remaining 81 isolates for performing conjugation between a rifampicin-sensitive plasmid bearing MAR strain and a plasmidless rifampicin resistant laboratory *E. coli* DH5α strain. Fifty-six rifampicin-sensitive MAR strains were thus used as donors in conjugation experiments.

2.2.9 Culture media

Luria-Bertani (LB) agar plates supplemented with the appropriate antimicrobial drugs were used for the maintenance of donor, recipient and transconjugant cultures at 4°C. Pure cultures were grown in LB broth for susceptibility tests and conjugal transfer assays.

2.2.10 Conjugation Study

Conjugation study was performed following the method described by Son *et al.* (1997). Rifampicin resistant recipients were used in all matings in order to use rifampicin along with another antibiotic, ampicillin (to which the donors remained resistant), for selection of transconjugants. 100 ml flasks containing 10 ml of LB medium were inoculated separately with overnight cultures of donors and recipient cells and incubated at 37°C with shaking to mid-log phase till the cell density became 10^7c.f.u ml^{-1}. Donor and recipient cells were mixed in 2:1 ratio and conjugal transfer was conducted at 37°C without shaking for a period of 5 hours.

Plating known number (x) of wild type cells onto appropriate selective agar plates for the recovery of mutant colonies (y) determined spontaneous mutation frequency (y/x) of the strains. The frequencies obtained were always less than 10^{-7}. The possible roles of transformation or viral transduction in mediating antibiotic resistance transfer were also investigated. Mating was performed between the recipient strain and the cell free supernatant from the donor culture in the same preparation. This was followed by spread plating onto the same selective agar plate for the selection of transconjugants. No transconjugants were collected in any of the culture media. The frequency of transfer was estimated as the number of presumptive transconjugants in relation to the initial number of donor cells (T/D).

2.2.11 Enumeration of bacteria

Following incubation, samples from in-vitro matings were serially diluted in 0.85% NaCl solution. A total viable cell count for each sample was determined by plating

100µl of appropriate dilutions of cell suspension on LB agar plates. The concentration of donor and recipient cells was estimated by plating appropriate dilutions on LB agar plates containing either ampicillin (100µg ml^{-1}) or rifampicin (100µg ml^{-1}). Transconjugants from each mating mix were selected and enumerated on LB agar plates supplemented with both ampicillin (100µg ml^{-1}) and rifampicin (100µg ml^{-1}). Just before plating for the recovery of the transconjugants, rifampicin (100µg ml^{-1}, final concentration) was added to the mating medium to inhibit gene transfer. All the plates were incubated at 37°C for 24 hours and colonies grown on agar plates were recovered as CFU per milliliter.

2.2.12 Genetic characterization of recovered cells

To verify that the cells recovered on selective media were donor, recipient, or transconjugant cells, 10 or more colonies of each were picked and tested for their biochemical and antimicrobial characterizations and for the presence of plasmid bands. Only if appropriate phenotypes were detected were the isolates considered to be recovered donor, recipient or transconjugant cells.

2.2.13 Size determination and restriction mapping of plasmids

Plasmids prepared from the purified transconjugants were digested with restriction enzymes, electrophoresed and the sizes of the restricted fragments were determined. All enzymatic treatments of DNA were performed as recommended by the manufacturers. All other DNA techniques were done according to the standard protocols (Sambrook 1989). To analyze the restriction fragments and to obtain single enzyme digestion pattern, several sets of agarose gel (of different agarose percentage) electrophoresis were done. To determine the orientation and contiguity of the restriction fragments, partial (incomplete) digestions of plasmid DNAs obtained from the specific hosts were also made.

2.2.14 Cloning of DNA fragments in pBluescript KS (+) vector

I. Preparation of vector and insert

The plasmid and the foreign DNAs were digested with appropriate restriction enzyme. The desired fragments were isolated by gel electrophoresis. The DNAs were purified by extraction with phenol, phenol : chloroform and ethanol precipitation. DNAs were redissolved in TE (pH 7.6) at a concentration of 0.1µg $µl^{-1}$ or less.

II. Setting up the ligation mixtures

0.1µg of the vector DNA was transferred to a sterile microfuge tube. Equimolar amount of foreign DNA was added to it. Sterile distilled water was added to make the volume to 8.5 µl and the solution was warmed to 45°C for 5 minutes to melt any cohesive termini that have re-annealed. The mixture was chilled to 0°C. To the mixture 1 µl of 10X T4 DNA ligase buffer [Tris.HCl – 600Mm; $MgCl_2$ – 50Mm, Dithioerythritol (DTT) – 10mM and ATP – 10 mM (pH 7.5)] and 0.1 Weiss unit T4 DNA ligase was added. The reaction was incubated for 4 hours at 16°C. 1-2 µl of each of the ligation reactions was used to transform competent *E.coli*.

III. Preparation of fresh competent *E. coli* using Calcium Chloride and Transformation

A single colony from a plate freshly grown for 16-20 hours at 37°C was picked and transferred into 100 ml of LB broth in a 1 L

flask. The culture was incubated for approximately 3 hours or more at 37°C with vigorous shaking to attain the viable cell number of 10^8 cells ml^{-1}. The cells were transferred aseptically to a sterile, ice-cold 50 ml polypropylene tube. The culture was cooled to 0°C by storing the tube on ice for 10 minutes. The cells were then recovered by centrifugation at 5000 rpm for 10 minutes at 4 °C. The media was decanted from the cell pellet. The pellet was resuspended in 10 ml of ice cold 0.1M $CaCl_2$ and stored on ice for 30 minutes. The cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4°C. The fluid was decanted from the cell pellet, and the tube was placed in an inverted position for 1 minute to drain away last traces of fluid. The pellet was resuspended in 2 ml of ice cold 0.1M $CaCl_2$ foe each 50 ml of original culture. Using a chilled, sterile pipette tip, 200 µl of each suspension of competent was transferred to a sterile microfuge tube. DNA (not more than 50 ng in a volume of 10 µl) was added to each tube. Gentle swirling of the microfuge tubes mixed the contents. The tubes were stored on ice for 30 minutes. The tubes were then transferred to a rack placed in a circulating water bath (42°C) for exactly 90 seconds followed by immediate transferring to an ice bath for chilling for 1-2 minutes. 800 µl of LB medium was added to each tube and incubated for 45 minutes in a water bath set at 37°C to allow the bacteria to recover and to express antibiotic resistance marker encoded by the plasmid. Appropriate volume (upto 200 µl per 9 cm petriplates) of competent cells was transferred onto LB agar plate containing appropriate antibiotic. A sterile bent glass rod was used to spread the transformed cells over the surface of the agar plate. The plates were left at room temperature until the liquid had been absorbed. Finally plates were inverted and incubated at 37°C for 12-16 hours for the appearance of colonies.

IV. Identification of bacterial colonies that contain recombinant plasmids by α- complementation method

To a pre-made Luria-Bartani agar plate containing 50 µg ml^{-1} ampicillin, 0.5 mM isopropylthiogalactosidase (IPTG) and X-gal (80µg $µl^{-1}$) transformed competent cells were plated as described earlier. It was possible to recognize colonies that carry putative recombinant plasmids by blue-white screening of the colonies. Insertion of foreign DNA into the polycloning site of plasmid pBluescript KS(+) leads to the incapability of complementation. Bacteria carrying recombinant colonies are therefore white colonies. Recircularized vectors containing bacteria could utilize chromogenic substrate X-Gal and thus form blue colonies.

2.3 Results

2.3.1 Antibiotic resistance profile of the 100 antibiotic resistant copiotrophic bacterial isolates

A total of twelve different antibiotics were used to check the antibiotic resistance profile of the selected bacterial isolates. The antibiotic resistant phenotypes exhibited by them have shown in Table 2.1. Sixty-four different combinations of MAR phenotypes were observed among the hundred isolates under study. The 100 MAR bacteria distributed into 8 groups according to the MAR index that ranged from 0.41-1.0.

Table 2.1 Antibiotic resistance profile of hundred gram-negative copiotrophic bacterial isolates

Sl. No.	Name of the Isolates	Ami	Amp	Cef	Cep	Chl	Gen	Kan	Net	Nit	Str	Tet	Tob
1.	TR 28	-	+	-	+	-	-	+	-	+	-	+	-
2.	TR 31	-	+	-	+	+	+	-	-	+	-	-	-
3.	TR 45	-	+	+	+	-	-	-	-	+	-	+	-
4.	TR 46	-	+	-	+	-	-	-	-	+	-	+	+
5.	TR 50	-	+	-	+	-	-	-	-	+	+	+	-
6.	TR 51	-	+	-	+	+	-	-	-	+	-	+	-
7.	TR 53	-	+	-	+	+	+	-	-	+	-	-	-
8.	TR 66	-	+	-	+	+	-	-	-	+	-	+	-
9.	TR 84	-	+	-	+	+	-	-	-	+	-	+	-
10.	TR 88	-	+	-	-	+	-	-	-	+	+	+	-
11.	TR 100	-	+	-	-	+	-	+	-	+	-	+	-
12.	TR 01	-	+	+	+	+	-	-	-	+	-	+	-
13.	TR 16	-	+	+	+	+	-	+	-	+	-	-	-
14.	TR 21	-	+	-	+	+	-	+	-	+	-	+	-
15.	TR 38	-	+	+	+	+	-	-	-	+	-	+	-
16.	TR 48	-	+	+	+	-	-	-	-	+	-	+	+
17.	TR 54	-	+	-	+	+	-	+	-	+	-	+	-
18.	TR 57	-	+	+	+	-	-	-	-	+	+	+	-
19.	TR 60	-	+	+	+	+	-	-	-	+	-	+	-
20.	TR 70	-	+	-	+	+	-	-	-	+	+	+	-
21.	TR 76	-	+	+	-	+	-	-	-	+	+	+	-
22.	TR 83	-	+	+	+	+	-	-	-	+	-	+	-
23.	TR 97	-	+	+	+	+	-	-	-	+	-	+	-
24.	TR 24	-	+	-	+	+	-	+	-	+	+	+	-
25.	TR 29	-	+	+	+	+	-	+	-	+	-	+	-
26.	TR 30	-	+	+	+	+	-	+	-	+	-	+	-
27.	TR 39	-	+	+	+	+	+	-	-	+	-	+	-
28.	TR 44	+	+	+	+	+	-	-	-	+	-	+	-
29.	TR 47	-	+	+	+	-	+	-	+	+	-	+	-
30.	TR 71	-	+	-	+	+	-	+	-	+	-	+	+
31.	TR 74	-	+	-	+	+	-	+	-	+	+	+	-
32.	TR 75	-	+	+	+	+	-	+	-	+	-	+	-
33.	TR 77	+	+	+	+	+	-	+	-	+	-	-	-
34.	TR 87	-	+	-	-	-	-	+	+	+	+	+	+
35.	TR 06	-	+	+	+	+	-	+	-	+	+	+	-
36.	TR 09	-	+	+	+	-	-	+	+	+	+	+	-
37.	TR 10	-	+	+	+	-	+	-	-	+	+	+	+
38.	TR 14	-	+	+	+	+	+	-	-	+	+	+	-
39.	TR 19	-	+	-	+	+	+	+	+	+	-	-	+
40.	TR 32	-	+	+	+	-	+	+	-	+	-	+	+
41.	TR 34	+	+	+	+	-	-	+	-	+	-	+	+
42.	TR 36	-	+	-	+	+	+	+	-	+	+	+	-
43.	TR 40	-	+	-	+	-	+	+	-	+	+	+	+
44.	TR 41	-	+	+	+	+	-	+	-	+	+	+	-
45.	TR 42	+	+	+	+	-	-	+	-	+	+	+	-
46.	TR 55	-	+	+	+	-	+	+	-	+	+	+	-
47.	TR 65	-	+	+	+	+	-	+	-	+	+	+	-
48.	TR 69	-	+	+	+	+	-	+	-	+	+	+	-
49.	TR 72	-	+	+	+	+	+	+	-	+	-	+	-
50.	TR 81	-	+	+	+	-	+	+	-	+	+	+	-
51.	TR 89	-	+	+	+	+	-	+	-	+	+	+	-
52.	TR 93	-	+	+	+	+	-	+	-	+	+	+	-
53.	TR 03	-	+	+	+	-	+	+	+	+	+	-	+
54.	TR 07	-	+	+	+	+	+	+	+	+	-	-	+
55.	TR 11	-	+	+	+	-	+	-	+	+	+	+	+
56.	TR 12	-	+	+	+	-	+	-	+	+	+	+	+
57.	TR 13	-	+	+	+	-	+	-	+	+	+	+	+
58.	TR 15	+	+	+	+	-	+	-	+	+	-	+	+
59.	TR 18	-	+	+	+	+	+	+	-	+	+	+	-
60.	TR 25	-	+	+	+	+	+	+	+	+	+	-	-

Sl. No.	Name of the Isolates	Ami	Amp	Cef	Cep	Chl	Gen	Kan	Net	Nit	Str	Tet	Tob
61.	TR 27	+	+	+	+	+	+	+	-	+	-	+	-
62.	TR 33	-	+	+	+	+	+	-	-	+	+	+	+
63.	TR 43	-	+	+	+	+	-	-	+	+	+	+	+
64.	TR 56	+	+	+	+	+	-	+	-	+	+	+	-
65.	TR 85	+	+	-	+	-	+	+	+	+	-	+	+
66.	TR 90	-	+	+	+	+	+	+	+	+	-	+	-
67.	TR 92	+	+	+	+	+	-	+	-	+	+	+	-
68.	TR 94	-	+	-	+	+	+	-	+	+	+	+	+
69.	TR 95	-	+	+	+	-	-	+	+	+	+	+	+
70.	TR 96	-	+	+	+	+	+	+	+	+	-	+	-
71.	TR 99	-	+	+	+	+	+	+	-	+	-	+	+
72.	TR 02	-	+	+	+	+	+	+	-	+	+	+	+
73.	TR 05	+	+	+	+	+	+	+	+	+	-	-	+
74.	TR 08	+	+	+	+	+	+	+	+	+	-	-	+
75.	TR 20	+	+	+	+	+	+	+	-	+	+	+	-
76.	TR 61	-	+	+	+	+	+	+	+	+	-	+	+
77.	TR 63	+	+	-	+	+	+	+	+	+	-	+	+
78.	TR 67	-	+	+	+	+	+	+	+	+	-	+	+
79.	TR 73	+	+	-	+	+	+	+	+	+	-	+	+
80.	TR 79	-	+	+	+	+	+	+	+	+	-	+	+
81.	TR 80	-	+	+	+	+	+	+	+	+	-	+	+
82.	TR 86	+	+	-	+	+	+	+	-	+	+	+	+
83.	TR 91	-	+	+	+	-	+	+	+	+	+	+	+
84.	TR 04	-	+	+	+	+	+	+	+	+	+	+	+
85.	TR 49	-	+	+	+	+	+	+	+	+	+	+	+
86.	TR 59	+	+	+	+	+	+	+	+	+	-	+	+
87.	TR 64	+	+	-	+	+	+	+	+	+	+	+	+
88.	TR 68	+	+	+	+	+	+	+	+	+	-	+	+
89.	TR 78	+	+	+	+	+	+	+	+	+	+	-	+
90.	TR 82	-	+	+	+	+	+	+	+	+	+	+	+
91.	TR 98	+	+	-	+	+	+	+	+	+	+	+	+
92.	TR 17	+	+	+	+	+	+	+	+	+	+	+	+
93.	TR 22	+	+	+	+	+	+	+	+	+	+	+	+
94.	TR 23	+	+	+	+	+	+	+	+	+	+	+	+
95.	TR 26	+	+	+	+	+	+	+	+	+	+	+	+
96.	TR 35	+	+	+	+	+	+	+	+	+	+	+	+
97.	TR 37	+	+	+	+	+	+	+	+	+	+	+	+
98.	TR 52	+	+	+	+	+	+	+	+	+	+	+	+
99.	TR 58	+	+	+	+	+	+	+	+	+	+	+	+
100.	TR 62	+	+	+	+	+	+	+	+	+	+	+	+

Ami: Amikacin; Amp: Ampicilin; Cef: Cefotaxim; Cep: Cephalexin; Chl: Chloramphenicol; Gen: Gentamicin; Kan: Kanamycin; Net: Netilmicin; Nit: Nitrofurantoin; Str: Streptomycin; Tet: Tetracycline; Tob: Tobramycin. Sl. No. 1-11: MAR index 0.41; 12-23: MAR index 0.50; 24-34: MAR index 0.58; 35-52: MAR index 0.66; 53-71: MAR index 0.75; 72-83: MAR index 0.83; 84-91: MAR index 0.91; 92-100: MAR index 1.0.

2.3.2 Detection of plasmid DNA

All the hundred isolates were screened for the presence of plasmid DNAs. Plasmid DNA bands were observed among seventy-seven isolates visualized by agarose gel electrophoresis. Most of these isolates had high molecular weight plasmids (molecular size >20 kb), but some were found to harbor more than one plasmid of varying sizes besides the large ones (Figure 2.1 and Figure 2.2).

2.3.3 Conjugation study

A total of fifty-six MAR isolates were examined for the presence of plasmid DNA in association with the antibiotic resistance for the bacterial hosts. All the 56 isolates were chosen to perform matings with the E.coli DH5α Rif^r as the potential recipient. Conjugation was found positive with 18 donor isolates. Table 2.2 and the Figure2.2 showed the resistance patterns and

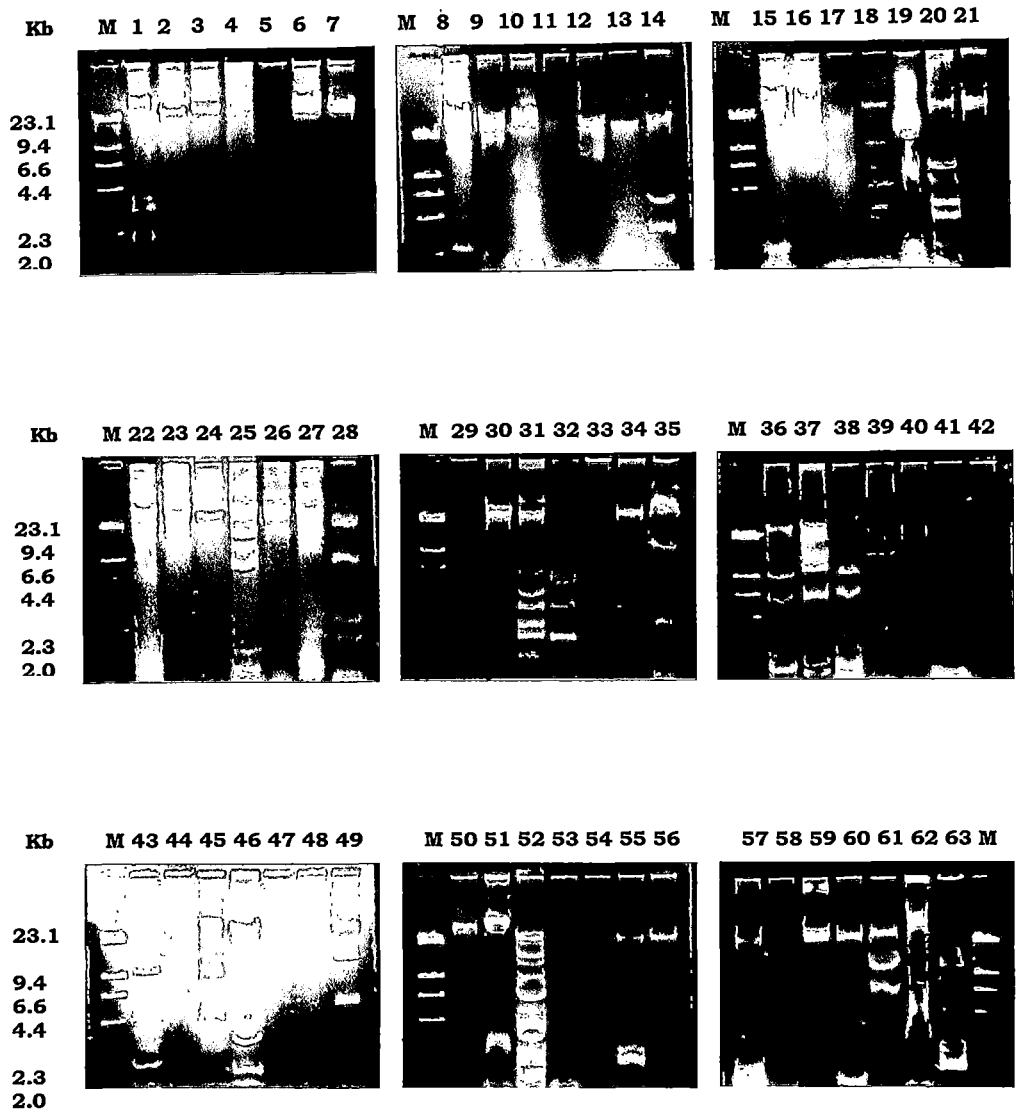


Figure 2.1. Agarose (0.7%) gel electrophoresis of plasmid DNA obtained from the gram-negative, multiple- antibiotic- resistant (MAR) copiotrophic bacterial isolates recovered from the water samples of river Torsa.

Lanes M, λ DNA digested with *Hind*III used as size standards; 1, TR 17; 2, TR 18; 3, TR 19; 4, TR 20; 5, TR 21, 6, TR 22; 7, TR 23; 8, TR 24; 9, TR 25; 10, TR 26; 11, TR 27; 12, TR 28; 13, TR 96; 14, TR 97; 15, TR 29; 16, TR 30; 17, TR 31; 18, TR 32; 19, TR 33; 20, TR 34; 21, TR 35; 22, TR 36; 23, TR 37; 24, TR 38; 25, TR 39, 26, TR 40; 27, TR 41; 28, TR 42; 29, TR 49; 30, TR 51; 31, TR 54; 32, TR 53; 33, TR 52; 34, TR 50, 35, TR 67; 36, TR 66; 37, TR 57, 38, TR 59; 39, TR 64; 40, TR 70; 41, TR 69; 42, TR 62; 43, TR 61; 44, TR 65; 45, TR 63; 46, TR 71; 47, TR 74; 48, TR 73; 49, TR 72; 50, TR 95; 51, TR 94; 52, TR 91, 53, TR 92; 54, TR 89; 55, TR 93; 56, TR 81; 57, TR 86; 58, TR 76; 59; TR 55; 60, TR 88; 61, TR 77; 62, TR 85; 63, TR 98.

Table 2.2: Characterization of the transconjugants

Donor	Resistance phenotype[a]	Resistance transferred	Gene transfer frequency*
TR 01	Amp^r Cef^r Cep^r Chl^r Tet^r	Amp^r Cef^r Cep^r Tet^r	5.0×10^{-3}
TR 02	Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r Str^r Tet^r Tob^r	Amp^r Cef^r Cep^r Gen^r Tet^r Tob^r	1.44×10^{-3}
TR 04	Ami^r Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r Net^r Str^r Tet^r Tob^r	Ami^r Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r Str^r Tet^r Tob^r	5.0×10^{-4}
TR 05	Ami^r Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r Net^r Tob^r	Amp^r Cef^r Cep^r	2.6×10^{-5}
TR 07	Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r Net^r Tob^r	Amp^r Cef^r Cep^r	4.2×10^{-5}
TR 08	Ami^r Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r Net^r Tob^r	Amp^r Cef^r Cep^r Kan^r Gen^r	6.6×10^{-5}
TR 10	Amp^r Cef^r Cep^r Gen^r Str^r Tet^r Tob^r	Amp^r Cef^r Cep^r Tet^r	4.5×10^{-4}
TR 13	Amp^r Cef^r Cep^r Gen^r Net^r Str^r Tet^r Tob^r	Amp^r Cef^r Cep^r Tet^r	7.0×10^{-5}
TR 16	Amp^r Cef^r Cep^r Chl^r Kan^r	Amp^r Cef^r Cep^r	1.75×10^{-4}
TR 17	Ami^r Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r Net^r Str^r Tet^r Tob^r	Ami^r Amp^r Cep^r Gen^r Kan^r Tet^r Tob^r	5.2×10^{-4}
TR 37	Ami^r Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r Net^r Str^r Tet^r Tob^r	Amp^r Cef^r Cep^r	6.0×10^{-4}
TR 48	Amp^r $Cef^r$$Cep^r$ Tet^r Tob^r	Amp^r Cep^r Tet^r	1.0×10^{-1}
TR 56	Ami^r Amp^r Cef^r Cep^r Chl^r Kan^r Str^r Tet^r	Amp^r Cep^r Tet^r	6.0×10^{-5}
TR 68	Ami^r Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r Net^r Tet^r Tob^r	Amp^r Cef^r Cep^r Kan^r Tet^r	1.5×10^{-5}
TR 79	Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r Net^r Tet^r *Tob*^{r}	Amp^r Cef^r Cep^r $Gen^r$$Tob^r$	3.75×10^{-6}
TR 81	Amp^r Cef^r Cep^r Gen^r Kan^r Str^r Tet^r	Amp^r Str^r Cef^r Cep^r	1.2×10^{-5}
TR 85	Ami^r Amp^r $Cep^r$$Gen^r$ Kan^r Net^r Tet^r Tob^r	Amp^r Cep^r	6.0×10^{-5}
TR 100	Amp^r Chl^r Kan^r Tet^r	Amp^r Tet^r	7.2×10^{-2}

[a]Ami: Amikacin; Amp: Ampicillin; Cef: Cefotaxim; Cep: Cephalexin; Chl: Chloramphenicol; Gen: Gentamicin; Kan:Kanamycin; Net: Netilmicin; Str: Streptomycin; Tet: Tetracycline; Tob: Tobramycin
*Frequencies are expressed as the number of transconjugants per input donor cells

plasmid content of the donor strains as well as the R factors transferred. The self-transmissibility of the transferred plasmids were confirmed by further conjugal transfer from *E. coli* DH5α transconjugants to another plasmidless *E coli* recipient strain, *E. coli* HB101. Frequencies of the conjugal transfer in broth matings ranged from 3.75×10^{-6} to 1.0×10^{-1} (Table 2.2). Acquisition of all resistance traits occurred by conjugation since no transconjugants were collected in any of the culture media used as transduction-transformation controls. Although, only plasmids of high molecular mass (>20 kb) were transferred to the recipient cells, mobilization of a small nonconjugative plasmid in donor isolate TR 04 was also noted. Highest rate

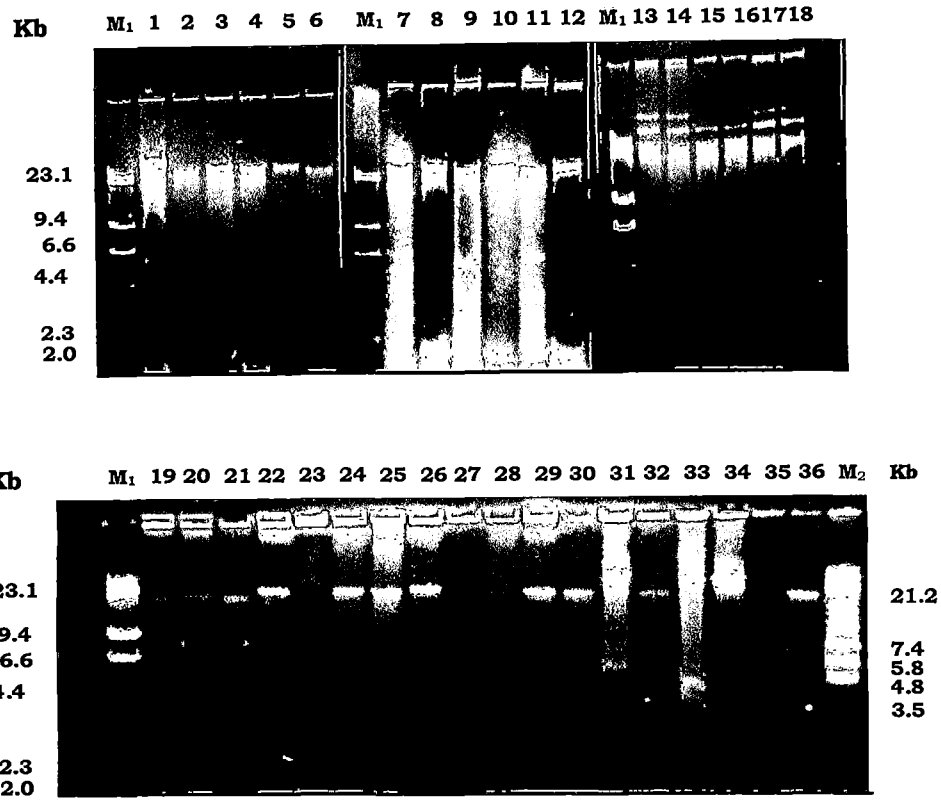


Figure 2.2. Agarose (0.7%) gel electrophoresis of plasmid DNA from donor isolates and their respective transconjugants.

Lanes M_1 and M_2, λ DNA digested with *Hind*III and *Eco*RI respectively, used as size standards; 1, TR 4; 2, Tc : TR 4; 3, TR 8; 4, Tc : TR 8; 5, TR 16; 6, Tc : TR 16; 7, TR 7; 8, Tc : TR 7; 9, TR 10; 10, Tc : TR 10; 11, TR 13; 12, Tc : TR 13; 13, TR 1; 14, Tc : TR 1; 15, TR 2; 16, Tc : TR 2; 17, TR 100; 18, Tc : TR 100; 19, TR 68; 20, Tc : TR 68; 21, TR 56; 22, Tc : TR 56; 23, TR 85; 24, Tc : TR 85; 25, TR 37; 26, Tc : TR 37; 27, TR 81; 28, Tc : TR 81; 29, TR 48; 30, Tc : TR 48; 31, TR 17; 32, Tc : TR 17; 33, TR 5; 34, Tc : TR 5; 35, TR 79; 36, Tc : TR 79.

of conjugal transfer was noted with the donor TR 48. The most common resistances transferred via transmissible R factors were ampicillin, cefotaxim and cephalexin (72.22%), followed by resistance to tetracycline (55.55%), gentamicin (27.7%). Transfer of kanamycin and tobramycin was recorded in 22.2% cases.

2.3.4 Genetic characterization of the recovered cells

Respective biochemical tests and the detection of plasmid DNA bands in donors, recipient as well as in transconjugants confirmed their respective status.

2.3.5 Restriction digestion of the plasmid DNAs obtained from respective transconjugants

Plasmid DNAs obtained from three transconjugants, namely, Tc : TR 01, Tc : TR 02 and Tc : TR 48, were digested with

*Eco*RI and *Bam*HI enzymes. The size and number of fragments generated after digestion have been given in Table 2.3 (A, B, C) and shown in respective figures. Partial digestions were performed with an aim to get the probable location of the restriction sites for the enzyme used [Table 2.4 (A, B); Figure 2.4].

Table 2.3 Size and number of fragments generated after digestion of plasmid DNAs with restriction endonucleases

A. Size and number of fragments generated after digestion of plasmid DNA of Tc : TR 01

Restriction enzyme	Number of fragments	Number of sites	Size of fragments (kb)	Total length (kb)
*Eco*RI	8	8	20.42, 19.05, 15.85, 12.02, 9.3, 7.2, 6.02, 3.46	93.32

B. Size and number of fragments generated after digestion of plasmid DNA of Tc : TR 02

Restriction enzyme	Number of fragments	Number of sites	Size of fragments (kb)	Total length (kb)
*Eco*RI	6	6	20.42, 17.78, 11.22, 8.91, 7.24, 6.02	71.59
*Bam*HI	5	5	24.55, 17.78, 16.60, 7.76, 3.80	70.49

C. Size and number of fragments generated after digestion of plasmid DNA of Tc : TR 48

Restriction enzyme	Number of fragments	Number of sites	Size of fragments (kb)	Total length (kb)
*Eco*RI	6	6	27.54, 11.48, 7.24, 5.99, 3.16, 2.88	58.29
*Bam*HI	7	7	27.54, 11.48, 5.49, 4.36, 4.16, 3.23, 2.95	59.21

Table 2.4 Size and number of fragments generated after partial digestion of plasmid DNAs with restriction endonuclease *Eco*RI

A. Size and number of fragments generated after partial digestion of plasmid DNA of Tc : TR 01

Restriction enzyme	Number of fragments	Size of fragments (kb)
*Eco*RI	8	24.55, 22.91, 19.05, 16.60, 14.45, 10.47, 8.12, 7.24, 5.8

B. Size and number of fragments generated after partial digestion of plasmid DNA of Tc : TR 02

Restriction enzyme	Number of fragments	Size of fragments (kb)
*Eco*RI	6	31.62, 25.12, 19.95, 15.49, 10.72, 7.94, 7.07, 6.16, 5.37

2.3.6 Cloning of *Eco*RI fragments of plasmid DNA of Tc : TR 48 in pBluescript KS$^+$

The plasmid DNAs obtained from the respective transconjugant and the vector were restricted with *Eco*RI and ligated at the *Eco*RI site of pBluescript KS$^+$. Total five different types of recombinant clones were obtained (Figure 2.7) for Tc : TR 48. One of the fragment (27.54 kb fragment from Tc : TR 48) could not be cloned.

2.4 Discussion

With wide spread use of antimicrobial drugs there has been a striking increase in the frequencies of multiple antibiotic resistant bacteria in the environment. Incidences of antibiotic resistant bacteria in fresh water sources have been reported

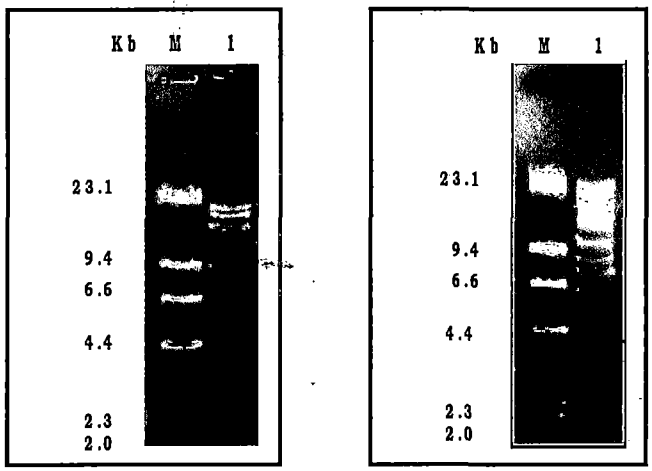


Figure 2.3. Restriction fragments separated in agarose gel (0.7%).
A. Single enzymatic digestion of plasmid DNA of Tc : TR 01 with *Eco*RI
B. Partial digestion of plasmid DNA of Tc : TR 01 with *Eco*RI
Lanes, M, λ DNA *Hind* III digest used as size standards

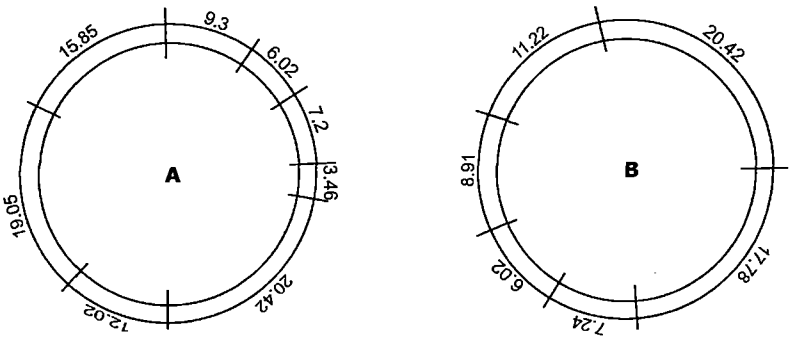


Figure 2.4. *Eco*RI restriction map of plasmid DNAs
A. Restriction map of plasmid DNA of Tc : TR 01
B. Restriction map of plasmid DNA of Tc : TR 02

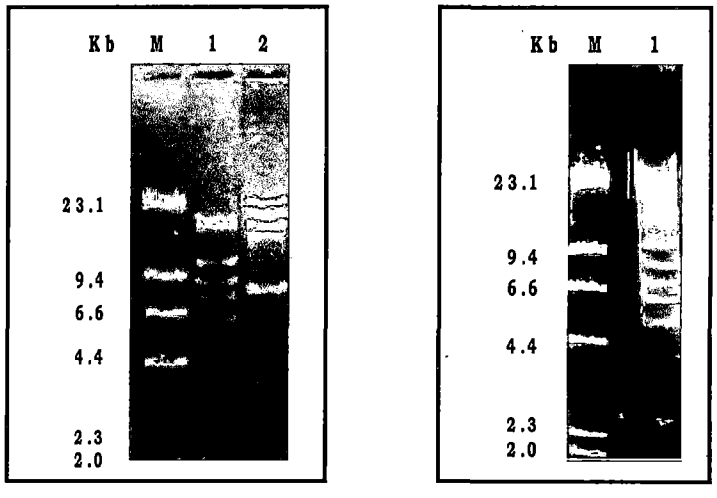


Figure 2.5. Restriction fragments separated in agarose gel (0.7%).
A. Enzymatic digestion of plasmid DNA of Tc : TR 02 with *Eco*RI and *Bam*HI
Lanes, 1, *Eco*RI digestion; 2, *Bam*HI digestion
B. Partial digestion of plasmid DNA of Tc : TR 02 with *Eco*RI
(Lanes M, λ DNA *Hind* III digest used as size standards)

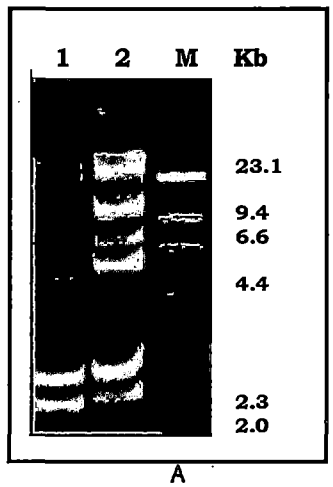
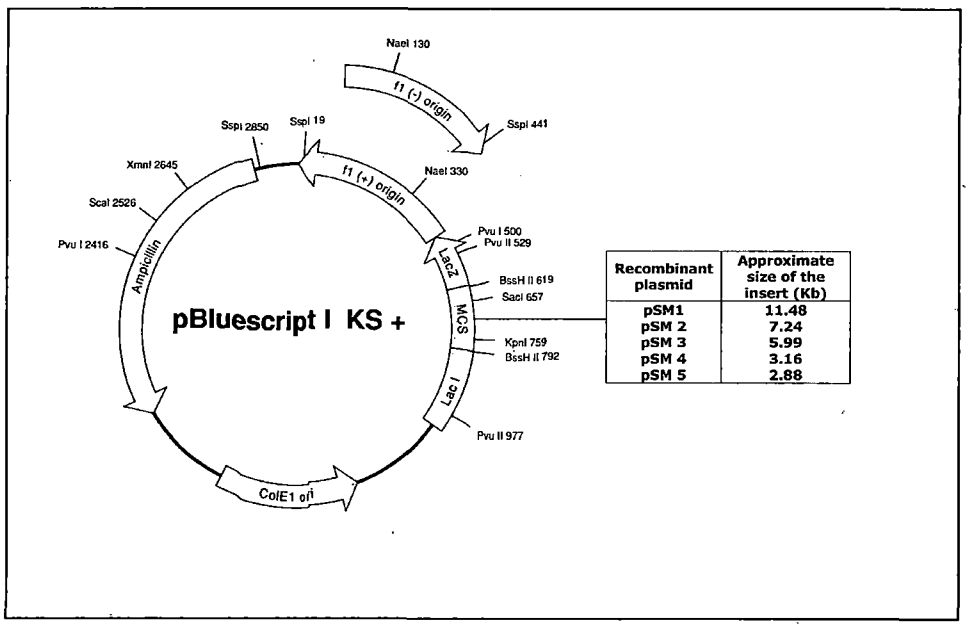


Figure 2.6. Restriction fragments separated in agarose gel (0.7%).
A. Enzymatic digestion of plasmid DNA of Tc : TR 48 with *Eco*RI and *Bam*HI
Lanes, M, λ DNA *Hind* III digest used as size standard; 1, *Bam*HI digestion; 2, *Eco*RI digestion



Recombinant plasmid	Approximate size of the insert (Kb)
pSM1	11.48
pSM 2	7.24
pSM 3	5.99
pSM 4	3.16
pSM 5	2.88

Figure 2.7. Cloning of EcoRI fragments of plasmid DNA of Tc : TR 48 in pBluescript I KS^+

in certain parts of the world (Kelch and Lee 1978, Niemi *et al.* 1983, French *et al.* 1987, Young 1993, Ogan *et al.* 1993, Ash *et al.* 2002, Roe *et al.* 2003). Earlier studies have shown that rivers in Australia, United States of America and South Africa have become major reservoirs for antibiotic resistant microbes (Boon *et al.* 1999, Ash *et al.* 2002, Lin *et al.* 2004). As river water serves as a major source of water for direct or indirect consumption by humans as well as animals, the presence of antibiotic resistant bacteria in river waterways offers cause for concern. The majority of the studies focused on transferable drug resistance because of its practical importance. There are only a few attempts of such study in India. A recent study on the river Mahananda, in the northern part of West Bengal, India, has shown that the river is highly contaminated with antibiotic resistant bacteria. It was found that a large proportion of these resistant organisms carried conjugative plasmids with antibiotic resistant traits (Mukherjee *et al.* 2005). Yearlong study on the Torsa river water revealed the occurrence of antibiotic-resistant-copiotrophic bacteria. Analysis of the antimicrobial resistance patterns of the isolates revealed their multi-drug resistant nature. Further investigation demanded the categorization of the antibiotic-resistant bacteria into two groups, one that possessed intrinsic resistance, i.e, species specific resistance and the other that acquired resistance due to either chromosomal mutations or incoming and thus transferable genes, mainly carried by plasmids or transposable elements. For copiotrophic bacterial isolates exhibiting distinct antibiotic resistance patterns, plasmid contents were analyzed and the transferability of the resistance determinants was examined.

The bacterial isolates used in this study were recovered from the water samples collected from the sampling station III. The choice of this sampling station was based on the fact that the higher frequency of isolation of multi-drug resistant strains from the said sampling station on river Torsa at Coochbehar compared with the other less urbanized sampling station is not surprising since the sewage load is larger in the river at that region. Previous studies reported that urban effluents contain high levels of antibiotic residues and antibiotic resistant bacteria belonging to the human and animal commensal flora, mainly Enterobacteriaceae (Halling-Sorensen *et al.* 1998, Bhattacherjee *et al.* 1988). Biological oxygen demand (BOD) is an expression of consumption of oxygen by microorganisms in aerobic degradation of the biodegradable organic waste present in water bodies. Therefore, it is an indirect measure of the organic waste load of the water and thus higher BOD will indicate high organic pollution of the water. Chemical oxygen demand (COD) may be defined as the measurement of oxygen that is required in oxidizing the organic compounds present in water by means of chemical reactions involving oxidizing substances. The average BOD values calculated from the water samples collected round the year from each sampling site, showed that the water of Torsa at SS III had more BOD (1.64 mg/l) followed by SS I (1.04 mg/l) and SS II (0.9 mg/l) (Bhadra *et al.* 2005). Another notable observation was that the COD of SS III was recorded higher in all sampling months compared to SS I and SS II. A previous study reported that the urban discharge resulted in the increase of resistant strains of riverine autochthonous

and allochthonous bacteria (Goni-Urizza *et al.* 2000).

One hundred, multiple antibiotic resistant, copiotrophic bacterial isolates were selected to study their plasmid content, as well as the transferability of those plasmids. These were isolated from the pool of 4254 antibiotic-resistant copiotrophs that were recovered from the water samples collected from SS III. The antibiotic sensitivity of the recovered copiotrophic bacterial isolates was primarily checked against a panel of five different antibiotics. The isolates that were ampicillin-resistant and exhibited a MAR index ranging from 0.40-1.0 were selected randomly from this pool of antibiotic resistant bacterial population and seven more antibiotics were introduced to check their antibiotic resistance profile. One hundred isolates that exhibited MAR index ranging from 0.41-1.0 were selected for further analysis. The rationality of selection of the antibiotics used was based on the findings of the earlier works. It has been observed that majority of the studies that dealt with antibiotic-resistant bacterial communities from aquatic environment used the antimicrobial compounds of several different groups including beta-lactams, aminoglycosides, cephalosporins, chloramphenicol, tetracyclines, sulfonamides etc. These hundred isolates, with MAR index ranging from 0.41-1.0, were further checked to see whether or not they carry class 1 integrons. The rationality of the selection of the antibiotics for the determination of antibiotic-resistance-patterns (ARPs) of the 100 isolates will be discussed in chapter 3 in more detail.

All the 100 isolates under study exhibited resistance to ampicillin and nitrofurantoin. Sixty-four different MAR combinations were recorded among them. The number of occurrences of different antibiotics in ARPs of the MAR isolates were as follows: Amikacin in 16, ampicillin in 64, cefotaxim in 43, cephalexin in 60, chloramphenicol in 43, gentamicin in 36, kanamycin in 43, netilmicin in 24, nitrofurantoin in 64, streptomycin in 32, tetracycline in 55 and tobramycin in 31. It was hinted by earlier authors (Hsu *et al.* 1992) that differences in percentage of bacterial resistance to various antibiotics may reflect the history of antibiotic application and hence there is a possibility of using bacterial drug resistance as an indicator of antibiotic pollution. In an earlier study on river Mahananda, it was found that out of a total 43 different ARPs of MAR, the number of occurrences of ampicillin, chloramphenicol, tetracycline, streptomycin, kanamycin, ciprofloxacin and netilmicin in the combinations were 36, 35, 27, 24, 24, 20 and 20 respectively which may indicate the chronology of usage. The resistance of natural isolates to antibiotics like cefotaxim and cephalexin, strongly suggests that these resistance organisms may produce extended spectrum beta lactamases (ESBL), since resistance to these third generation cephalosporins is considered the single most important indicator of ESBL (Ash *et al.* 2002).

The MAR index of the 100 isolates under study ranged from 0.41-1.0. It was thought that the isolates with higher MAR indices would be the potential carriers of conjugative R plasmids. According to the results of earlier studies, transmissible resistance determinants (R plasmids) were found to be associated with the isolates having higher MAR index. In a previous study it was observed that no transmissive

r-determinants were found in *Escherichia coli* strains resistant to a single antibiotic. However, 85% of the multi-resistant *E. coli* carried conjugative R plasmids (Rudneva *et al.* 1980). Another study reported the incidence of conjugative plasmids among some multiple antibiotic resistant *E. coli* strains isolated from river waters. 65% of the said *E. coli* strains exhibited MAR index of 1.0 (resisted all the eight antibiotics tested) and were the carriers of potential conjugative plasmids (Cernat *et al.* 2002).

The term plasmid was introduced as a generic term for any extrachromosomal genetic particle. It was intended to clarify the classification of the agents that had been thought of disjunctively a parasite, symbionts, organelles or genes (Lederberg 1998). Starting from about 1970, plasmids became important reagents in molecular genetic research and biotechnology. Molecular and genetic analysis of bacterial plasmids led to basic concepts such as the operon and the replicon and has provided essential information on DNA conjugation and fertility, control of gene expression, gene transfer and genetic recombination, and transposable elements. Studies of essential plasmid functions have resulted in revelations about basic aspects of initiation of DNA replication and its regulation, DNA partitioning, and plasmid copy number and plasmid incompatibility. In addition numerous studies have shown the role played by plasmids in bacteria of importance in other areas such as agriculture and plant molecular biology. In a more applied vein, plasmids play a central role in the initial development of recombinant DNA technology, gene cloning and contributed to the evolution of molecular biology.

One of the features that keep plasmids at the forefront of microbiology is their ability to carry and transmit genes encoding resistance to antimicrobial compounds. These plasmids are known as R plasmids. They represent the most common genetic instrument for resistance among bacteria and are often self-transmissible. Due to their transmissibility, they are abundant in environments with the greatest potential for significant contamination by antimicrobial agents as well as from apparently nonselective environments including estuaries, deep ocean water, sediment, surface water and drinking water. Isolation of R plasmid bearing bacteria from natural environmental sources has led to such speculation about the possibility of *insitu* resistance transfer, the frequency with which it occurs and its effect on public health.

A total of seventy-seven isolates under this study harbored plasmids. Only alkaline lysis method was used for plasmid isolation. It is possible that some of the bacteria may have been refractory to the used procedure or large or low copy number plasmids were not observed. In a previous study, two methods, boiling lysis and alkaline lysis, were used for plasmid isolation from the culturable antibiotic resistant bacteria from river waters and plasmid bands were detected among 40% of them (Ash *et al.* 2002). Another study presented a correlation between the number of antibiotic resistance markers and the numbers of plasmid bands detected. However, in this study, no such correlation was scored between the number of antibiotic markers and the number of plasmid bands (Chang *et al.* 1987). Such observation was similar to the observation made by earlier authors (Goni-urizza *et al.* 2000). Such findings suggest

that most of these plasmids encoded characters other than antibiotic resistance or were cryptic.

The loss of antibiotic efficacy through the emergence and transfer of bacterial antibiotic resistance is an increasing reality (Kruse and Sorum 1994, Salyers 1997). In fact, resistant bacteria were detected soon after the introduction of commercial antimicrobials, but it was not until the 1960s when transferable drug resistance was described (Salyers 1997). The resistance factors have now been found in a host of different environments and in a variety of different organisms. The rapid dissemination of antibiotic resistance genes in bacterial populations can be partly attributed to plasmid mediated horizontal transfer. Plasmids capable of being transferred and stably maintained in a wide range of bacteria are of special interest with respect to interspecies gene exchange (Gotz *et al.* 1996). The presence in waterways of both potentially pathogenic gram-negative bacteria and fecal coliforms containing transferable R plasmids raises the question whether resistance transfer may actually occur in streams, rivers, bays and other waterways. Harsh environmental conditions in these areas would seem to minimize the likelihood of transfer but certain microenvironments may provide conditions where resistance transfer could occur. With the passage of antibiotic resistance genes from resistant to formerly sensitive bacteria, maintenance of antibiotic resistance in pathogenic native bacteria could provide a reservoir for antibiotic resistance genes. These antibiotic resistant bacteria are significant environmental contaminants. The results of earlier works suggested that antibiotic resistant bacteria survive better than the sensitive bacteria in surface waters (Kelch and Lee 1978). It was observed that R factor mediated antibiotic resistance increased the survival ability of those antibiotic resistant bacteria. The greater survival of these strains in aquatic systems may be due to the fact that in some cases, the resistance to antibiotics seems to be associated with resistance to environmental factors such as light and metals.

To confirm that the antibiotic resistance was mediated by R factor, a conjugation experiment was conducted between randomly selected, gram-negative, rifampicin sensitive, ampicillin resistant bacterial isolates and an *Escherichia coli* DH5α recipient. Conjugation was found positive among 18 of the 56 mating experiments having a different donor isolate in each set-up.. One of the donor isolate, TR 04, was found to transfer a small plasmid along with the large one to the recipient cell. Such observations are in well accordance with the results of the previous studies (Son *et al.* 1997, Mukherjee *et al.* 2005). It was apparent from this study that copiotrophic bacterial isolates harbored R plasmid DNA. Acquisition of the phenotypic characteristics of the donor plasmids by the recipient cell was circumstantial evidence of actual plasmid transfer. Isolation of plasmid DNA from donor and recipient cells confirmed that transfer had taken place. 'Amp^r Cef^r Cep^r' combination was the most common resistance pattern that had been transferred from donor to the recipient.

Some of the donor isolates, TR 01, TR 02, TR 100, exhibited very high rate of conjugal transfer frequency. Increase in the rate of transfer was scored when

primarily formed *E. coli* transconjugants were further mated with another plasmid less *E. coli* recipient. The donor isolate TR 02 transferred its antibiotic resistance determinants to *E. coli* DH5α with a frequency of 1.44×10^{-3}. The respective transconjugant Tc: TR 02 had transferred its determinants with a frequency of 4.5×10^{-2} to the recipient *E. coli* HB101. Such findings indicated that conjugal transfer took place with a very high frequency between the bacteria of same genus. In an earlier study, R plasmid transfer frequencies were estimated by conjugation of drug resistant *E. coli* strains isolated from river water with *E. coli* DH5α recipient marked with chromosomal resistance to nalidixic acid. It was found that 80% of the R plasmid containing *E. coli* strains transferred two or more of their resistance markers at frequency of about 10^{-4} (Cernat *et al.* 2002). In another study, conjugation between rifampicin resistant *E. coli* K12 and MRE (multidrug resistant Enterobacteriaceae) clinical strains resulted in the transfer of complete resistance patterns at frequencies ranging from 10^{-4} to 10^{-2} (Leverstein van Hall *et al.* 2002). A fairly high rate of transfer (10^{-3}) of an antibiotic resistance plasmid from an *E. coli* donor to *Yersinia pestis* was observed in the flea midgut (Hinnebusch *et al.* 2002). The abundance of antibiotic resistant strains in an environmental setting where bacteria presumably do not come into contact with antibiotics, suggests that resistance genes can also be stably maintained in the absence of antibiotic selection. Conjugal transfer itself plays a vital role in favor of stable maintenance of antibiotic resistance genes in a bacterial population by transferring the resistance determinants to a plasmid less cell. Any successful attempt to curb the spread of resistance will have to take into account the fact that those resistance genes and transmissible elements that carry them could be easily received but difficult to get rid of.

Characterization of naturally occurring antibiotic resistance plasmids and understanding their self-transmissible nature helps to address the long term problem of increasing prevalence of antibiotic resistance and strategies aimed against conjugative gene transfer are needed. The development of such strategies will require a greater understanding of molecular event of plasmid mediated conjugative resistance gene acquisition. By understanding the rates of the emergence of new resistance genotypes, one can determine the lifespan of a given anti microbial agent developed with considerable outlay of intellectual and financial investment. In view of the recent advancements in Genetic engineering strategies and technologies, the understanding the role of R plasmids and their horizontal dissemination in the environment is essential for the evaluation of the possible consequences of the deliberate environmental release of recombinant bacteria.

The construction of gene banks for naturally occurring resistance plasmids (R plasmids) has tremendous biotechnological potential. As most of these naturally occurring R plasmids are self-transmissible and often have broad host range, the origin of replication sequences characterized from these plasmids are of immense value in the construction of shuttle vectors which can shuttle between distant groups of bacteria and for the construction of shuttle vectors between bacteria and lower eukaryotes like yeasts.

Novel gene cassettes characterized from the naturally occurring R plasmids like antibiotic resistance genes against newer antibiotics and antimicrobial agents, help us in designing and construction of novel vectors with more and advanced selection strategies and higher insert capacities.

Strong promoter consensus characterized from broad host range resistance plasmids are of considerable interest for designing efficient expression strategies among diverse groups of bacteria and yeast. Further characterizations of regulatable promoters that can express only in presence of selection pressures, characterized from these resistance plasmids are of immense importance in industrial strain construction and heterologous protein production. Finally cloning, characterization and sequencing of novel genes from resistance plasmids helps in unraveling the mysteries of microbial evolution and genome diversity. Sequence data available from such work also help in analyzing gene structure, function predictions using the tools of genetic engineering and bioinformatics.

2.5 Conclusion

An understanding of horizontal gene transfer is necessary for defining the diversity of bacterial genomes and the range of ecological adaptations exhibited by bacteria. Although plasmid mediated conjugative gene transfer has been widely recognized as prominent genetic route towards microbial resistance in many organisms, this subject often gets perfunctory treatment in review articles. This is because of the fact that our understanding of the basic science of gene transfer by conjugation was insufficient to provide the concepts for the development of long term or short term strategies for the problem of prevalence of antibiotic resistance genes among pathogenic bacteria. Considerable progress has been achieved over the years; the slowly evolving models have yet to offer innovative applications that address the problem of drug resistance transfer. Hence, greater insights into biochemical and molecular events of conjugative gene transfer are needed to identify the unique molecular activities that can be targeted, which in turn can become a part of complex clinical regimes. Further in view of the extensive biotechnological potential, characterization and sequencing of novel antibiotic resistance R plasmids and gene cassettes should be undertaken on a large scale to avail the use of natural antibiotic resistance genes carried by bacterial plasmids.

2.6 Summary of chapter 2

One hundred multiple-antibiotic-resistant (MAR) copiotrophic bacterial isolates from a population of 3786 ampicillin-resistant bacteria were checked for Gram reaction. The MAR index of the isolates under study ranged between 0.41 to 1.0. They exhibited 64 different combinations of antibiotic resistance patterns. A total of seventy-seven isolates were found to carry plasmids of varying sizes. By performing specific biochemical tests these isolates were grouped as pseudomonads and members of Enterobacteriaceae. Conjugal transfer assays were performed to evaluate the potential of the resident plasmids of the enteric members for transferring antibiotic resistance genes. Countable multiple antibiotic resistant transconjugants arose readily and conjugal transfer frequency was in the range of 3.75×10^{-6} to 1.0×10^{-1}. Amp^r Cef^r Cep^r was the most common carriage of resistances in transmissible R plasmids. The plasmids obtained from the transconjugant Tc : TR 48 were used to construct plasmid gene bank for future genomics.

2.7 References

Adhikari, R.P., K. C. Kalpana., P. Shears, and A.P. Sharma. 2000. Antibiotic resistance conferred by conjugative plasmid in *Escherichia coli* isolated from Kathmandu valley. J. Health. Popul. Nutr. **18**: 57-59.

Al-Jebouri, M. M, and N. S. Al-Meshhadani. 1985. A note on antibiotic-resistant *Escherichia coli* in adult man, raw sewage and sewage polluted river Tigris in Mosul, Nineva. J. Appl. Bacteriol. **59**: 513-518.

Amaro, C., R. Aznar., E. Garay, and E. Alcaide. 1988. R plasmids in environmental *Vibrio cholerae* non-O1 strains. Appl. Environ. Microbiol. **54**: 2771-2776.

Amyes, S. G. B., S. Tait., C. J. Thomson., D. J. Payne., L. S. Nandivada., M. V. Jesudason., U. D. Mukundan, and H. K. Young. 1992. The incidence of antibiotic resistance in faecal flora in South India. J. Antimicrob. Chemother. **29** : 415-425.

Andersen, S. R, and R. A. Sandaa. 1994. Distribution of tetracycline resistance determinants among gram-negative bacteria isolated from polluted and unpolluted marine sediments. Appl. Environ. Microbiol. **60**: 908-912.

Ash, R. J., B. Mauck, and M. Morgan. 2002. Antibiotic resistance of gram-negative bacteria in rivers, United States. Emerg. Infect. Dis. **8** : 713-716.

Baquero, F., M. C. Negri., M. I. Morosini, and J. Blazquez. 1998. Antibiotic selective environments. Clin. Infect. Dis. **27**: S5-S11.

Barbosa, T. M, and S. B. Levy. 2000. The impact of antibiotic use on resistance development and persistence. Drug Resistance Updates. **3**: 303-311.

Barrett, F. F., R. F. McGhee, and M. Finland. 1968. Methicillin-resistant *Staphylococcus aureus* at Boston City Hospital. N. Engl. J. Med. **279**: 441.

Bell, J. B., W. R. Macrae, and G. E. Elliott. 1980. Incidence of R factors in coliform, fecal coliform, and *Salmonella* populations of the red river in Canada. Appl. Environ. Microbiol. **40**: 486-491.

Bell, J. B., G. E. Elliott, and D. W. Smith. 1983. Influence of sewage treatment and urbanization on selection of multiple resistance in fecal coliform populations. Appl. Environ. Microbiol. **46**: 227-232.

Bhadra, B., S. Mukherjee., R. Chakraborty, and A. K. Nanda. 2003. Physicochemical and bacteriological investigation on the river Torsa of North Bengal. J. Environ. Biol. **24**:125-133.

Bhadra, B., S. Das., R. Chakraborty and A. K. Nanda. 2005. Investigation of some basic water quality parameter of the North Bengal Terai river Kaljani- a tributary of river Torsa, and comparison their of with the main stream. J. Environ. Biol. **26** : 277-286.

Bhattacherjee, J. W., S. P. Pathak, and A. Gaur. 1988. Antibiotic resistance and metal tolerance of coliform bacteria isolated from Gomati river water at Lucknow city. J. Gen. Appl. Microbiol. **34** : 391-399.

Birnboim, H.C, and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic. Acid. Res. **7**, 1513-1523.

Biyela, P.T., J. Lin, and C.C. Bezuidenhout. 2004. The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. Water. Sci. Technol. **50**: 45-50.

Blondeau, J. M, and G. S. Tillotson. 2000. Antimicrobial susceptibility patterns of respiratory pathogens-a global perspective. Semin Respir Infect **15**:195-207.

Boon, P. I, and M. Cattanach. 1999. Antibiotic resistance of native and fecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, south-eastern Australia. Lett. Appl. Microbiol. **28**: 164-168.

Boyd, E. F., C. W. Hill., S. M. Rich, and D. L. Hartl. 1996 Mosaic structure of plasmids from natural populations of *Escherichia coli*. Genetics. **143**: 1091-1100.

Boyd, E. F, and D. L. Hartl. 1997. Horizontal transmission of plasmids between natural populations of *Escherichia coli* and *Salmonella enterica*. J. Bact. **179**: 1622-1627.

Bradley, D. E. 1980. Morphology and serological relationship of conjugative pili. Plasmid. **4** : 155-169.

Bradley, D. E., D. E. Taylor., and D. R. Cohen. 1980. Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. J. Bacteriol. **143** : 1466-1470.

Bremner, D. A. 1979. Transfer of plasmid mediated antibiotic resistance in strains of *Pseudomonas aeruginosa* isolated in Auckland. J. Med. Microbiol. **12** : 303-310.

Burton, N. F., M. J. Day, and A. T. Bull. 1982. Distribution of bacterial plasmids in clean and

polluted sites in a Southwales river. Appl. Environ. Microbiol. **44**: 1026-1029.

Cernat, R., V. Lazar., C. Balotescu., A.Cotar., E. Coipan, and C. Cojocaru. 2002. Distribution and diversity of conjugative plasmids among some multiple antibiotic resistant *E. coli* strains isolated from river waters. Bacteriol. Virusol. Parazitol. Epidemiol. **47**: 147-153.

Chang, B. J, and S. M. Bolton. 1987. Plasmids and resistance to antimicrobial agents in *Aeromonas sobria* and *Aeromonas hydrophila* clinical isolates. Antimicrob. Agents. Chemother. **31** : 1281-1282.

Chaurasia, S. 1994. Water pollution from mass bathing in river Mandakini during Chitrakoot Deepawali Mela 1993. Indian Journal of Environmental Protection. **14** : 758-765.

Cohen M. L. 1992. Epidemiology of drug resistance: Implications for a post antimicrobial era. Science. **257** : 1050-1055.

Dancer, S. J., P.Shears, and D. J. Platt. 1997. Isolation and characterization of coliforms from glacial ice and water in Canadas High Arctic. J. Appl. Bacteriol. **82**: 597-609.

Dionsio, F., I. Matic., M. Radman., O. R. Rodrigues, and F. Taddei. 2002. Conjugative plasmids can mediate gene transfer between taxa in diverse environments. Genetics. **162**: 1525-1532.

Doolittle, W. F. 1999. Lateral genomics. Trends. Genet. **15**: M5-M8.

Doolittle, W. F., Y. Boucher., C. L. Nesbo., C. J. Douady., J. O. Andersson, and A. J. Roger. 2003. How big is the iceberg of which organellar genes in nuclear genomes are but the tip? Philos. Trans. R. Soc. Lond. B. Biol. Sci. **358**: 39-57.

Eisen, J. A. 2000. Assessing evolutionary relationships among microbes from whole genome analysis. Curr. Opin. Microbiol. **3**: 475-480.

Falkow, S., P. Guerry., R. W. Hedges, and N. Datta. 1974. Polynucleotide sequence relationships among plasmids of the I compatibility complex. J. Gen. Microbiol. **85** : 65-76.

French, G. L., J. Ling., K. I. Chow, and K. K. Mark. 1987. Occurrence of multiple antibiotic resistance and R plasmid in gram negative bacteria isolated from fecally contaminated fresh water streams. Epidemiol. Infect. **98**: 285-299.

Galani. I., E. Xirouchaki, K. Kanellakopoulou., G. Petrikkos, and H.Giamarellou. 2002. Transferable plasmid mediated resistance to multiple antimicrobial agents in *Klebsiella pneumoniae* isolates in Greece. 2002. Clin. Microbiol. Infect. **8**: 579-588.

Garnier, F., S. Taourit., P. Glaser., P. Courvalin, and M. Galimand. 2000. Characterization of transposon Tn*1549*, conferring VanB-type resistance in *Enterococcus* spp. Microbiology **146**:1481-1489.

Gogarten, J. P., W. F. Doolittle, and J. G. Lawrence. 2002. Prokaryotic evolution in light of gene transfer. Mol. Biol. Evol. **19**: 2226-2238.

Gold, H. S, and R. C. Moellering. 1996. Antimicrobial drug resistance. N. Eng. J. Med. **335**: 1445-1453.

Golet, E. M., A. Strehler., A. C. Alder, and W. Giger. 2002. Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. Analytical Chemistry. **74**: 5455-5462.

Goni-Urizza, M., M. Capdepuy., C. Arpin., N. Raymond., P. Caumette, and C. Quentin. 2000. Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and *Aeromonas* spp. Appl. Environ. Microbiol. **66**: 125-132.

Gotz, A., R. Pukall., E. Smit., E. Tietze., R. Prager., H. Tschape., J. D. van Elsas, and K. Smalla. 1996. Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. Appl. Environ. Microbiol. **62** : 2621-2628.

Grohmann, E., G. Muth, and M. Espinosa. 2003. Conjugative Plasmid Transfer in Gram-Positive Bacteria. Microbiology and Molecular Biology Reviews. **67** : 277-301.

Hacker, J, and E. Carniel. 2001. Ecological fitness, genomic islands and bacterial pathogenicity: A Darwinian view of the evolution of microbes. EMBO reports. **21** : 376-381.

Halling-Sorensen, B., S. Nors Nielsen., P. F. Lanzky., F. Ingerslev., H. C. Holtenlutzhoft, and S. E. Jorgensen. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment-a review. Chemosphere. **36** : 357-393.

Hilario, E, and J. P. Gogarten. 1993. Horizontal transfer of ATPase genes-the tree of life becomes a net of life. Biosystems. **31**: 111-119.

Hinnebusch, B. J., M. L. Rosso., T. G. Schwan, and E. Carniel. 2002. High frequency conjugative

transfer of antibiotic resistance genes to *Yersinia pestis* in the flea midgut. Mol. Microbiol. **46**: 349-354.

Hooper, D. C, and J. S. Wolfson. 1993. Mechanisms of bacterial resistance to quinilones. In: Hooper, D. C., Wolfson, J. S., eds. Quinolone antimicrobial agents. 2nd ed. Washington DC. American Society for Microbiology, 97-118.

Hsu, C. H., S. C. Hwang, and J. K. Liu. 1992. Succession of bacterial drug resistance as an indicator of antibiotic application in aquaculture. J. Fish. Soc. Taiwan. **19** : 55-64.

Jain, R., M. C. Rivera, and J. A. Lake. 1999. Horizontal gene transfer among genomes: the complexity hypothsis. Proc. Natl. Acad. Sci. USA. **96**: 3801-3806.

Jain, R., M. C. Rivera., J. E. Moore, and J. A. Lake. 2003. Horizontal gene transfer accelerates genome innovation and evolution. Mol. Biol. Evol. **20**: 1598-1602.

Johnson, A. P., L. Burns., N. Woodford., E. J. Threfall., J. Naidoo., E. M. Cooke, and R. C. George. 1994. Gentamicin resistance in clinical isolates of *Escherichia coli* encoded by genes of veterinary origin. J. Med. Microbiol. **40** : 221-226.

Jorgensen, S. T., A. Lester., J. W. Hansen, and A. Jorgensen. 1989. Molecular epidemiology of antibiotic resistant *Haemophilus influenzae* isolated in Denmark 1981-87. APMIS. **97** : 619-624.

Kelch, W. J, and J. S. Lee. 1978. Antibiotic resistance patterns of gram negative bacteria isolated from environmental sources. Appl. Environ. Microbiol. **36** : 450-456.

Klimke, W. A, and L. S. Frost. 1998. Genetic analysis of the role of the transfer gene *tra* N of the F and R 100-1 plasmids in mating pair stabilization during conjugation. J. Bact. **180** : 4036-4043.

Kontny, I, and U. Thielebeule. 1988. The occurrence of antibiotic-resistant *Aeromonas* and *Vibrio* species in river water. Zentralbl. Bakteriol. Mikrobiol. Hyg (B).**186**: 55-66.

Kralikova, K., V. Krcmery. and V. Jr. Krcmery. 1984. Transferable resistance to gentamicin and other antibiotics in Enterobacteriaceae isolates from municipal wastewater. J. Hyg. Epidemiol. Microbiol. Immunol. **28** : 161-166.

Krumperman, P.H. 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microbiol. **46** : 165-170.

Kummerer, K. 2004. Resistance in the environment. J. Antimicrob. Chemother. **54** : 311-320.

Kurland, C. G. 2000. Something for everyone. Horizontal gene transfer in evolution. EMBO.Rep. **1** : 92-95.

Kruse, H, and H. Sorum. 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl. Environ. Microbiol. **60** : 4015-4021.

Lanka, E, and B. H. Wilkins. 1995. DNA processing reactions in bacterial conjugation. Ann. Rev. Biochem. **64** : 141-169.

Lantos, J., M. Hegedus, and M. Zsigo. 1982. *Escherichia coli* strains isolated from surface waters. Distribution by resistance to antibiotics and R plasmid transfer. Acta. Microbiol. Acad. Sci. Hung. **29**: 161-171.

Lawrence, J. G. 1997. Selfish operons and speciation by gene transfer. Trends. Microbiol. **5**: 355-359.

Lawrence, J. G. 1999. Gene transfer, speciation, and the evolution of bacterial genomes. Curr. Op. Microbiol. **2**: 519-523.

Lawrence, J. G. 1999. Selfish operons: the evolutionary impact of gene clustering in prokaryotes and eukaryotes. Curr. Op. Genet. Dev. **9**: 642-648.

Lawrence, J. G, and J. R. Roth. 1998. Roles of horizontal transfer in bacterial evolution. Pp 208-225 in Horizontal Gene Transfer, M. Syvanen and C. I. Kado, eds. Chapman and Hall, London.

Lawrence, J. G, and H. Ochman. 1998. The molecular archaeology of bacterial genomes. Proc. Natl. Acad. Sci. USA. **95**: 9413-9417.

Leverstein–van Hall, M. A., A. T. A. Box., H. E. M. Blok., A. Paauw., A. C. Fluit, and J. Verhoef. 2002. Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant Enterobacteriaceae in a clinical setting. J. Infect. Dis. **186** : 49-56.

Lederberg, J. 1998. Plasmid (1952-1997).Plasmid. **39** : 1-9.

Levy, S. B. 1998. The challenge of antibiotic resistance. Scientific American. **275**: 46-53.

Lin. J., P. T. Biyela, and T. Puckree. 2004. Antibiotic resistance profiles of environmental isolates from Mhlathuze River, KwaZulu- Natal (RSA). Water SA. **30** : 23-28.

Mach, P. A, and D. J. Grimes. 1982. R plasmid transfer in a waste water treatment plant. Appl. Environ. Microbiol. **44**: 1395-1403.

Maiden, M. C. J. 1998. Horizontal genetic exchange, evolution and spread of antibiotic resistance in bacteria. Clin. Infect. Dis. **27**: S12-S20.

Marranzano, M., A. Nastasi., G. Scarlata., S. Salvo, and A. Falcidia. 1976. Resistance to antibiotics and resistance factors in *Salmonella wien* strains isolated in Sicily. Boll. Ist. Sieroter. Milan. **55**: 187-190.

Marshall, B., D. Petrowski, and S. B. Levy. 1990. Inter and intraspecies spread of *Escherichia coli* in a farm environment in the absence of antibiotic usage. Proc. Natl. Acad. Sci. **87** : 6609-6613.

Martin, W. 1999. Mosaic bacterial chromosomes: A challenge on route to a tree of genomes. Bio-Essays. **21**: 99-104.

Mayer, K. H., M. E. Fling., J. D. Hopkins, and T. F. Obrien. 1985. Trimethoprim resistance in multiple genera of Enterobacteriaceae at a U. S. Hospital: spread of the type II dihydrofolate reductase gene by a single plasmid. J. Infect. Dis. **151**: 783-789.

Medigue, C., T. Rouxel., P. Vigier., A. Henaut, and A. Danchin. 1991. Evidence for horizontal gene transfer in *Escherichia coli* speciation. J. Mol. Biol. **222** : 851-856.

Mergeay, M., P. Lejeune., A. Sadouk., A. Gertis, and L. Fabry. 1987. Shuttle transfer (or retrotransfer) of chromosomal markers mediated by plasmid pULB113. Mol. Gen. Genet. **209** : 61-70.

Minkley, E. G. Jr. 1986. The conjugation system of F, the fertility factor of *E. coli*. Ann. Rev. Genetics. **20**: 593-624.

Mirkin, B. G., T. I. Fenner., M. Y. Galperin, and E. V. Koonin. 2003. Algorithms for computing parsimonious evolutionary scenarios for genome evolution, the last universal common ancestor and dominance of horizontal gene transfer in the evolution of prokaryotes. BMC. Evol. Biol. **3**: 2.

Moissenko, N. N. 1994. Antibiotic resistant bacteria in sewage and the water of surface reservoirs. Antibio. Khimioter. **39** : 64-68.

Mukherjee, S., B. Bhadra., R. Chakraborty., A. Gurung., S. Some, and R. Chakraborty. 2005. Unregulated use of antibiotics in Siliguri city vis-à-vis occurrence of MAR bacteria in community waste water and river Mahananda, and their potential for resistance gene transfer. J. Environ. Biol. **26**: 229-238.

Murray, B. E. 1997. Antibiotic resistance. Advances in lateral medicine. **42** : 339-367.

Niemi, M., M. Sibakov, and S. Niemala. 1983. Antibiotic resistance among different species of fecal coliforms isolated from water samples. Appl. Environ. Microbiol. **45** : 79-83.

Normark, B. H, and S. Normark. 2002. The evolution and spread of antibiotic resistance. J. Int. Med. **252**: 91-106.

Ochman, H., J. G. Lawrence, and E. A. Groisman. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature. **405** : 299-304.

Ogan, M.T, and D. E. Nwiika. 1993. Studies on the ecology of aquatic bacteria of the Lower Niger Delta: multiple antibiotic resistance among the standard plate count organisms. J. Appl. Bacteriol. **74** : 595-602.

Ohlsen, K., T. Ternes., G. Werner., U. Wallner., D. Loffler., W. Zilbuhr., W. Witte, and J. Hacker. 2003. Impact of antibiotics on conjugational resistance gene transfer in *Staphylococcus aureus* in sewage. Environ. Microbiol. **5** : 711-716.

Okumara, J., K. Osaka, and N. Okabe. Widespread Multi-Antimicrobial-Resistant *Shigella* in Asia: What does it mean? Abstract No. AM011, International Conference on Improving use of Medicines, 2004.

Oppegaard, H., T. M. Steinum, and Y. Wasteson. 2001. Horizontal transfer of a multi-drug resistance plasmid between coliform bacteria of human and bovine origin in a farm environment. Appl. Environ. Microbiol. **67** : 3732-3734.

Orencia, M. C., J. S. Yoon., J. E. Ness., W. P. C. Stemmer, and R. C. Stevens. 2001. Predicting the emergence of antibiotic resistance by directed evolution and structural analysis. Nature Structural Biology. **8** : 238-242.

Pathak, S. P., S. Kumar., P. W. Ramteke., R. C. Murthy., K. P. Singh., J. W. Bhattacharjee, and P.K. Ray. 1992. Riverine pollution in some northern and north eastern states in India. Environ. Monitor. Ass. **22** : 227-236.

Phillips, I. 1998. Lessons from the past: A personal view. Clinical Infectious Diseases. **27** : S2-S4.

Reiman, C, and D. Haas. 1993. Mobilisation of chromosomes and nonconjugative plasmids by cointegrate mechanisms, p137-188. In D. B. Clewell (ed.) Bacterial Conjugation. Plenum Publishing, New York.

Richmond, A. S., M. S. Simberkoff., J. J. Rahal. Jr, and S. Schaefler. 1975. R factors in gentamicin-resistant organisms causing hospital infection. Lancet. **2** : 1176-1178.

Rivera, M. C., R. Jain., J. E. Moore, and J. A. Lake. 1998. Genomic evidence for two functionally distinct gene classes. Proc. Natl. Acad. Sci. USA **95**: 6239-6244.

Rinker, A. G. Jr., A. L. Boyd., N. D. Gary, and W. Kundig. 1988. Isolation of multiple antibiotic resistant Enterobacteriaceae from river water. Microbios. **56**: 169-175.

Roe, M. T., E. Vega, and S. D. Pillai. 2003. Antimicrobial resistance markers of class 1 and class 2 integron bearing *Escherichia coli* from irrigation water and sediments. Emerg. Infect. Dis. **9** : 822-826.

Rudneva, S. N., L. G. Stoliarova., A. A. Ershov, and V. V. Pantiukhova. 1980. Spread and transfer of plasmid resistance in *Escherichia coli*. Antibiotiki. **25** : 429-432.

Salyers, A. A, and C. F. Amiable-Cuevas. 1997. Why are antibiotic resistance genes so resistant to elimination? Antimicrob. Agents. Chemother. **41** : 2321-2325.

Sambrook, J., E. E. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A laboratory manual. Cold Spring harbor, New York, Cold Spring Harbor Laboratory Press.

Shears, P., M. A. Hussain., A. H. Chowdhury, and K. Z. Mamun. 1995. Water sources and environmental transmission of multiply resistant enteric bacteria in rural Bangladesh. Ann. Trop. Med. Parasitol. **89** : 297-303.

Smith, D. H. 1967. R factor infection of *Escherichia coli* lyophilized in 1946. J. Bacteriol. **94**: 2071-2072.

Smith, R. D, and J. Coast. 2002. Antimicrobial resistance: a global response, Bulletin of the World Health Organisation. **80**: 126-133.

Snel, B., P. Bork, and M. A. Huynen. 2002. Genomes in flux: the evolution of archaeal and proteobacterial gene content. Genome. Res. **12**: 17-25.

Snider, D. E. Jr., G. M. Cauthen., L. S. Farer., G. D. Kelly., J. O. Kilburn., R. C. Good, and S. W. Dooley. 1991. Drug resistant tuberculosis. Am. Rev. Respir. Dis. **144**: 732.

Solodovnikov, IuP., T. N. Mamontova., M. V. Turchinskaia., V. P. Timina, and M. M. Voskoboinikova. 1979. Antibiotic resistance of Shigellae in different regions of the USSR. Antibiotiki. **24** : 100-105.

Son, R., G. Rusul, and M. I. A. Karim. 1997. Conjugal transfer of plasmids and antibiotic resistance among *Escherichia coli* isolated from animals in a rural area in Sarawak (Malaysia). J. Appl. Microbiol. **82** : 240-244.

Spratt, B. G., L. D. Bowler., Q. Y. Zhang., J. J. Zhou, and J. M. Smith. 1992. Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal Neisserria species. J. Mol. Evol. **34**: 115-125.

Stelzer, W, and E. Ziegert. 1986. Occurrence of gentamicin-resistant coliforms in river water samples. Zentralbl. Mikrobiol. **141** : 3-9.

Syvanen, M. 1994. Horizontal gene transfer-evidence and possible consequences. Annu. Rev. Genet. **28**: 237-261.

Szpirer, C., E. Top., M. Couturier, and M. Mergeay. 1999. Retrotransfer or gene capture: a feature of conjugative plasmids, with ecological and evolutionary significance. Microbiology. **145** : 3321-3329.

Trichomirov, E. D., T. N. Mamontova., M. V. Turchinskaia., V. S. Burkin, and Ibragimov, FKh. 1985. Formation of drug resistance in *Vibrio cholerae* of non-O1 group inhabiting surface-water reservoirs. Zh. Mikrobiol. Epidemiol. Immunobiol. **9** : 40-46.

Tolls, J. 2001. Sorption of veterinary pharmaceuticals in soils: a review. Environ. Sci. Technol. **35**: 3397-3406.

White, D. G, and P. F. McDermott. 2001. Emergence and transfer of antibacterial resistance. J. Dairy. Sci. **84** : E151-E155.

Willetts, N, and R. Skurray. 1987. Structure and function of the F factor and mechanism of conjugation, p1110-1133. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter and H. E. Umbarger (ed.), E. coli and S. typhimurium: cellular and molecular biology. American Society for Microbiology, Washington, DC.

Young, H. K. 1993 Antimicrobial resistance spread in the aquatic environments. Antimicrob. Agents. Chemother. **31**:627-635.

Chapter 3

Molecular characterization of antibiotic resistance gene cassettes associated with class 1 integrons in Gram-negative multiple-antibiotic-resistant copiotrophic bacterial isolates from river Torsa and determination of the carriage of class 1 integrons on conjugative plasmids

3.1 Introduction

Sequence based identification of antibiotic resistance genes along with the flanking DNA region from different mobile DNA elements, including plasmids and transposons, their alignments and comparison have eventually led to the discovery of more recent device for resistance gene acquisition and transfer- the "integrons". In 1986, the DNA sequences of several seemingly unrelated antibiotic resistance genes heralded the first hints regarding integrons. Common regions were noted upstream and downstream of various antibiotic resistance genes. These regions were found to be in different places on various plasmids, suggesting that, like transposons, these elements are mobile. 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 new elements were not repeats, and (ii) the elements 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 and were named integrons (Martinez and de la Cruz 1990, Ouellette and Roy 1987, Stokes and Hall 1989, Sundstrom *et al.* 1988).

3.1.1 Integrons as tool of natural genetic engineering system

The present day definition of integrons has been 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 (the so called 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, 98, Rowe-Magnus *et al.* 1999, Rowe-Magnus *et al.* 2002). It is these gene cassettes that encode 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 IS26, IS1999, IS2000 and IS6100. Most frequently occurring IS element at the 3' - end of integrons is IS6100.

3.1.2 Types and structural organization

In general, integrons are of two types- resistance integrons (RI) and super integrons (SI). Three classes of resistance integrons, namely class 1, class 2 and class 3 have been defined on the basis of the divergence among their integrase genes. The integrase gene is responsible for the synthesis of the enzyme, integrase,

which helps in the integration of gene cassettes and excision of gene cassettes in and from the integron. In other words, the enzyme integrase is responsible for integration and excision of gene cassettes. The enzyme recognizes site-specific recombination sites for integration. Nearly 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). *Tn7* transposon family is associated with the class 2 integron platform (Hall and Stokes 1993). Arakawa *et al.* (1995) identified class 3 integron platform on a large transferable plasmid in a *Serratia marcescens* strain. Recently, the work of 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 γ-proteobacterial 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.

3.1.3 Class 1 integrons

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) and environmental isolates (Gebreyes *et al.* 2002, Chen *et al.* 2004, Nandi *et al.* 2004). 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 (*sulI*) and an open reading frame (ORF5) of unknown function.

Integron integrases, as stated earlier, being members of tyrosine recombinase family, possesses four invariant residues (RHRY) and conserved motifs (boxes I and II; patches I, II, and III). Multiple alignments of integron itegrases with tyrosine recombinases has 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 *intII* seem to play a role in DNA binding (K219) which others are implicated in the recombination activity (Stokes *et al.* 1994).

The 59 base element (*att*C) is the recombination site, present on the gene cassette, recognized by the integron integrase (Stokes *et al.* 1997). 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). Boundaries of each inserted gene cassettes are defined by two core sites in the same orientation with the sequence GTTRRRY (R = Purine, Y = Pyrimidine) that are the target of the recombination process (recombination occurs between the G and the 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 (*att*C) 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 sites 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 length 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.

3.1.4 Gene cassettes

The antibiotic resistance genes that integrons capture are located on the mobile gene cassettes. The cassettes consist of a promoterless coding sequence (most commonly an antibiotic resistance gene) 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 integrase, apart from catalyzing the recombination between *attI*1 and *attC* sites, also facilitates excessive recombination 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 (P_{ANT}) region located in the 5' CS of the integron. This promoter is located 214 bases from the inner boundary of the 5' conserved segment (Collis and Hall 1995). In fact, the P_{ANT} 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. This codon often position near the 5' end of the gene cassette and the supposed ribosome binding sites are weak at best. Some 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-

1. Gene cassettes are discrete genetic elements that may exist as free, circular, non- replicating DNA molecules when moving from one genetic site to other, but are normally found as linear sequence that contribute part of a larger DNA molecule such as plasmid or bacterial chromosome.
2. Gene cassettes normally contain only a single gene and an additional short sequence, called 59 be, that functions as a specific recombination site.
3. The cassettes are small, normally ranging from 500-1000 bp.
4. The genes carried on gene cassettes usually lack promoters and are expressed from a promoter on integron.
5. In rare cases, a cassette may carry two genes; these exceptions are likely to have been 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 (Figure 3.1 and Figure 3.2).

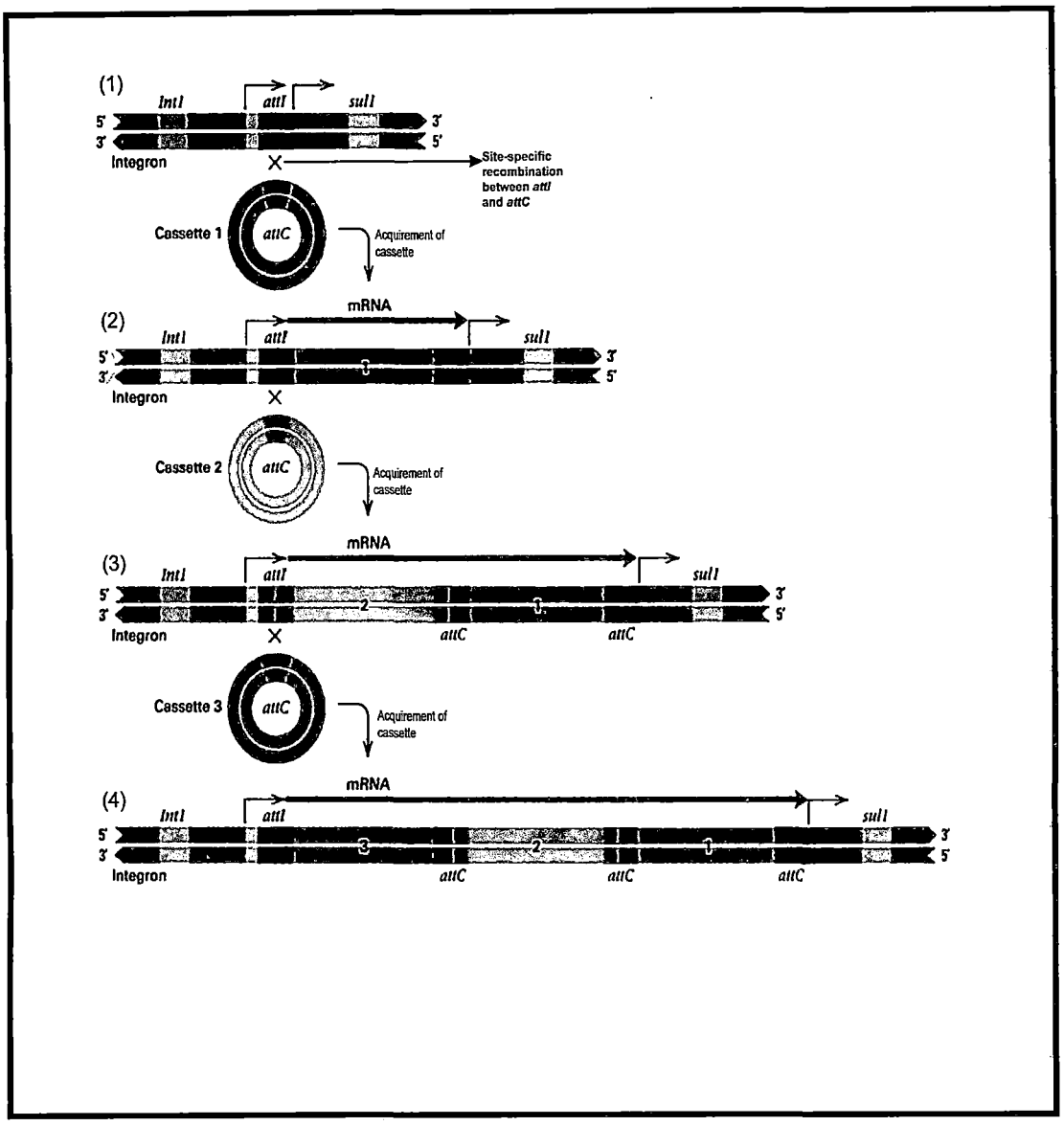


Figure 3.1. Sequential integration of antibiotic resistance gene cassettes within integron structure following site-specific recombination between *attI* site on integron and *attC* site on gene cassettes.

1. Showing site-specific recombination between *attI* and *attC* sites.
2. Acquirement of the second cassette by using the same *attI* site.
3. Addition of the third cassette into the integron.
4. Integron structure with three inserted gene cassettes

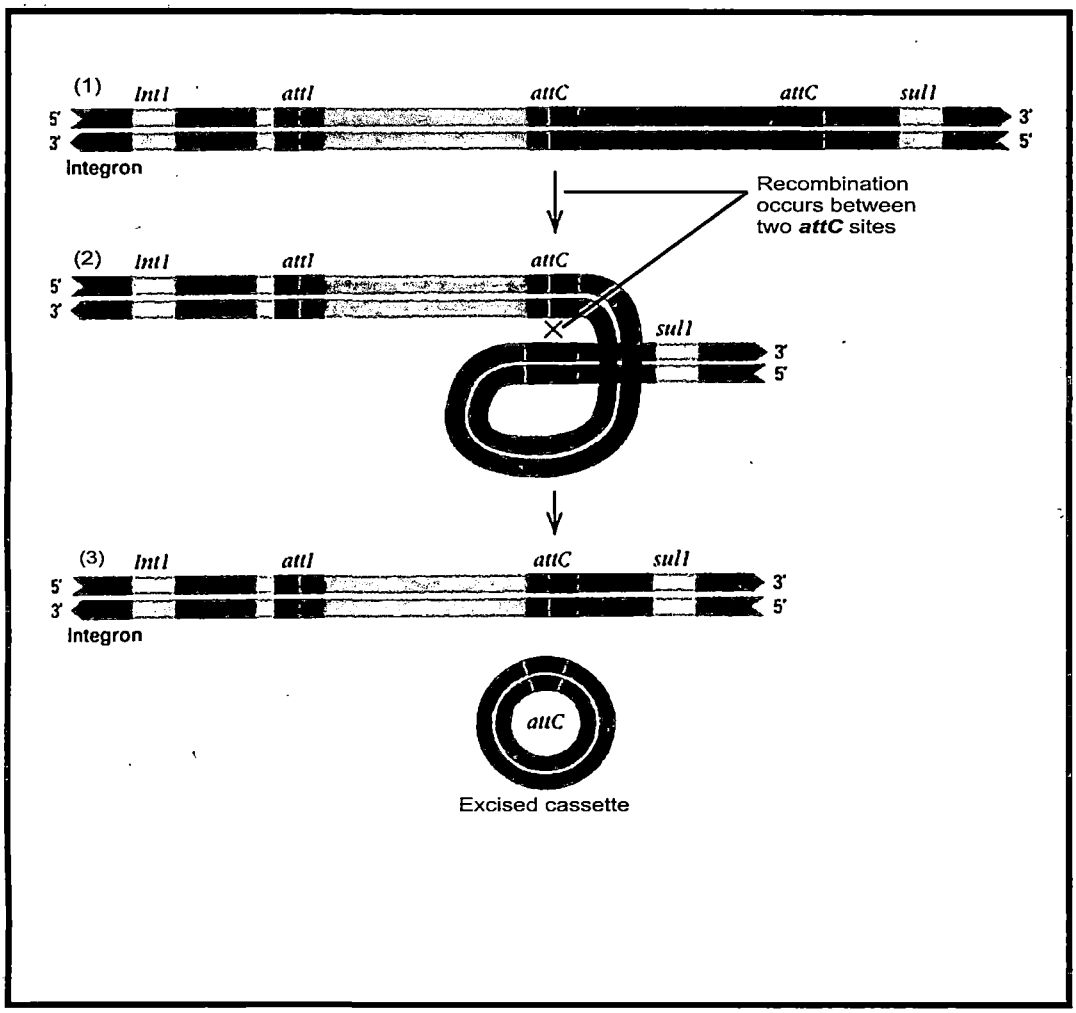


Figure 3.2. Diagrammatic sequence illustrating the excision of gene cassettes from an integron involving site-specific recombination between two *attC* sites

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. The most notable gene cassettes identified between the resistance integrons are those conferring resistance to antibiotics. More than 70 different antibiotic resistance genes, covering most classes of antimicrobials presently in use, are structured as gene cassettes (Fluit *et al.* 2004) and the list is growing.

3.1.4.1 Spread of gene cassettes

Dissemination 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-

1. Mobilization of individual cassette by the integron encoded integrase.
2. Movement when the integron containing the cassette relocates-probably by targeted transposition.
3. Dissemination of large transposons such as *Tn21* carrying integrons and
4. Movement of conjugative plasmids containing integrons among different bacterial species.

Several studies have shown the role of integrons in mediating antibiotic resistance in a variety of enteric bacteria by lateral gene transfer. The spread of resistance genes is greatly enhanced following the movement of conjugative plasmids containing integrons among different bacterial species. In a study integrons from clinical isolates was chosen as a marker of resistance because of its association with multi-resistance. Conjugation experiments with these strains resulted in the transfer of complete resistance patterns at high frequencies (10^{-2} to 10^{-4}). These findings provide strong evidence in favor of the contribution of horizontal gene transfer to the emergence of multi-drug resistance (Leverstein van Hall *et al.* 2002). In a Korean study conjugal transfer and southern hybridization were performed to determine genetic localization of class 1 integrons found in *Escherichia coli* isolates from human and animal. The results revealed the role of horizontal transfer of class 1 integrons through conjugative plasmids for their wide dissemination (Kang *et al.* 2005). Shiga toxin producing *E. coli* (STEC) recovered from poultry, cattle, swine and humans were assayed for integron transfer by conjugation. Transfer of integrons by conjugation between strains of *E. coli* resulted in the transfer of antimicrobial resistance phenotypes. It was concluded that class 1 integrons, located on mobile plasmids, had facilitated the emergence and dissemination of antimicrobial resistance among STEC in humans and food animals (Singh *et al.* 2005). Another study reported that *Salmonella* genomic island 1 (SGI1) could be conjugally transferred from *Salmonella enterica* donor strains to non SGEI1 *S. enterica* and *E. coli* recipient strains (Doublet *et al.* 2005).

3.1.5 Integron Epidemiology

3.1.5.1 Integrons in clinical settings

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. One of the first such study was conducted by Sallen *et al.* (1995), who systematically screened 49 clinical enterobacterial isolates from one location in France and incidence of integrons was detected in 59% of them. Several studies have demonstrated wide 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. A similar result was reported from Netherlands where 135 strains belonging to seven species of Enterobacteriaceae carried an integron (Jones *et al.* 1997). The revelations from such studies could be extended to the rest of Western and Central Europe. It was shown 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 pervasiveness 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 integron carrying isolates were found (Gonzalez *et al.* 1998). In Taiwan, the presence of class 1 integrons was detected in 52% of the tested clinical isolates of *Escherichia coli* (Chang *et al.* 2000). Ploy *et al.* (2000) analyzed twenty *Acinetobacter baumannii* strains for integron content. 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 ascertained by Guerra *et al.* (2000). Study by 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, on the characterization of class 1 integrons in clinical strains of *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Enteritidis, from Norwegian hospitals, was made by Lindstedt *et al.* (2003). 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.

3.1.5.2 Integrons in non-clinical samples

It is worth mentioning that 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 purpose, 72 *Escherichia coli* strains of the EcoR collection, 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), and fish (L'abee-Lund *et al.* 2001). 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'Halloram *et al.* 2004. Their presence have also been described in isolates obtained from pets such as dogs (Sanchez *et al.* 2002) and zoo animals (Mazel *et al.* 2000). Integron sequences have also been detected from soils (Neild *et al.* 2001). Using culture independent methods, they detected several novel integrase genes. However, complete gene cassettes were not identified.

3.1.5.3 Integrons in aquatic environment

Prevalences of class 1 integrons have also been evidenced in various environmental samples including fish farms, estuary, irrigation water sources, and other aquatic environments. In a study, gram-negative bacteria isolated from an estuarine environment were analyzed to detect the presence of class 1 integrons. Three thousand isolates were examined for the said purpose and the presence of *intI*1 gene was detected among 109 of them. 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 was 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). Coliform bacteria isolated from the aquatic environment were investigated for antibiotic susceptibilities and detailed structures of class 1 integrons. 24% of the isolates under study 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 also multiple antibiotic resistant (MAR). Conjugative plasmids were also isolated from a small percentage of the isolates. The study identified the Mhlathuze River as a reservoir of resistance genes and also as a medium for the spread of those genes (Biyela *et al.* 2004).

3.1.5.4 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 gluutamicum* 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.

3.1.6 Integron study in India

All these data suggested that integrons were common worldwide, especially in Enterobacteriaceae, and that they contribute to antimicrobial resistance. Few studies in India have shown the prevalence and distribution of integrons from distinct clinical settings. Thungapathra *et al.* 2002, investigated the molecular mechanisms of multidrug resistance in *Vibrio cholerae* belonging to non-O1, non-O139 serogroups isolated during 1997 to 1998 in Calcutta, India. Twenty-two out of ninety-four strains were found to have class 1 integrons. The gene cassettes identified were *dfrA1*, *dfrA15*, *dfrA5*, *dfrA12*, *aac(6')-Ib*, *aadA1*, *aadA2* and *ereA2*. Their results indicated that besides class 1 integrons and plasmids, a conjugative transposon element, SXT, possibly contributed to the multiple antibiotic resistance. Other authors have also examined examined the distribution of class 1 integrons and SXT elements in *Vibrio cholerae* O1 E1 Tor strains, isolated in Calcutta, India, before and after the *V. cholerae* O139 outbreak in 1992 (Amita *et al.* 2003). Class 1 integrons, with *aadA1* gene cassettes, were detected primarily in the pre-O139 strains; the SXT element was found mainly in the post-O139 strains. The first report of identification of antibiotic resistance genes and a class 1 integron in *Vibrio fluvalis* H-O8942, isolated from a hospitalized infant aged 6 months suffering from cholera-like diarrhoea was made from India in 2002 (Ahmed *et al.* 2004). This isolate was found to carry a class 1 integron carrying a novel aminoglycoside adenyltransferase gene, *aac-(3')-Id*, and aminoglycoside adenyltransferase gene, *aadA7*. Phylogenetic analyses suggested that the *aac-(3')-Id* represents a fourth evolutionary lineage in the aminoglycoside acetyl transferase genes. 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. In addition, the same report indicated that a bla_{VEB-1} like gene might be located outside a class 1 integron structure. The results pointed toward a fact that integrons may constitute a reservoir for the spread of antibiotic resistance genes that may be located and expressed either inside or outside an integron structure.

In this study, 100 Gram-negative multiple-antibiotic-resistant (MAR) isolates from the river Torsa which resisted 5 or more antibiotics were examined for the presence of class 1 integrons, employing a highly reproducible PCR strategy. Presence of class 1 integrons on conjugative plasmids of MAR isolates was demonstrated by conjugal transfer assay. The residence of class 1 integrons on conjugative R plasmids was confirmed in only six out of eleven integron-positive donor isolates. The sequences of the gene cassettes from the transconjugants were analyzed. Nucleotide sequence determination of 12

amplicons enabled to characterize the nature of gene cassettes of 20 isolates out of a total of 40 integron-positive MAR strains. The descriptions of ORFs, encoding proteins responsible for antibiotic resistance, have been presented.

3.2 Materials and Methods

3.2.1 MAR isolates for detecting the presence of class 1 integrons.

One hundred isolates were examined for the presence of class 1 integrons. The selected MAR isolates were primarily differentiated into pseudomonads and representatives of Enterobacteriaceae by oxidase and glucose fermentation tests before attempting the search for class 1 integron.

3.2.2 Antibiotic resistance determination

Antibiotic resistance was determined by the method described earlier in chapter 1. The antibiotics and the concentrations used were as follows: amikacin (25μg ml^{-1}), ampicillin (100μg ml^{-1}), cefotaxim (25μg ml^{-1}), cephalexin (25μg ml^{-1}), chloramphenicol (100μg ml^{-1}), gentamicin (25μg ml^{-1}), kanamycin (50μg ml^{-1}), netilmicin (25μg ml^{-1}), nitrofurantoin (25μg ml^{-1}), streptomycin (100μg ml^{-1}), tetracycline (20μg ml^{-1}), and tobramycin (25μg ml^{-1}). The isolates were considered multiple-antibiotic-resistant (MAR) if growth on at least two different antibiotic containing plates was at least equal to that on the growth control without antibiotics.

The MAR index of each individual isolate was scored by dividing the number of antibiotics (a) to which the isolate was resistant with the total number of antibiotics (b) to which the isolate was exposed, i.e., a/b (Krumperman 1983).

3.2.3 Detection of class 1 integrons by polymerase chain reaction (PCR) amplification

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 [Leverstein van Hall *et al.* 2002]. Primer Int_2F (5′-TCTCGGGTAACATCAAGG-3′), specific for the 3′ region of the integrase gene (approximately 600 bp upstream from the 5′ CS primer site) was 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 DNA templates for PCR were prepared as described by Levesque *et al.* 1995. MAR bacterial isolates were grown in 5ml LB in presence of a selective antibiotic at 37°C overnight, then 200μl of the overnight grown culture was added to 800μl of distilled water and boiled for 10 minutes. The bacterial suspension was then centrifuged at 6000 rpm for 5 minutes and the supernatant obtained was used as the template.

PCR amplification was performed using 'PCR Amplification Kit' (GENEI, India), in 50 μl reaction volume, following instructions provided by supplier. Each 50 μl PCR mix contains; 3 μl of 10mM dNTP mix, 5 μl of 10X buffer containing 15 mM $MgCl_2$, 12.5 pmol of each forward and

reverse primer, 15 µl of template DNA and 3U *Taq* DNA Polymerase. Taq DNA polymerase was added [1 µl of the 3U/ µl diluted solution] after 12 minutes at 94°C (hot start method). The PCR was done in a GenAmp PCR system (Applied Biosystems). The profile followed for the amplification: 1 minute of denaturation at 94°C, annealing for 1 minute at 55°C, and 2-3 minutes of extension at 72°C for a total of 30 cycles. In all reactions, PCR set up containing genomic DNA of *E. coli* XL1 Blue and sterile distilled water were used as target, which served as negative controls.

3.2.4 Size determination of the CS-PCR products

After PCR amplification, 10 µl of the amplification reactions were electrophoresed on 1% agarose (SRL, India) gel [1 gm agarose was added in 100 ml 1X TAE (Sambrook *et al.* 1989)] and run in 1X TAE buffer [Tris-acetate-EDTA, pH 7.8, prepared as recommended in Molecular Cloning Techniques (Sambrook *et al.* 1989)] containing 0.5 µg/ml ethidium bromide. A 500 bp ladder (Bangalore Genei, India) was used as the molecular size marker. After agarose gel electrophoresis, for visualization and documentation of the electrophoresed bands, a UV-transilluminator (Gibco-BRL, USA) and KD1SD-Software package (Kodak Digital Science, Japan) were used respectively.

3.2.5 Characterization of integrons by restriction fragment length polymorphism (RFLP) typing

To determine whether different isolates carried identical integrons, the amplicons of similar sizes were compared by RFLP typing. Selected amplified products generated by 5′ CS and 3′ CS primers were digested with *Eco*RI (New England Biolabs) restriction enzyme. Restriction analysis of the PCR products were done in a 20 µl reaction volume, containing 15 µl of the amplified PCR mix, 2 µl enzymes, 2 µl restriction buffer and 1 µl sterile distilled water, for 16 h at 30 ^{0}C. If the amplicons from two strains yielded the same RFLP pattern, two integrons were considered to be identical. If the PCR product contained a different RFLP pattern, the new product was sequenced as well.

3.2.6 Statistical analysis

The observations were classified simultaneously according to two attributes, MAR index [Low (0.41 – 0.58) and High (0.66–1.0)] and occurrence of integrons [presence (+) or absence (-)]. The frequencies in the different categories were arranged in a two-way table (known as 2 × 2 contingency table) (Table 3.1). The chi-square (χ^2) distribution was then used as test for independence of attributes, i.e., to test whether the two attributes are associated or not (Gupta 2001).

Table 3.1. 2×2 Contingency Table for χ^2 test

MAR index	*Incidence of class 1 integrons*		Total
	No. of integron + isolates	No. of integron – isolates	
Low (0.41 – 0.58)	6(a)	28 (b)	34 (R1)
High (0.66 – 1.0)	34 (c)	32 (d)	66 (R2)
	40 (C1)	60 (C2)	100 (N)

3.2.7 Identification of class 1 integron-positive MAR isolates

The cultures were identified according to Bergey's manual of systematic bacteriology [Brenner 1984]. Isolates were placed into genera or groups on the basis of cell and colonial morphology, Gram stain, motility, catalase and oxidase reactions, indole, Voges Proskauer, methyl red, citrate reactions, gelatin liquefaction, nitrate reduction, urease test, glucose oxidation and carbohydrate fermentations. The detailed descriptions of the biochemical tests and the analysis of the results have been made in chapter 4.

3.2.8 Detection of class 1 integrons on the conjugative plasmids of transconjugants by CS-PCR

The detailed description of selection of the potential donor isolates for conducting the conjugation experiment have been described in detail in section 2.2.8 of Chapter 2. The donors were the members of the pool of 81 MAR isolates that were grouped primarily as members of Enterobacteriaceae. The presence of class 1 integrons in both the donors and their respective transconjugants were detected by the same CS-PCR procedure as is described in section 3.2.3. The template DNA was prepared from both donors and transconjugants by boiling lysis. Plasmid DNAs isolated from the transconjugants was also used as template (~10ng) for the same PCR reaction. Genomic DNA prepared from the plasmid less *E.coli* DH5α was used as the negative control.

3.2.9 Identification of the amplicons generated from donors and their respective transconjugants

The amplicons were compared by RFLP typing to determine whether both donors and their respective transconjugants carried identical integrons. For this purpose enzymes *Eco*RI, *Pvu*I and *Nco*I were used. Restriction analysis of the PCR products was done in the same way as described in section 3.2.5. If the amplicons generated from both donor and transconjugant yielded the same RFLP pattern compared to that of the amplicon derived from the plasmid DNA isolated from the transconjugant, all three were considered to be identical.

3.2.10 Cloning of amplicons generated with 5′ CS and 3′ CS primers

3.2.10.1 Chemicals and reagents

All the chemicals used in the experiment were purchased from SRL Fine Chemicals (SRL India Ltd.). De-ionized double distilled water was used for the preparation of reagents. The reagents were either filter-sterilized or autoclaved, wherever required, before use and preserved in glass containers.

3.2.10.2 Elution of the PCR products from low melting point agarose

Approximately 100 µl of PCR products (generated by 5′ CS and 3′ CS primers) were directly precipitated by dehydrated ethanol and were 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 ^{0}C in 1X TAE following techniques stated in 'Basic Methods in Molecular Biology' (Davis *et al.* 1987). The required band (s) were sliced and taken in an 2 ml microcentrifuge tube, and were melted at 65 ^{0}C 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 (10mM Tris-Cl: 0.1 mM EDTA, pH 7.5) and quantified.

3.2.10.3 Cloning of the PCR product

The pGEM-T Easy Vector System II, purchased form Promega Corporation, Madison, USA, was used for the cloning of PCR products following manufacturer's instructions.

I. Ligation

The vector (25 ng) and the insert DNA were taken in the molar ratio of 1:2 and were suspended in 4µl sterile double distilled water. 5 µl of 2X rapid ligation buffer and 1 µl of T4 DNA ligase were added and thoroughly mixed by vortexing and centrifugation. The mixture was kept at 4 ^{0}C for at least 16 h, heated at 60 ^{0}C for 10 min and 5-7 µl was used to transform competent *E. coli* cells.

II. Transformation

The competent cells of *Escherichia coli* XL1-Blue were transformed by the process described earlier in the section 2.2.13 of chapter 2.

III. Selection of recombinants

The recombinants were selected by α-complementation method (by blue-white screening). Recombinants were confirmed for the presence of the required insert by PCR reaction using 5′ CS and 3′ CS primers. Recombinant-plasmids were isolated and screened by alkaline lysis method (Birnboim and Doly, 1979). Purified clones were checked by *Eco*RI restriction digestion followed by agarose gel electrophoresis.

3.2.11 DNA sequencing and computer analysis of the sequence data

The recombinant plasmids were directly used for sequencing of the inserts using primers for T7 and SP6 promoters. Nucleotide sequencing was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kits (Parkin-Elmer) using specific primer and the reaction was analyzed in an 'AB1 PRISM 377 DNA Sequencer'.

3.2.12 Analysis of the sequence

Analysis of nucleotide sequences were done with several bioinformatics tools.

I. *Restriction analysis*: For determination of restriction map of the sequence, the software package NEB CUTTER v. 2.0 of New England Biolabs Inc. was used from the website http://tools.neb.com/NEBcutter2.

II. *Determination of open reading frame*: To determine the largest possible protein-coding region, the software package 'Fast PCR v 3.3.67' of the Institute of Biotechnology, University of Helsinki, Finland, was used.

III. *Similarity Search*: For similarity search studies of nucleic acid and amino acid sequences the BLAST N and BLAST P programs were used (Altschul *et al.* 1997) from the website http://www.ncbi.nlm.nih.

IV. *Conserve domain search* (CDD): The Domain analysis of the amino acid sequence of the inserts was done using the program of Marchler-Bauer and Bryant (2004) of 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.embl-heigelberg.de and 'SWISS-MODEL' (Schwede *et al.* 2003,

Guex and Peitsch, 1997) was used. The function of the peptides was analyzed using the ProtFun 2.1 software of the website http://www.cbs.dtu.dk.

3.2.13 Accession numbers

The nucleotide sequences of *dfrA1,* the *dfrA1* variant, *dfrA5, dfrA7,* the *dfrA12* variant, *dfrA17, aac-(6')-Ib, aadA1* and *aadA6* were assigned EMBL and GenBank accession numbers (Table. 3.4).

3.3 Results

3.3.1 Antimicrobial resistance pattern of MAR isolates

The antimicrobial resistance pattern of 100 MAR isolates was analyzed. All 100 isolates were resistant to both ampicillin and nitrofurantoin. The detail antimicrobial resistance pattern exhibited by the isolates has been presented in Table 3.2. Twenty nine were resistant to amikacin, 73 were resistant to cefotaxim, 96 were resistant to cephalexin, 76 were resistant to chloramphenicol, 57 were resistant to gentamicin, 70 were resistant to kanamycin, 43 were resistant to netilmicin, 53 were resistant to streptomycin, 89 were resistant to tetracycline and 49 were resistant to tobramycin. Among the 100 MAR bacteria distributed into 8 groups according to the MAR index, 9 isolates were resistant to all 12 antibiotics tested.

3.3.2 Detection of class 1 integrons and RFLP typing of the CS-PCR product

Class 1 integrons were sought in 100 MAR isolates. 40 (40%) of the isolates were identified to carry detectable class 1 integron structures. The remaining 60 isolates did not yield a product. The amplicon lengths, corresponding to the approximate sizes of the inserted cassette DNA, varied from 0.7 to 3.2 kb (Figure 3.3 and Table 3.3). The largest amplicon of 3.2 kb was detected singly in *Serratia* sp. TR 40. Among the 40 isolates, 32 yielded single amplicon and 8 yielded two amplicons of different sizes (Figure 3.3 and Table 3.3). PCR with a primer combination of Int_2F and 3' CS resulted in amplicons larger by an approximate size of 600 bp than the amplicons derived from 5' CS and 3' CS primers (Figure 3.4). Five isolates, TR 18, TR 40, TR 68,TR 76, TR 82 and TR 99 did not give any product when amplified with Int_2F and 3' CS primer set.

A 2.0 kb amplicon, recorded in eight strains (TR 06, TR 10, TR 11, TR 12, TR 13, TR 14, TR 15 and TR 17), yielded the same RFLP pattern upon digestion with *Eco*RI enzyme (Figure 3.5). Similarly, a 1.4 kb amplicon of TR 52 and TR 62 digested with *Eco*RI also yielded identical RFLP patterns.

3.3.3 Identification of integron-positive isolates

Biochemical characterization of integron-positive isolates revealed that 32 out of 40 isolates belong to the family Enterobacteriaceae. The other 8 gram-negative copiotrophic isolates did not belong to this family (Table 3.3). The isolates were identified up to the genus level following the principles of numerical taxonomy (data has been presented in chapter 4).

3.3.4 Test of significance

The number of integron positive isolates in low and high MAR index categories was 6 and 34; similarly, the number of integron negative isolates in the said two categories was 28 and 32 (Table 3.1). On the hypothesis of independence, the test statistic followed χ^2 distribution with 1

degree of freedom [χ^2 (corrected) = N {|ad – bc| - N/2}^{2} / R_1. R_2. C_1. C_2]. Since the observed value of the statistic, 12.18, was found greater than the tabulated value (value for χ^2 for 1 d.f at 5% level is 3.84), it was significant. We therefore rejected the null hypothesis at the 5% level of significance and concluded that the attributes were not independent; i.e. the data supported the alternative hypothesis that MAR index and integron carriage were associated.

3.3.5 Identification of integron-borne gene cassettes

Purified CS-PCR products of TR 02, TR 17, TR 40, TR 48, TR 52, TR 58, TR 59, TR 63, TR 85, TR 90, TR 95 and TR 97, cloned in pGEM-T Easy Vector (Figure 3.6), were subjected to DNA sequencing and gene cassettes thereby identified by sequence analysis (Table 3.3). Characterization of the gene cassettes revealed a significant association between the nature of the gene cassettes and the corresponding antibiotic resistance phenotype of the isolates. All integron-carrying strains expressed resistance to cotrimoxazole (trimethoprim + sulphonamide). The most common carriage by the integron-positive isolates involved dihydrofolate reductase cassettes conferring resistance to trimethoprim. These cassettes (*dfrA1, dfrA5, dfrA7, dfrA17* and two novel ORFs showing partial homology with *dfrA1* and *dfrA12* respectively) represented 70% of the cassettes detected (Table 3.3). Aminoglycoside adenyl transferase (*aad*A1 and *aad*A6) gene cassettes responsible for conferring resistance to streptomycin and spectinomycin were detected in four isolates, while the aminoglycoside acetyltransferase gene cassette (*aac*-(6')-*Ib*) was detected in only three isolates (Table 3.4). Resistance to other antibiotics like ampicillin, cephalosporins, chloramphenicol, nitrofurantoin and tetracycline did not correspond to identified gene cassettes. The sequence derived from 1.2 kb amplicon of TR 58 (Ac. No. AJ937774) showed 100% identity with *V.cholerae* class 1 integron *dfrA1* gene (Ac. No. AF221901). The sequence derivative from *E. coli* TR 02 (Ac. No. AJ620333) showed 100% identity with *E. coli* class 1 integron *dfrA5* gene (Ac. No. AJ419169), while the *Enterobacter* sp. TR 85 (Ac. No. AJ867252) revealed 98% identity with *E. coli* class 1 integron *dfrA5* gene (Ac. No. AJ419169); the sequence from *Salmonella* sp. TR 95 (Ac. No. AY604170) shared 98% identity with *Salmonella enterica* subsp. *enterica* serovar Typhi dihydrofolate reductase type *VII* gene (Ac. No. AY245101). The resulting sequence from *Serratia* sp. TR 48 (Ac. No. AJ868226) showed 98% identity with *Salmonella* sp. S126 class 1 integron *dfrA17* gene (Ac. No. AY263739). The BLASTP analysis of the sequence derived from the *Pseudomonas* sp. TR 52 (Ac. No. AJ620334) showed 100% identity with amino glycoside adenyl transferase AAD A6 of *Pseudomonas aeruginosa* (Ac. No. AF140629). The translated partial *aadA1* sequence of *Kluyvera* sp. TR 97 (Ac. No. AJ698461) showed 97% (189/193) identity with AAD A1 of *E. coli* isolate Ec 1484R (Ac. No. AY224185) and *S. typhimurium* (Ac. No. AJ496285). The partial CDS of *aadA1* gene as derived from *Serratia* sp. TR 40 (Ac. No.AJ938160) expressed 100% identity with the same gene from *Escherichia coli* isolate Ec1484R class 1 integron (Ac. No. AY224185). The complete CDS of *aac-(6')-Ib* from *Pseudomonas* sp. TR 59 (Ac. No. AJ697861) shared identity of 98 and 97% at nucleotide and protein level with amino glycoside 6'-N- acetyl transferase of *Vibrio*

cholerae class 1integron (Ac. No. AY103455) while the partial CDS of the same gene from *Yersinia* sp. TR 63 (Ac. No. AJ937775) shared 99% identity with the same *V. cholerae* (Ac. No. AY103455) class 1 integron. The sequence derived from the 0.8 kb amplicon of TR 58 (Ac. No.AJ938159) showed 99% identity (564/569) with *aac*-(6')-*Ib* gene of *Burkholderia cepacia* class 1 integron (Ac. No. AF371964).

3.3.5.1 Sequence analysis of CS-PCR product derived from TR 02

A 0.8 kb amplicon was derived from the isolate TR 02. The amplicon was cloned (pTR02) and the primer T7 was used for sequencing of the cloned fragment. Sequence analysis of the cloned fragment revealed the presence of a single ORF. This ORF represented an uninterrupted polypeptide sequence of 147 amino acids. The start of the ORF began with a GTG start codon coding valine at positions 101 to 103 (Figure 3.7) [Valine may also act as start codon in some cases (Adrian *et al.* 1998, Chang *et al.* 2000)]. A typical *dfrV* gene cassette yields a polypeptide of 157 amino acids, as is found in plasmid pLMO2O. Sequence of the gene cassette of TR 02 was read upto 147th amino acid and the ORF was expected to extend further by 10 amino acids in the region yet to be sequenced. Even if the chain length is reduced by 10 amino acids, it will not hamper the activity or the stability of the functional protein, since these amino acids are not in contact with many other residues that participate in numerous interactions and thereby do not affect the reaction rate by several orders of magnitude (Saraf *et al.* 2003). The amino acid at the 27th position of the putative translated product of TR 02 sequence was glutamate like other members of family1 DHFRs and the typical signature sequence of DHFR was also present at the N terminal end. The Blast N analysis of the sequence revealed 100% homology at the nucleotide level with plasmid pLMO20 *dhfr* V gene cassette (Accession no: X12868, cds: from 1306 to 1779), *E. coli* class 1 integron *dfr* V gene cassette (Accession no: AJ419169; cds: from 117 to 590), *Vibrio cholerae* class 1 integron bearing *dfr* V gene cassette (Accession no: AJ512546; cds: from 92 to 565). The translated polypeptide sequence was compared with other amino acids sequences in the Swiss Prot database. The result revealed that the translation product bears 99% identity with *dfr* V.

3.3.5.2 Sequence analysis of CS-PCR product derived from TR 17

A 2.0 kb CS-PCR product of *Citrobacter* sp. TR 17 as insert in pTR17 was sequenced with T7 promoter primer. The analysis of the partial sequence of the insert revealed the presence of one unpunctuated ORF of 154 amino acids (Figure 3.8). The translated polypeptide in BlastP analysis yielded best scores with all DHFR sequences and highest identity was observed with *dfrA12* protein. However a typical *dfrA12* gene cassette codes for a protein of 165 amino acids. The TR 17 ORF was short of the first 13 amino acids that were present in *dfrA12*. Typically a *dfrA12* polypeptide starts with methionine and has a valine residue at 11th position followed by two successive A residues before another M at position 14. In case of TR 17, ATG codon at nucleotide position 112 to114 was predicted to be the start codon. Though there could be the coding of a V followed by two A residues situated immediate upstream of the ATG codon, but this valine coded by GTT is most unlikely to be the start codon because reports of

using alternative start codons for various type I DHFRs is restricted to the using of either GTG or TTG. The first 120 amino acid stretch of TR 17 ORF bears 100% identity when aligned with *dfrA12* protein sequences while the remaining 34 amino acids of the partial sequence did not match with any of the existing sequences of the protein databases. The nucleotide sequence of TR 17 when multiply aligned with *dfrA12* sequences of *V. cholerae* (Ac. No AY103459), *Salmonella enterica* (Ac. No. AY126944), *E. coli* (Ac. No. Z21672), and *Citrobacter freundii* (Ac. No. AF550415), revealed single base pair deletion(s) at nucleotide position after 470 and 505 and two transversion events at c.561 and 562 that has resulted in a frame-shift leading to the alteration of protein sequence coded after the 120th amino acid of the TR 17 ORF. The DHFR profile-based multiple alignment, generated by MaxHom algorithm (PredictProtein), using TR 17 protein sequence as a query against SWISS-PROT database was subsequently fed into the neural network for secondary structure prediction by PHD. The predicted secondary structure of TR 17 protein was composed of 22.73% helix, 22.08% extended sheet and others 55.19%. The predicted secondary structure of dfrA12 protein is composed of 16.36% helix, 34.55% extended sheet and others 49.09%. The protein appeared as compact as a globular domain.

3.3.5.3 Sequence analysis of CS-PCR product derived from TR 40

A 3.2 kb CS-PCR product of isolate TR 40 cloned in pGEM-T easy vector (pTR40) was sequenced with SP6 promoter primer. Nucleotide sequence analysis revealed the presence of a single ORF of 469 bp encoding a polypeptide of 155 amino acids (Figure 3.9). The 5′ end of the ORF was not complete. The ORF was terminated with a TAA stop codon at positions 467 to 469. A core site, GTTRRRY (GTTAGAT at positions 524 to 530), was present at the 3′ end of the said ORF. The putative coding sequence bears 100% identity with *aadA1* gene sequence.

3.3.5.4 Sequence analysis of CS-PCR product derived from TR 48

An amplicon of 1600 bp was obtained by CS-PCR and was cloned in T vector. The insert from the respective clone pTR48 was sequenced with T7 promoter primer. Sequence analysis of the insert revealed the presence of an ORF, yielding a putative protein of 134 amino acids. The ORF began with GTG codon (coding for valine) at nucleotide positions 81 to 83 and ended with the stop codon TGA at positions 483 to 485 (Figure 3.10). The translated polypeptide in BlastP analysis yielded best scores with all DHFR sequences and highest identity was observed with dfrA17 protein. This polypeptide sequence was 23 amino acids shorter than the normal dfrA17. A normal *dfrA17* encoded protein is 157 amino acids long. The 30 amino acid residues present at the C-terminal end (128th to 157th) of the normal dfrA17 protein were not present in the polypeptide under study. Moreover, the last four amino acid residues of this polypeptide were completely different. The estimated half-life and the instability index (II) of the predicted protein were computed to be 1.2 hours (mammalian reticulocytes, in vitro) and 32.42 respectively, which classified the protein as stable. Predicted secondary structure (PHD) composition of this protein was: *H* (denoting α- helix) = 18.94 %; *E* (denoting extended β- strand/sheet) = 31.06 % and *L* (denoting others, loop) =

50.0 %, which was comparable to typical dfrA17 protein composed of *H* =17.20 %, *E* =36.94 % and *L* =45.86 %.

3.3.5.5 Sequence analysis of CS-PCR product derived from TR 52

The partial sequencing of the cloned fragment of *Pseudomonas* sp. TR 52 was done by using the promoter primer T7. Sequence analysis revealed the presence of one ORF of 94 amino acids. ATG was the start codon for this ORF (positions 91 to 93) and the termination codon was TGA at positions 373 to 375 (Figure 3.11). The 5′ conserved region was represented with a stretch of 72 nucleotides (from positions 9 to 80) located 10 bp upstream of the start of the ORF. The instability index of this putative translated product was computed to be 41.43, which classified the protein as unstable. The predicted secondary structure of this protein was H =32.98%; E =21.28% and L =45.74%. The protein may be globular but not as compact as a globular domain. The protein bears 100% identity with aminoglycoside adenyltransferase AAD A6 protein. An AAD A6 protein sequence typically constituted of 281 amino acids with a predicted secondary structure composition of *H* =49.47%, *E* =7.83 % and *L* =42.70% and appeared as a compact globular domain.

3.3.5.6 Sequence analysis of CS-PCR product derived from TR 58

Providencia sp. TR 58 yielded two amplicons of different sizes (1.2 kb and 0.8 kb) with primers 5′ CS and 3′ CS. The amplicons were separately cloned in pGEM–T Easy Vector. Sequencing analysis confirmed the presence of different gene cassettes in these two amplicons.

The 1.2 kb insert from pTR5801 was sequenced with T7 primer. The sequence analysis showed the presence of an ORF of 445 bp encoding a polypeptide of 148 amino acids. The ORF began with GTG start codon at positions 93 to 95 (Figure 3.12). The 5′ conserved region was represented with a stretch of 75 nucleotides (from positions 1 to 74) located 20 bp upstream of the ORF. The 3′ end of this ORF was not complete. The BlastP analysis of the coding sequence revealed 99% identity with dihydrofolate reductase type I sequences of the existing protein database.

The primer SP6 was used for sequencing of the 0.8 kb insert of the clone pTR5802. A potential ORF of 441 bp encoding a polypeptide of 146 amino acids was identified. The ORF began with ATG start codon at positions 94 to 96 and terminated with a TAA stop codon at positions 532 to 534 (Figure 3.13). Blast analysis of the sequence identified it as *aac-6′-Ib*, showing 99% identity with the existing aminoglycoside-6'-N-acetyl transferase gene sequences.

3.3.5.7 Sequence analysis of CS-PCR product derived from TR 59

The 811 bp insert from pTR59 was sequenced with both T7 and SP6 promoter primers. A stretch of 67 nucleotides (from 12 to 78) represented the 5' conserved segment. Further 68 nucleotides downstream of that conserved region, a 579 bp ORF potentially encoding a polypeptide of 192 amino acids was identified. The ORF began with GTG start codon at positions 147 to 149 and terminated with a TAA stop codon at positions 723 to 725 (Figure 3.14). No typical *E. coli* ribosome binding sites were identified upstream of the ATG start codon. Blast analysis of the coding sequence identified it as *aac-6′-Ib*, showing 97% identity with the existing aminoglycoside-6'-N-acetyl transferase

gene sequences. The nucleotide sequence immediately after the 3' end of *aac-6'-Ib* encoded a structure of 59 nucleotides (725 to 783) which was recognizable as an *att*C site (59 base element). The predicted secondary structure of this putative translated product was *H*=25.00%, *E*= 29.17% and *L*=45.83% which was comparable to the predicted secondary structure of the AAC-6-Ib protein (*H*=26.56%, *E*=27.60% and *L*=45.83%). The instability index of this protein was computed to be 40.74 that classified the protein as instable.

3.3.5.8 Sequence analysis of CS-PCR product derived from TR 63

The primer T7 was used for the sequencing of the cloned insert of the 900 bp in the recombinant plasmid pTR63. Further analysis revealed the presence of one unpunctuated ORF of 140 amino acids (Figure 3.15). A 75 nucleotides long 5' conserved region was located 142 nucleotides upstream of the ORF. The ORF began with the start codon ATG at positions 143 to 145. The 3' end of this ORF was not complete. The translated polypeptide of this ORF revealed maximum homology (99%) with the *aac-6'-Ib* gene product.

3.3.5.9 Sequence analysis of CS-PCR product derived from TR 85

The primer T7 was used for sequencing the 800 bp insert of pTR85. Nucleotide sequence analysis of the insert revealed the presence of a potential ORF encoding a polypeptide of 157 amino acids. The ORF began with GTG start codon at positions 51 to 53 and terminated with a TAA stop codon at positions 522 to 524 (Figure 3.16). The translated polypeptide sequence was compared with other amino acid sequences in the Swiss-Prot database. Sequences with the best scores were all dhfr sequences. The amino acid sequence of this ORF shared 99% identity with the normal dfrV protein.

3.3.5.10 Sequence analysis of CS-PCR product derived from TR 90

The complete sequence of 1177 bp insert was derived from pTR90 by using T7 and SP6 promoter primers. BlastN analysis of that sequence showed highest identity of 98% (1131/1150) with *Vibrio cholerae* class 1 integron sequence (Ac. No. AF455254) reported from India. Two ORFs, ORF1 and ORF2 were predicted from the sequence. ORF1 was found to code for the longest uninterrupted polypeptide sequence of 218 amino acids while the translation product of ORF2 led to possible hypothetical protein sequence of 148 amino acids. The ORF1 began with the atypical *E. coli* start codon GTG at position 97 to 99 and ended with stop codon TGA at positions 751 to 753 (Figure 3.17). Despite the unusual start codon, which normally codes for valine, it was the only codon, which was preceded by a plausible SD sequence (TGAGGAAGA). Since the ATG codon that was located four amino acids downstream of the GTG codon was not preceded by such a sequence, it was unlikely that this was the start codon. The ORF1 was flanked by a core element (GTTAACC) located 14 nucleotides 5′ to the start of the reading frame and differed from the consensus sequence of the core element (GTTRRRY) by a single nucleotide. Like most gene cassettes no recognizable promoter was present between the core element and the start of the ORF. The translated polypeptide sequence of ORF1 was compared with amino acid sequences in the Swissprot database. The first 152 amino acid sequence of ORF1 which yielded best scores were all DHFR type I

sequences. It is interesting to note that all dfrA1 protein sequences are 157 amino acids long and the first 152 residues of this novel ORF shared 95% identity with the existing *dfrA1* type. A stretch of 33 amino acid residues, from 153rd to 185th residue, did not find any resemblance with the existing sequences of the protein databases. Furthermore, another stretch of 25 amino acid residues, from residue 186 to 210, bore 60% identity with E1 protein of *E. coli*. Again the remaining C-terminal amino acids did not match with any of the existing protein sequences (Figure 3.18A).

Complete sequence alignment between *V. cholerae* (Ac. No. AF455254; 1179 bp) and *Morganella* sp. TR 90 (Ac. No. AJ698460; 1177 bp) gene cassettes revealed a DNA region/ mutation window of 216 bp [494 to 709 nucleotide position of *V. cholerae* sequence aligned to nucleotide position- 497 to 697 in *Morganella* sp. TR 90] that has presumably suffered 16 single base pair deletion and one base replacement event giving rise to the CDS of ORF1 protein (Figure 3.18B). The CDS, which was restricted to coding of 157 amino acids of *dfrA1* protein in *V. cholerae*, was found extended to a length of 218 amino acid residues before encountering a stop codon in *Morganella* sp. TR 90 sequence. Within the mutation window recombination crossover region (hs1) covers the sequence block from c.590 – 644 where six separate single base pair deletion events have been noted in the pair-wise sequence alignment (Fig 3.18B). Intrinsic polymerase pausing or pausing induced by a region of DNA secondary structure may initiate or promote misalignment. Once initiated, misalignment is directed by DNA sequence complementarity and therefore involves repetitive DNA sequence elements, such as direct repeats, inverted repeats, or other repetitive tracts including runs of mono-, di-, and tri-nucleotide (or other) repeats. The possible frame shifts due to the above mentioned reasons can fit into a model where deletion events of a typical *V. cholerae* like sequence may give rise to *Morganella* sp. TR 90 type sequence, resulting into read through across the non-coding region and merging with N-terminal coding region of the next ORF. The possible mutational events due to replication slippage have been illustrated in the Box 3.1.

Using ProtParam (ExPASy) tool, the estimated half-life and the instability index (II) of the predicted protein was computed to be 10 h (*E. coli* in vivo) and 32.08 respectively, which classified the protein as stable. The dihydrofolate reductase signature sequence of this protein revealed from PROSITE motif search [Bairoch *et al.* 1997] was 'VIGngpdIPWsakg.EqIIFkaiT'. The predicted secondary structure (PHD) composition of ORF1 protein was: *H* (denoting α- helix) = 19.25%, *E* (denoting extended β- strand/sheet) = 29.58%, and *L* (denoting others, loop) = 51.17%, which was comparable to *dfrA1* protein composed of *H* = 19.75%, *E* = 31.21% and *L* = 49.04%. Both the ORF1 protein and *dfrA1* protein appeared as compact globular domains in Globe prediction algorithm and no bonded cysteine was found in them as revealed by CYSPRED algorithm (Predict Protein). The predicted secondary structure and the presence of a dihydrofolate reductase signature indicated that the TR 90 protein might function like other DHFRs, similarly to the dfrA1 protein and might also confer resistance to trimethoprim.

3.3.5.11 Sequence analysis of CS-PCR product derived from TR 95

Both T7 and SP6 promoter primers were used for sequencing of the 718 bp long insert of pTR 95. The nucleotide sequence analysis showed the presence of a single gene, *dfr* gene. A 5' CS region (from positions 21 to 77) and a 59 base element at the 3' end (from positions 566 to 693) flanked the gene. This suggests that *dfr* gene is present as a gene cassette inserted into the variable region between the 5' and 3' conserved segments of a class 1 integron. This gene cassette contained an ORF of 324 bp encoding 107 amino acids. The ORF began with the start codon ATG at positions 248 to 250 and terminated with TAA stop codon at positions 569 to 571(Fig 3.19). The coding sequence, designated *dfrA7*, showed 98% identity with the dihydrofolate reductase type VII gene product. This polypeptide lacked a 50 amino acids long stretch at the N-terminal end, which is present in the normal dfrA7 protein. The estimated half-life and the instability index (II) of the predicted protein was computed to be 30 h (mammalian reticulocytes invivo) and 33.79 respectively, which classified the protein as stable. Predicted secondary structure (PHD) composition for the putative translated product was: *H* (denoting α- helix) = 15.89%; *E* (denoting extended β- strand/sheet) = 40.19%; and *L* (denoting others, loop) = 43.93%. The protein appears as compact, as a globular domain. On the contrary, the ideal dfrA7 protein may be globular but is not as compact as a domain with the predicted secondary structure composed of *H*=17.20%, *E*=35.03% and *L*=47.77%. The instability index of the dfrA7 protein is computed to be 30.48, which classifies the protein as stable.

3.3.5.12 Sequence analysis of CS-PCR product derived from TR 97

The 2.0 kb insert of pTR97 was sequenced with SP6 promoter primer. Partial sequencing of the said insert followed by nucleotide sequence analysis revealed the presence of a single ORF of 579 bp, encoding 192 amino acids (terminated with TAA stop codon at positions 584 to 586) (Figure 3.20). The 5' end of this ORF is not complete. The 3' end of the said ORF was flanked by a GTTRRRY core site (GTTAGAT at positions 641 to 647). Nucleotides from positions 588 to 641 represented the 3' portion of the 59 be. An inverse core site, RYYAAC (GTCTAAC at positions 588 to 594, differed from the consensus sequence by a single nucleotide), was also detected at the 3' end. The coding sequence shared 97% identity with the existing aminoglycoside adenyl transferase (*aadA1*) gene sequences.

3.3.6 Location of class 1 integrons on the conjugative plasmids

Out of the eighteen successful donor isolates (Table 2.2 of chapter 2) (all of which were the members of the said pool of 81 MAR isolates), eleven (TR 02, TR 04, TR 10, TR 13, TR 17, TR 37, TR 48, TR 56, TR 68, TR 79, TR 81 and TR 85) were found to carry detectable class 1 integron structures. When plasmid DNAs from the transconjugants was used as the template DNA for the PCR reaction, specific amplicons were produced from six (TR 02, TR 04, TR 10, TR 13, TR 17 and TR 85) of them (Table 3.5). The result confirmed the location of class 1 integrons on the conjugative plasmid that was transferred from the donor to the recipient. Upon digestion with restriction enzymes, CS-PCR products derived from the donor, its

Table 3.5. Class 1 integrons in donors and respective transconjugants

Donor	Class 1 integron in donor	Presence of integron in transconjugant	Nature of the gene cassette and accession no.
TR 02	+	+	*dfrA5* AJ620333
TR 10	+	+	*dfrA12*
TR 13	+	+	*dfrA12*
TR 17	+	+	*dfrA12* AY604169
TR 37	+	-	
TR 48	+	+	*dfrA17* AJ868226
TR 56	+	-	
TR 68	+	-	
TR 79	+	-	
TR 81	+	-	
TR 85	+	+	*dfrA5* AJ867252

transconjugant and from the plasmid isolated from the respective transconjugant, yielded identical RFLP patterns. The complete sequence analysis of the cloned amplicons of transconjugants produced 100% identity with the class 1 integron sequences derived from the respective donor isolates.

Table 3.2 Multidrug resistance combinations in 100 gram-negative bacterial isolates of River Torsa

Mar Index	No. of strains[a]	Resistance[b]											
		Ami	Amp	Cef	Cep	Chl	Gen	Kan	Net	Nit	Str	Tet	Tob
0. 41	1	-	+	-	+	-	-	+	-	+	-	+	-
	2	-	+	-	+	+	+	-	-	+	-	-	-
	1	-	+	+	+	-	-	-	-	+	-	+	-
	1	-	+	-	+	-	-	-	-	+	-	+	+
	1	-	+	-	+	-	-	-	-	+	+	+	-
	3	-	+	-	+	+	-	-	-	+	-	+	-
	1	-	+	-	-	+	-	-	-	+	+	+	-
	1	-	+	-	-	+	-	+	-	+	-	+	-
0. 50	5	-	+	+	+	+	-	-	-	+	-	+	-
	1	-	+	+	+	+	-	+	-	+	-	-	-
	1	-	+	-	+	+	-	+	-	+	-	+	-
	1	-	+	+	+	-	-	-	-	+	-	+	+
	1	-	+	-	+	+	-	+	-	+	-	+	-
	1	-	+	+	+	-	-	-	-	+	+	+	-
	1	-	+	-	+	+	-	-	-	+	+	+	-
	1	-	+	+	-	+	-	-	-	+	+	+	-
0. 58	3	-	+	+	+	+	-	+	-	+	-	+	-
	2	-	+	-	+	+	-	+	-	+	+	+	-
	1	-	+	+	+	+	+	-	-	+	-	+	-
	1	+	+	+	+	+	-	-	-	+	-	+	-
	1	-	+	+	+	-	+	-	+	+	-	+	-
	1	-	+	-	+	+	-	+	-	+	-	+	+
	1	+	+	+	+	+	-	+	-	+	-	-	-
	1	-	+	-	-	-	-	+	+	+	+	+	+
0. 66	6	-	+	+	+	+	-	+	-	+	+	+	-
	2	-	+	+	+	-	+	+	-	+	+	+	-
	1	-	+	+	+	-	-	+	+	+	+	+	-
	1	-	+	+	+	-	+	-	-	+	+	+	+
	1	-	+	+	+	+	+	-	-	+	+	+	-
	1	-	+	-	+	+	+	+	+	+	-	-	+
	1	-	+	+	+	-	+	+	-	+	-	+	+
	1	+	+	+	+	-	-	+	-	+	-	+	+
	1	-	+	-	+	+	+	+	-	+	+	+	-
	1	-	+	-	+	-	+	+	-	+	+	+	+
	1	+	+	+	+	-	-	+	-	+	+	+	-
	1	-	+	+	+	+	+	+	-	+	-	+	-
0. 75	1	-	+	+	+	-	+	+	+	+	+	-	+
	1	-	+	+	+	+	+	+	+	+	-	-	+
	3	-	+	+	+	-	+	-	+	+	+	+	+
	1	+	+	+	+	-	+	-	+	+	-	+	+
	1	-	+	+	+	+	+	+	-	+	+	+	-
	1	-	+	+	+	+	+	+	+	+	-	-	-
	1	+	+	+	+	+	+	+	-	+	-	+	-
	1	-	+	+	+	+	+	-	-	+	+	+	+
	1	-	+	+	+	+	-	-	+	+	+	+	+
	2	+	+	+	+	+	-	+	-	+	+	+	-
	1	+	+	-	+	-	+	+	+	+	-	+	+
	2	-	+	+	+	+	+	+	+	+	-	+	-
	1	-	+	-	+	+	+	-	+	+	+	+	+
	1	-	+	+	+	-	-	+	+	+	+	+	+
	1	-	+	+	+	+	+	+	-	+	-	+	+
0. 83	1	-	+	+	+	+	+	+	-	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	-	-	+
	1	+	+	+	+	+	+	+	-	+	+	+	-
	2	-	+	+	+	+	+	+	+	+	-	+	+
	2	+	+	-	+	+	+	+	+	+	-	+	+
	2	-	+	+	+	+	+	+	+	+	-	+	+
	1	+	+	-	+	+	+	+	-	+	+	+	+
	1	-	+	+	+	-	+	+	+	+	+	+	+
0. 91	3	-	+	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	-	+	+
	2	+	+	-	+	+	+	+	+	+	+	+	+
	1	+	+	+	+	+	+	+	+	+	+	-	+
1. 0	9	+	+	+	+	+	+	+	+	+	+	+	+

[a] Total number of isolates with a particular combination of antibiotic resistance.
[b]Ami: Amikacin; Amp: Ampicilin; Cef: Cefotaxim; Cep: Cephalexin; Chl: Chloramphenicol; Gen:Gentamicin; Kan: Kanamycin; Net: Netilmicin; Nit: Nitrofurantoin; Str: Streptomycin; Tet: Tetracycline; Tob: Tobramycin.

Table 3.3 MAR indices and sizes of inserted gene cassettes for forty gram-negative integron positive isolates of River Torsa

Mar Index	Isolates carrying class 1 integron	Identification	Approx. length of the amplicons generated with 5'cs and 3'cs (bp)
0.41	TR 53	*Escherichia* sp.	2700
0.50	TR 97	*Kluyvera* sp.	2000
	TR 76	*Proteus* sp.	2000
	TR 48	*Serratia* sp.	1600
0.58	TR 77	*Proteus* sp.	1200,1800
	TR 39	*Serratia* sp.	1900,3000
0.66	TR 6, TR 10 and TR 14	*Citrobacter* spp.	2000
	TR 81	*Providencia* sp.	1300
	TR 40	*Serratia* sp.	3200
0.75	TR 11, TR 12 , TR 13 and TR 15	*Citrobacter* spp.	2000
	TR 85	*Enterobacter* sp.	800
	TR 90	*Morganella* sp.	1177
	TR 56	*Proteus* sp.	1200,1800
	TR 18	*Providencia* sp.	1200
	TR 95	*Salmonella* sp.	718
	TR 27 and TR 92	*Pseudomonas* spp.	1200,1800
	TR 99	*Pseudomonas* sp.	1200
0.83	TR 91	*Citrobacter* sp.	800
	TR 02	*Escherichia* sp.	750
	TR 73	*Providencia* sp.	900
	TR 79	*Salmonella* sp.	900
	TR 63	*Yersinia* sp.	900
	TR20	*Acinetobacter* sp.	1300
0.91	TR 04	*Enterobacter* sp.	800
	TR 68	*Providencia* sp.	1200, 800
	TR 78	*Serratia* sp.	900
	TR 82	*Serratia* sp.	1200
	TR 49	*Acinetobacter* sp.	1100,900
	TR 59	*Pseudomonas* sp.	811
1.0	TR 17	*Citrobacter* sp.	2000
	TR 37	*Kluyvera* sp.	3000
	TR 58	*Providencia* sp.	1200,800
	TR 52	*Pseudomonas* sp.	1400
	TR 62	*Pseudomonas* sp.	1400

Table 3.4 Characterization of the gene cassettes in MAR isolates of river Torsa

Isolate	Antimicrobial resistance profile[a]	Nature of the gene cassette	Cassette encoded antibiotic resistance	Accession No.
TR 97	AMP, CEF, CEP, CHL, COT, NIT, TET,	*aad*A1	STR	AJ698461
TR 48	AMP, CEF, CEP, COT, NIT, TET, TOB	*dfrA17*	TMP	AJ868226
TR 6	AMP, CEF, CEP, CHL, COT, KAN, NIT, STR, TET	*dfrA12*	TMP	-[b]
TR 10	AMP, CEF, CEP, COT, GEN, NIT, STR, TET, TOB	*dfrA12*	TMP	-[b]
TR 14	AMP, CEF, CEP, CHL, COT, GEN, NIT, STR, TET	*dfrA12*	TMP	-[b]
TR 40	AMP, CEP, COT, GEN, KAN, NIT, STR, TET, TOB	*aad A1*	STR	AJ938160
TR 11, TR 12, TR 13	AMP, CEF, CEP, COT, GEN, NET, NIT, STR, TET, TOB	*dfrA12*	TMP	-[b]
TR 15	AMI, AMP, CEF, CEP, COT, GEN, NET, NIT, TET, TOB	*dfrA12*	TMP	-[b]
TR 85	AMI, AMP, CEP, COT, GEN, KAN, NET, NIT, TET, TOB	*dfrA5*	TMP	AJ867252
TR 90	AMP, CEF, CEP, CHL, COT, GEN, KAN, NET, NIT, TET	*dfrA1* variant	TMP	AJ698460
TR 95	AMP, CEF, CEP, COT, KAN, NET, NIT, STR, TET, TOB	*dfrA7*	TMP	AY604170
TR 02	AMP, CEF, CEP, CHL, COT, GEN, KAN, STR, TET, NIT, TOB	*dfrA5*	TMP	AJ620333
TR 63	AMI, AMP, CEP, CHL, COT, GEN, KAN, NET,NIT, TET, TOB	*aac*-(6')- *Ib*	AMI	AJ937775
TR 59	AMI, AMP, CEF, CEP, CHL, COT, GEN, KAN, NET,NIT, TET, TOB	*aac*-(6')- *Ib*	AMI	AJ697861
TR 17	AMI, AMP, CEF, CEP, CHL, COT, GEN, KAN, NET,NIT, STR, TET, TOB	*dfrA12*	TMP	AY604169
TR 58	AMI, AMP, CEF, CEP, CHL, COT, GEN, KAN, NET,NIT, STR, TET, TOB	*dfrA1*, *aac*-(6')- *Ib*	TMP AMI	AJ937774, AJ938159
TR 52	AMI, AMP, CEF, CEP, CHL, COT, GEN, KAN, NET,NIT, STR, TET, TOB	*aadA6*	STR	AJ620334
TR 62	AMI, AMP, CEF, CEP, CHL, COT, GEN, KAN, NET,NIT, STR, TET, TOB	*aadA6*	STR	-[c]

[a]AMI: Amikacin; AMP: Ampicillin; CEF: Cefotaxim; CEP: Cephalexin; CHL: Chloramphenicol; COT: Cotrimoxazole, GEN: Gentamicin; KAN:Kanamycin; NET: Netilmicin; NIT: Nitrofurantoin; STR: Streptomycin; TET: Tetracycline; TMP: Trimethoprim; TOB: Tobramycin

[b]RFLP pattern similar to TR 17 amplicon

[c]RFLP pattern similar to TR 52 amplicon

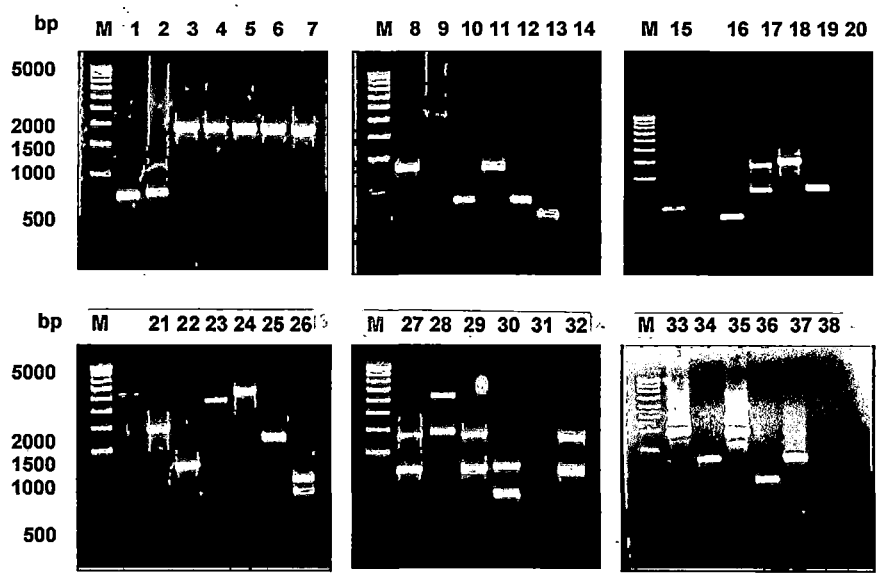


Figure 3.3. PCR amplification, using the 5' CS and 3' CS primers, of variable regions of class 1 integrons from Torsa isolates. The PCR products were separated by electrophoresis in 1.0%agarose.

Lane M, 500 bp DNA ladder; 1, TR 02; 2, TR 04; 3, TR 06; 4, TR 10; 5, TR 11; 6, TR 12; 7, TR 13; 8, TR 52; 9, TR 53; 10, TR 59; 11, TR 62; 12, TR 63; 13, TR 91; 14, TR 73; 15, TR 79; 16, TR 85; 17, TR 77; 18, TR 97; 19, TR 82; 20, TR 95; 21, TR 17; 22, TR 20; TR 37; 24, TR 40; 25, TR 48; 26, TR 49; 27, TR 27; 28, TR 39; 29, TR 56; 30, TR 58; 31, TR 68; 32, TR 92; 33, TR 14; 34, TR 18; 35, TR 76; 36, TR 78; 37, TR 90; 38, TR 73.

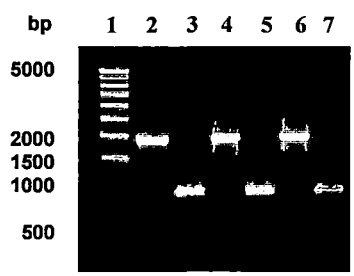


Figure 3.5. Agarose gel electrophoresis of *Eco*RI restricted 2000 bp amplicons generated with 5' CS and 3' CS primers. Lane 1, 500 bp DNA ladder; 2, TR 06 ; 3, *Eco* RI digest of TR 06 ; 4, TR 10 ; 5, *Eco* RI digest of TR 10 ; 6, TR 17 ; 7, *Eco* RI digest of TR 17

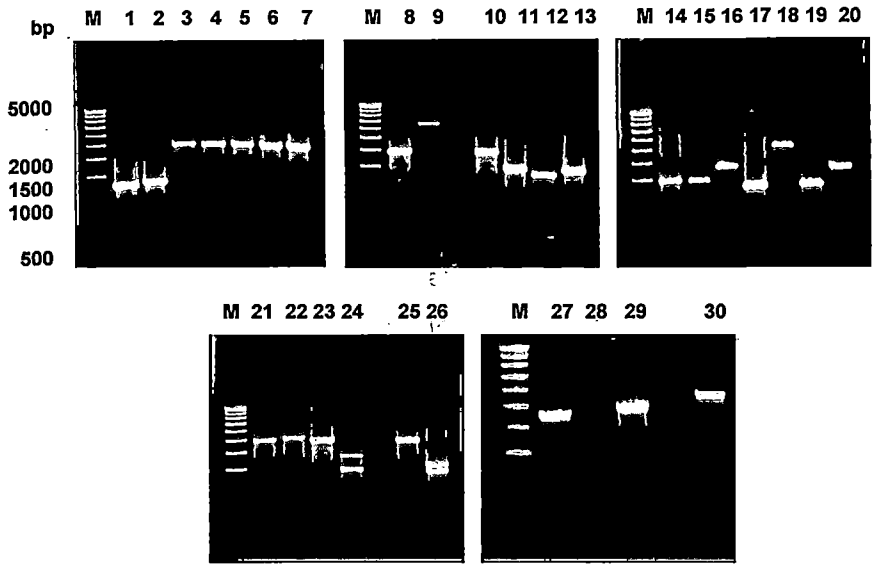
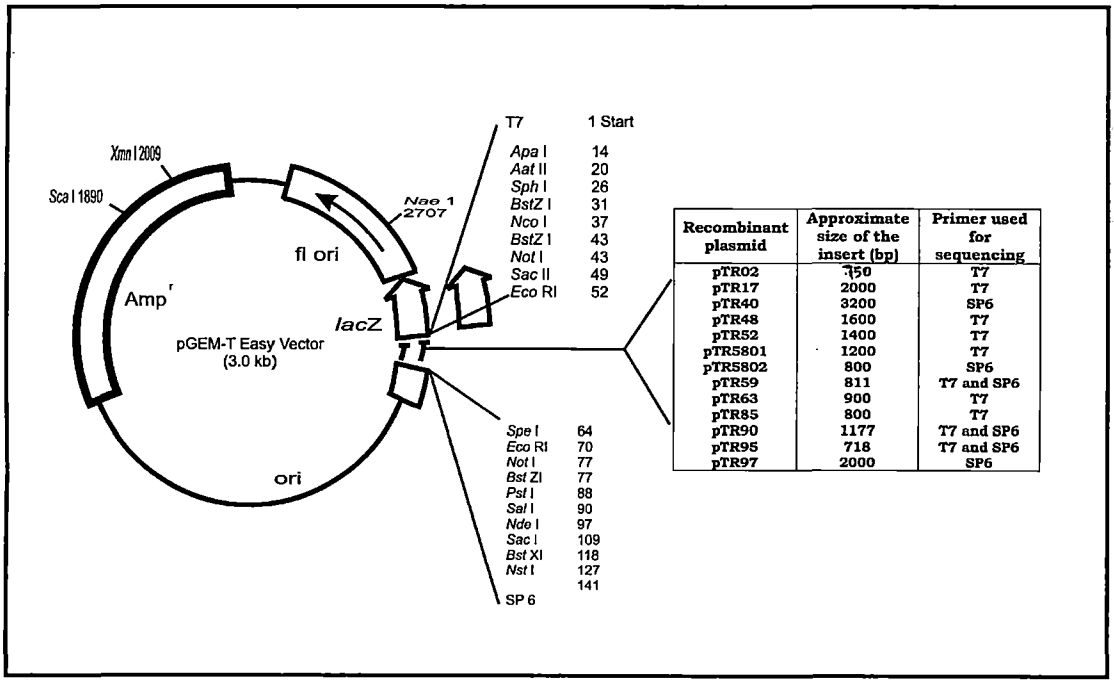


Figure 3.4. PCR amplification, using the Int_2F and 3' CS primers, of variable regions of class 1 integrons from Torsa isolates. The PCR products were separated by electrophoresis in 1.0% agarose. Lanes M, 500 bp DNA ladder; 1,TR 02 ; 2, TR 04 ; 3, TR 06 ; 4, TR 10 ; 5, TR 11 ; 6, TR 12 ; 7, TR 13 ; 8, TR 52 ; 9, TR 53 ; 10, TR 62 ; 11, TR 63 ; 12, TR 91 ; 13, TR 73 ; 14, TR 78 ; 15, TR 79 ; 16, TR 81 ; 17, TR 85 ; 18, TR 97 ; 19, TR 95 ; 20, TR 90 ; 21, TR 27 ; 22, TR 39 ; 23, TR 56 ; 24, TR 58 ; 25, TR 77 ; 26, TR 49 ; 27, TR 48; 28, TR 37; 29, TR 92; 30, TR 20.



Recombinant plasmid	Approximate size of the insert (bp)	Primer used for sequencing
pTR02	150	T7
pTR17	2000	T7
pTR40	3200	SP6
pTR48	1600	T7
pTR52	1400	T7
pTR5801	1200	T7
pTR5802	800	SP6
pTR59	811	T7 and SP6
pTR63	900	T7
pTR85	800	T7
pTR90	1177	T7 and SP6
pTR95	718	T7 and SP6
pTR97	2000	SP6

Figure 3.6. Cloning of CS-PCR product(s) in pGEM-T Easy Vector
Amp^r, gene for ampicillin resistance; *ori*, origin of replication; *lacZ*, gene for the synthesis of β-galactosidase.

```
CGCTGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAG 60

GGCAGTCGCCCTAAAACAAAGTTAACCCGGAACCAAAATTGTGAAAGTATCATTAATGGC 120
                                          V  K  V  S  L  M  A
TGCAAAAGCGAAAAACGGAGTGATTGGTTGCGGTCCACACATACCCTGGTCCGCGAAAGG 180
 A  K  A  K  N  G  V  I  G  C  G  P  H  I  P  W  S  A  K  G
AGAGCAGCTACTCTTTAAAGCCTTGACGTACAACCAGTGGCTTTTGGTGGGCCGCAAGAC 240
 E  Q  L  L  F  K  A  L  T  Y  N  Q  W  L  L  V  G  R  K  T
GTTCGAATCTATGGGAGCACTCCCTAATAGGAAATACGCGGTCGTTACTCGCTCAGCCTG 300
 F  E  S  M  G  A  L  P  N  R  K  Y  A  V  V  T  R  S  A  W
GACGGCCGATAATGACAACGTAATAGTATTCCCGTCGATCGAAGAGGCCATGTACGGGCT 360
 T  A  D  N  D  N  V  I  V  F  P  S  I  E  E  A  M  Y  G  L
GGCTGAACTCACCGATCACGTTATAGTGTCTGGTGGCGGGGAGATTTACAGAGAAACATT 420
 A  E  L  T  D  H  V  I  V  S  G  G  G  E  I  Y  R  E  T  L
GCCCATGGCCTCTACGCTCCATATATCGACGATTGATATTGAGCCGGAAGGAGATGTTTT 480
 P  M  A  S  T  L  H  I  S  T  I  D  I  E  P  E  G  D  V  F
CTTTCCGAATATTCCCAATACCTTCGAAGTTGTTTTTGAGCAACACTTTAGCTCAAACAT 540
 F  P  N  I  P  N  T  F  E  V  V  F  E  Q  H  F  S  S  N  I
```

Figure 3.7 [A]. Partial nucleotide sequence of CS-PCR product of TR 02 showing ORF and the putative translated product

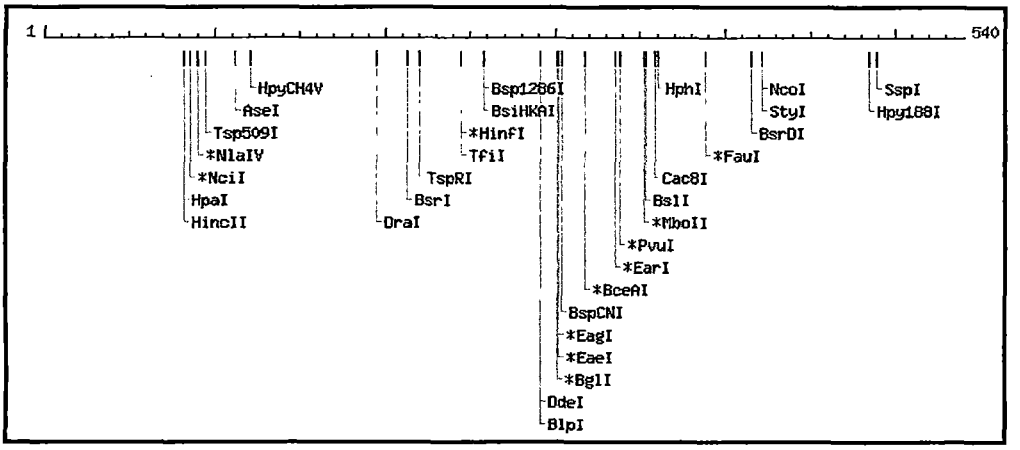


Figure 3.7 [B]. Restriction site analysis of the partial sequence generated from cloned CS-PCR product of TR 02. *, cleavage affected by methylations

```
GGCAAGTCTGCTAAACCCGTAANCAGGNTCNGGNTGCANAACAACAGGGTNTGTAGTCAG 60


GTCAAGTCTGCTTAAACTCGGGAATCAGTACGCATTCATCTCGTTGCTGCGATGGGAGCC 120
                                                      M  G  A
AATCGGGTTATTGGCAATGGTCCTAATATCCCCTGGAAAATTCCGGGTGAGCAGAAGATT 180
N  R  V  I  G  N  G  P  N  I  P  W  K  I  P  G  E  Q  K  I
TTTCGCAGACTCACTGAGGGAAAAGTCGTTGTCATGGGGCGAAAGACCTTTGAGTCTATC 240
F  R  R  L  T  E  G  K  V  V  V  M  G  R  K  T  F  E  S  I
GGCAAGCCTCTACCGAACCGTCACACATTGGTAATCTCACGCCAAGCTAACTACCGCGCC 300
G  K  P  L  P  N  R  H  T  L  V  I  S  R  Q  A  N  Y  R  A
ACTGGCTGCGTAGTTGTTTCAACGCTGTCGCACGCTATCGCTTTGGCATCCGAACTCGGC 360
T  G  C  V  V  V  S  T  L  S  H  A  I  A  L  A  S  E  L  G
AATGAACTCTACGTCGCGGGCGGAGCTGAGATATACACTCTGGCACTACCTCACGCCCAC 420
N  E  L  Y  V  A  G  G  A  E  I  Y  T  L  A  L  P  H  A  H
GGCGTGTTTCTATCTGAGGTACATCAAACCTTCGAGGGTGACGCCTTCTTCCAATGCTCA 480
G  V  F  L  S  E  V  H  Q  T  F  E  G  D  A  F  F  Q  C  S
ACGAAACAGAATTCGAGCTTGTCTCACCGAAACCATTCAAGCTGTAATTCCGTACACCCA 540
T  K  Q  N  S  S  L  S  H  R  N  H  S  S  C  N  S  V  H  P
CTCCGTTTATGCGCGTCGAACGGGCTAACATTCCG                          575
L  R  L  C  A  S  N  G  L  T  F
```

Figure 3.8 [A]. Partial nucleotide sequence of CS-PCR product of TR 17 showing ORF and the putative translated product.

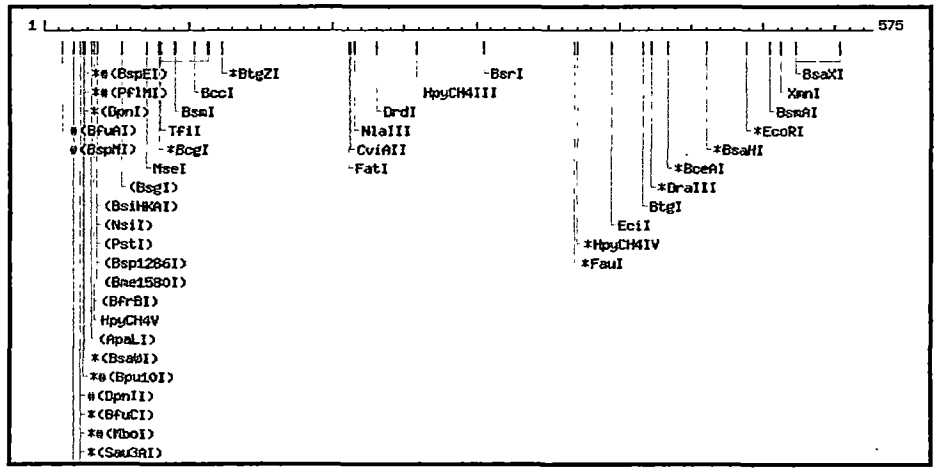
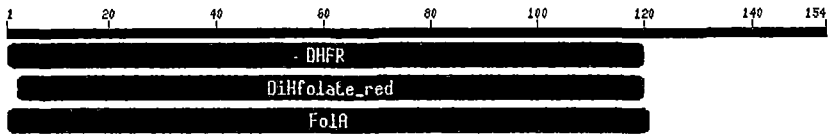


Figure 3.8 [B]. Restriction site analysis of the partial sequence generated from cloned CS-PCR product of TR 17. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
        ....,....1....,....2....,....3....,....4....,....5....,....6
AA      MGANRVIGNGPNIPWKIPGEQKIFRRLTEGKVVVMGRKTFESIGKPLPNRHTLVISRQAN
PHD_sec                    HHHHHHHHH      EEEE               EEEEEEE

        ....,....7....,....8....,....9....,....10.1.,....11.1.,....12.1
AA      YRATGCVVVSTLSHAIALASELGNELYVAGGAEIYTLALPHAHGVFLSEVHQTFEGDAFFQCS
PHD_sec      EEEE  HHHHHHHHH      EEEEE HHHHHHHHHH    EEEEEEE

        .,....13.1.,....14.1.,....15.1.
AA      TKQNSSLSHRNHSSCNSVHPLRLCASNGLTF
PHD_sec      EEEEEE
```

Figure 3.8 [C]. Predicted secondary structure of the putative translated product of ORF of TR 17 amplicon

Result of conserve domain search for the putative translated product obtained from ORF of TR 17




```
gnl|CDD|27775 cd00209, DHFR, Dihydrofolate reductase (DHFR).
              CD-Length = 158 residues, 77.2% aligned
              Score =  166 bits (423), Expect = 9e-43
Query:  1     MGANRVIGNGPNIPWKIPGEQKIFRRLTEGKVVVMGRKTFESIGK-PLPNRHTLVISRQA  59
Sbjct:  7     VDENGVIGKDNKLPWHLPEDLKHFKKTTTGNPVIMGRKTFESIPRRPLPGRTNIVLSRQL  66

Query:  60    NYR-ATGCVVVSTLSHAIALASELGNELYVAGGAEIYTLALPHAHGVFLSEVHQTFEGDA  118
Sbjct:  67    DYQDAEGVEVVHSLEEALELAENTVEEIFVIGGAEIYKQALPYADRLYLTRIHAEFEGDT  126

Query:  119   FF  120
Sbjct:  127   FF  128


gnl|CDD|22940 pfam00186, DiHfolate_red, Dihydrofolate reductase..
              CD-Length = 173 residues,  75.1% aligned
              Score =  140 bits (353), Expect = 1e-34
Query:  3     ANRVIGNGPNIPWKIPGEQKIFRRLTEGK---VVVMGRKTFESIG---KPLPNRHTLVIS  56
Sbjct:  10    KNGGIGKDGDLPWRLPNDLKYFKAVTTGTPRNAVIMGRKTWESIPEKFRPLPGRLNIVLS  69

Query:  57    RQANYRATGCVVVSTLSHAIALAS------ELGNELYVAGGAEIYTLALPHAHGVFLSEV  110
Sbjct:  70    RSEDYDAQGDNVVVSSSIEAALDLLAEPPEASIERVFVIGGAQLYAAALPLADRLYLTRI  129

Query:  111   HQTFEGDAFF  120
Sbjct:  130   DGEFEGDTFF  139


gnl|CDD|10137 COG0262, FolA, Dihydrofolate reductase [Coenzyme metabolism].
              CD-Length = 167 residues,  77.2% aligned
              Score =  110 bits (277), Expect = 7e-26
Query:  1     MGANRVIGNGPNIPWKIPGEQKIFRRLTEGKVVVMGRKTFESIG---KPLPNRHTLVISR  57
Sbjct:  8     VSLDGVIGRDNSLPWHLPEDLAHFKATTLGKPVIMGRKTYESLPGEWRPLPGRKNIVLSR  67

Query:  58    QANYRATGCVVVS-TLSHAIALA-SELGNELYVAGGAEIYTLALPH--AHGVFLSEVHQT  113
Sbjct:  68    NPDLKTEGGVEVVDSIEEALLLLLKEEGEDIFIIGGGELYRQFLPAGLADELILTIIPVL  127

Query:  114   F-EGDAFFQ  121
Sbjct:  128   LGEGDTLFP  136
```

```
ATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGA 60
 F  G  E  W  Q  R  N  D  I  L  A  G  I  F  E  P  A  T  I  D
CATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCC 120
 I  D  L  A  I  L  L  T  K  A  R  E  H  S  V  A  L  V  G  P
AGCGGCGGAGGAACTCTTTGATCCGGTTCCTGAGCAGGATCTATTTGAGGCGCTAAATGA 180
 A  A  E  E  L  F  D  P  V  P  E  Q  D  L  F  E  A  L  N  E
AACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCT 240
 T  L  T  L  W  N  S  P  P  D  W  A  G  D  E  R  N  V  V  L
TACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGC 300
 T  L  S  R  I  W  Y  S  A  V  T  G  K  I  A  P  K  D  V  A
TGCCGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAG 360
 A  D  W  A  M  E  R  L  P  A  Q  Y  Q  P  V  I  L  E  A  R
ACAGGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCTCGCGTGCAGATCAGTTGGAAGA 420
 Q  A  Y  L  G  Q  E  E  D  R  L  A  S  R  A  D  Q  L  E  E
ATTTGTTCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCTAACAAT 480
 F  V  H  Y  V  K  G  E  I  T  K  V  V  G  K  *(3' end of ORF)
TCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAAGCGTTAGATGCACTAAGCA 540

CATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTTA                     581
```

Figure 3.9 [A]. Partial nucleotide sequence of CS-PCR product of TR 40 showing ORF and the putative translated product (* indicate stop codon).

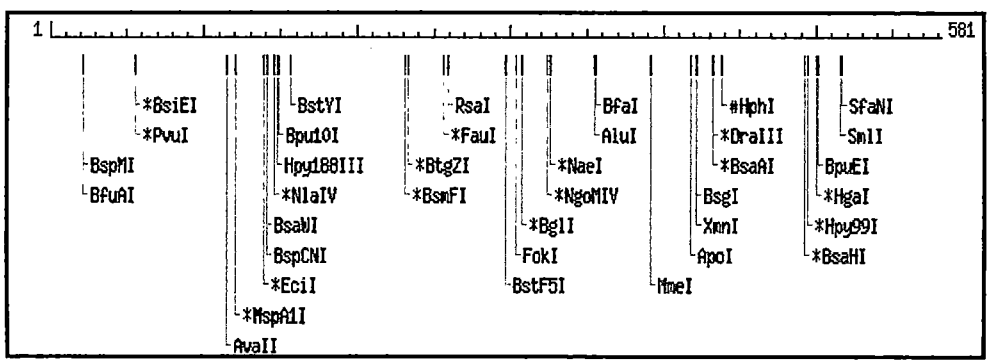


Figure 3.9 [B]. Restriction site analysis of the partial sequence generated from cloned CS-PCR product of TR 40. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
CGCGGTCGACGTATGGAGCAGCACGATCGTGTACTGCAGCAGGGCAGGTCGCCCTCNACA 60

CAGAGTTAGCCATTAAGGGAGTGAAATTGAAAATATCATTGATTTCTGCAGTGTCAGAAA 120
                    V  K  L  K  I  S  L  I  S  A  V  S  E  N
ATGGCGTAATCGGTAGTGGTCCTGATATCCCGTGGTCAGTAAAAGGTGAGCAACTACTCT 180
  G  V  I  G  S  G  P  D  I  P  W  S  V  K  G  E  Q  L  L  F
TTAAAGCGCTCACATATAATCAATGGCTCCTTGTCGGAAGAAAAACATTTGACTCTATGG 240
  K  A  L  T  Y  N  Q  W  L  L  V  G  R  K  T  F  D  S  M  G
GTGTTCTTCCAAATCGCAAATATGCAGTAGTGTCAAAGAACGGAATTTCAAGCTCAAATG 300
  V  L  P  N  R  K  Y  A  V  V  S  K  N  G  I  S  S  S  N  E
AAAACGTCCTAGTTTTTCCTTCAATAGAAAATGCTTTGAAAGAGCTATCAAAAGTTACAG 360
  N  V  L  V  F  P  S  I  E  N  A  L  K  E  L  S  K  V  T  D
ATCATGTATATGTCTCTGGCGGGGGTCAAATCTATAATAGCCTTATTGAAAAAGCAGATA 420
  H  V  Y  V  S  G  G  G  Q  I  Y  N  S  L  I  E  K  A  D  I
TAATTCATTTGTCTACTGTTCACGTTGAAGTCGAAGGTGATATCAAAATTCCCTATAATG 480
  I  H  L  S  T  V  H  V  E  V  E  G  D  I  K  I  P  Y  N  A
CCTGAGAATTTCAATTTGGTTTTTGAACAGTTTTTTATGTCTAATATAAATTATACATAC 540
  *(3' end of ORF)
CAGATTTGGAAAAAAGGCTAACAATGCGTTGCAGCACCAGTCGCTTCGCTCCTTGGACAG 600

CTTTTAAGTCGCGTCTTTGGGGGTTTN                                   627
```

Figure 3.10 [A]. Partial nucleotide sequence of CS-PCR product of TR 48 showing ORF and the putative translated product (* indicate stop codon).

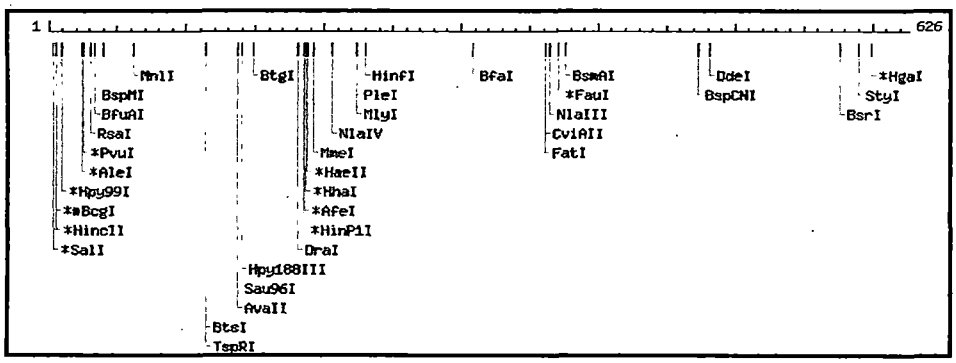
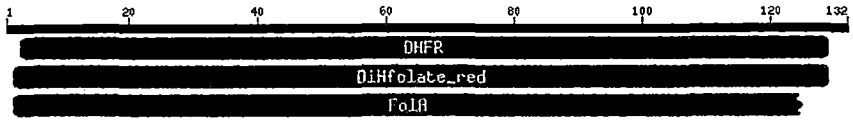


Figure 3.10 [B]. Restriction site analysis of the partial sequence generated from cloned CS-PCR product of TR 48. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
        ....,....1....,....2....,....3....,....4....,....5....,....6
AA      LKISLISAVSENGVIGSGPDIPWSVKGEQLLFKALTYNQWLLVGRKTFDSMGVLPNRKYA
PHD_sec  EEEEEEEE    EEE           HHHHHHHH     EEE               EEE

        ....,....7....,....8....,....9....,....10.1.,....11.1.,....12.1
AA      VVSKNGISSSNENVLVFPSIENALKELSKVTDHVYVSGGGQIYNSLIEKADIIHLSTVHVEVE
PHD_sec EEEE         EEEE  HHHHHHHHH    EEEEE  HHHHHHHH    EEEEEEEEEEE

        .,....13.
AA      GDIKIPYNA
PHD_sec
```

Figure 3.10 [C]. Predicted secondary structure of the putative translated product of ORF of TR 48 amplicon

Result of conserve domain search for the putative translated product obtained from ORF of TR 48



gnl|CDD|27775 cd00209, DHFR, Dihydrofolate reductase (DHFR).
CD-Length = 158 residues, 81.6% aligned
Score = 137 bits (346), Expect = 5e-34

```
Query:  3    ISLISAVSENGVIGSGPDIPWSVKGEQLLFKALTYNQWLLVGRKTFDSMG--VLPNRKYA  60
Sbjct:  1    ISLIVAVDENGVIGKDNKLPWHLPEDLKHFKKTTTGNPVIMGRKTFESIPRRPLPGRTNI  60

Query:  61   VVSKNGISSSNENVLVFPSIENALKELSKVTDHVYVSGGGQIYNSLIEKADIIHLSTVHV  120
Sbjct:  61   VLSRQLDYQDAEGVEVVHSLEEALELAENTVEEIFVIGGAEIYKQALPYADRLYLTRIHA  120

Query:  121  EVEGDIKIP  129
Sbjct:  121  EFEGDTFFP  129
```

gnl|CDD|22940 pfam00186, DiHfolate_red, Dihydrofolate reductase..
CD-Length = 173 residues, 80.9% aligned
Score = 132 bits (333), Expect = 1e-32

```
Query:  2    KISLISAVSENGVIGSGPDIPWSVKGEQLLFKALTYNQW---LLVGRKTFDSMGV----L  54
Sbjct:  1    MISLIVAVDKNGGIGKDGDLPWRLPNDLKYFKAVTTGTPRNAVIMGRKTWESIPEKFRPL  60

Query:  55   PNRKYAVVSKNGISSSNE-NVLVFPSIENALKELSKVT----DHVYVSGGGQIYNSLIEK  109
Sbjct:  61   PGRLNIVLSRSEDYDAQGDNVVVSSSIEAALDLLAEPPEASIERVFVIGGAQLYAAALPL  120

Query:  110  ADIIHLSTVHVEVEGDIKIP  129
Sbjct:  121  ADRLYLTRIDGEFEGDTFFP  140
```

gnl|CDD|10137 COG0262, FolA, Dihydrofolate reductase [Coenzyme metabolism].
CD-Length = 167 residues, only 79.0% aligned
Score = 103 bits (259), Expect = 6e-24

```
Query:  2    KISLISAVSENGVIGSGPDIPWSVKGEQLLFKALTYNQWLLVGRKTFDSMG----VLPNR  57
Sbjct:  1    MIILIVAVSLDGVIGRDNSLPWHLPEDLAHFKATTLGKPVIMGRKTYESLPGEWRPLPGR  60

Query:  58   KYAVVSKNGISSSNENVLVFPSIENALKELSKVTD-HVYVSGGGQIYNSLIE--KADIIH  114
Sbjct:  61   KNIVLSRNPDLKTEGGVEVVDSIEEALLLLLKEEGEDIFIIGGGELYRQFLPAGLADELI  120

Query:  115  LSTVHVEV-EGD  125
Sbjct:  121  LTIIPVLLGEGD  132
```

```
CCGCGCTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAG 60

GGCAGTCGCCCTAAAACAAAGTTAGACATCATGAGTAACGCAGTACCCGCCGAGATTTCG 120
                                M  S  N  A  V  P  A  E  I  S
GTACAGCTATCACTGGCTCTCAACGCCATCGAGCGTCATCTGGAATCAACGTTGCTGGCC 180
V  Q  L  S  L  A  L  N  A  I  E  R  H  L  E  S  T  L  L  A
GTGCATTTGTACGGCTCTGCACTGGACGGTGGCCTGAAGCCATACAGTGATATTGATTTG 240
V  H  L  Y  G  S  A  L  D  G  G  L  K  P  Y  S  D  I  D  L
CTGGTTACTGTGGCTGCACGGCTCGATGAGACTGTCCGACAAGCCCTGGTCGTAGATCTC 300
L  V  T  V  A  A  R  L  D  E  T  V  R  Q  A  L  V  V  D  L
TTGGAAATTTCTGCCTCCCCTGGCCAAAGTGAAGCTCTCCGCGCCTTGGAAGTTACCATC 360
L  E  I  S  A  S  P  G  Q  S  E  A  L  R  A  L  E  V  T  I
GTCCGTGCATGGTGATGTTGTCCCTTGGCGTTATCCGGCCAGACGGGAACTGCAATTCGG 420
V  R  A  W  *  (3' end of ORF)
GGAGTGGCAGCGTAAGGACATTCTTGGCGGGCATCTTCGAGCCCGCCACAACCGATGTTG 480

ATCTTGGCTATTCTGGCTTAACTTAAAGTAAGCAACATAAGCCTTGCATTTGCAGGGTCG 540
```

Figure 3.11 [A]. Partial nucleotide sequence of CS-PCR product of TR 52 showing ORF and the putative translated product (* indicate stop codon).

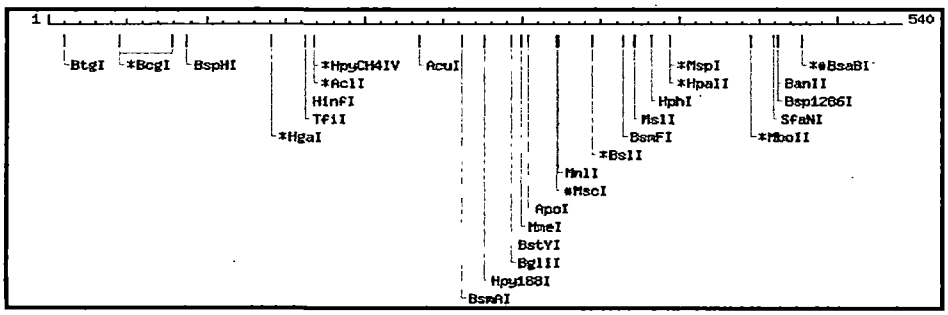
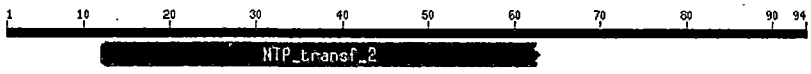


Figure 3.11 [B]. Restriction site analysis of the partial sequence generated from cloned CS-PCR product of TR 52. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
          ....,....1....,....2....,....3....,....4....,....5....,....6
AA        MSNAVPAEISVQLSLALNAIERHLESTLLAVHLYGSALDGGLKPYSDIDLLVTVAARLDE
PHD_sec         HHHHHHHHHHHHHHHHHH    EEEEEEE              EEEEE     H

          ....,....7....,....8....,....9....
AA        TVRQALVVDLLEISASPGQSEALRALEVTIVRAW
PHD_sec HHHHHHHHHHHH            EEEEEEEE
```

Figure 3.11 [C]. Predicted secondary structure of the putative translated product of ORF of TR 52 amplicon

Result of conserve domain search for the putative translated product obtained from ORF of TR 52



gnl|CDD|25840 pfam01909, NTP_transf_2, Nucleotidyltransferase domain. Members of this family belong to a large family of nucleotidyltransferases. This family includes kanamycin nucleotidyltransferase (KNTase) which is a plasmid-coded enzyme responsible for some types of bacterial resistance to aminoglycosides. KNTase in-activates antibiotics by catalysing the addition of a nucleotidyl group onto the drug..

CD-Length = 94 residues, only 53.2% aligned
Score = 38.2 bits (88), Expect = 2e-04

```
Query:  12  QLSLALNAIERHLESTLLAVHLYGSALDGGLKPYSDIDLLVTVAARLDETVR  63
Sbjct:  4   ILSKIKEALKELYG--VAEVYLFGSYARGTYLPGSDIDLLVVVPDPVELFLE  53
```

```
GCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGC 60

CCTAAAACAAAGTTAACCTCTGAGGAAGAATTGTGAAACTATCACTAATGGTAGCTATAT  120
                                  V  K  L  S  L  M  V  A  I  S
CGAAGAATGGAGTTATCGGGAATGGCCCTGATATTCCATGGAGTGCCAAAGGTGAACAGC  180
  K  N  G  V  I  G  N  G  P  D  I  P  W  S  A  K  G  E  Q  L
TCCTGTTTAAAGCTATTACCTATAACCAATGGCTGTTGGTTGGACGCAAGACTTTTGAAT  240
  L  F  K  A  I  T  Y  N  Q  W  L  L  V  G  R  K  T  F  E  S
CAATGGGAGCATTACCCAACCGAAAGTATGCGGTCGTAACACGTTCAAGTTTTACATCTG  300
  M  G  A  L  P  N  R  K  Y  A  V  V  T  R  S  S  F  T  S  D
ACAATGAGAACGTATTGATCTTTCCATCAATTAAAGATGCTTTAACCAACCTAAAGAAAA  360
  N  E  N  V  L  I  F  P  S  I  K  D  A  L  T  N  L  K  K  I
TAACGGATCATGTCATTGTTTCAGGTGGTGGGGAGATATACAAAAGCCTGATCGATCAAG  420
  T  D  H  V  I  V  S  G  G  G  E  I  Y  K  S  L  I  D  Q  V
TAGATACACTACATATATCTACAATAGACATCGAGCCGGAAGGTGATGTTTACTTTCCTG  480
  D  T  L  H  I  S  T  I  D  I  E  P  E  G  D  V  Y  F  P  E
AAATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGACTTCGCCTCTAACATAAATT     537
  I  P  S  N  F  R  P  V  F  T  Q  D  F  A  S  N  I  N
```

Figure 3.12 [A]. Partial nucleotide sequence of CS-PCR product of TR 5801 showing ORF and the putative translated product.

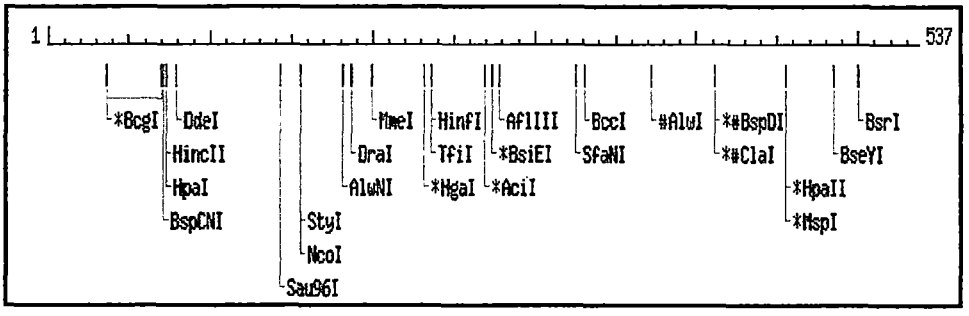


Figure 3.12 [B]. Restriction site analysis of the partial sequence generated from cloned CS-PCR product of TR 5801. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
GGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAACAGTACTTGCCAAGCGAT 60

TTAGCGCAAGAGTCCGTCACTCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTAT 120
                                  M  L  N  G  E  P  I  G  Y
GCCCAGTCGTACGTTGCTCTTGGAAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCA 180
A  Q  S  Y  V  A  L  G  S  G  D  G  W  W  E  E  E  T  D  P
GGAGTACGCGGAATAGACCAGTTACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGA 240
G  V  R  G  I  D  Q  L  L  A  N  A  S  Q  L  G  K  G  L  G
ACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAGATC 300
T  K  L  V  R  A  L  V  E  L  L  F  N  D  P  E  V  T  K  I
CAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCTACGAGAAAGCGGGGTTT 360
Q  T  D  P  S  P  S  N  L  R  A  I  R  C  Y  E  K  A  G  F
GAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGC 420
E  R  Q  G  T  V  T  T  P  D  G  P  A  V  Y  M  V  Q  T  R
CAGGCATTCGAGCGAACACGCAGTGATGCCTATCCCTTCCATCGAGGGGGACGTCCAAGG 480
Q  A  F  E  R  T  R  S  D  A  Y  P  F  H  R  G  G  R  P  R
GCTGGCGCCCTTGGCGGCCCCTCATGTCAAACGCTAGATGCACTAAGCACATAATTGCTC 540
A  G  A  L  G  G  P  S  C  Q  T  L  D  A  L  S  T  *(3'end of ORF)
ACAGCCAAACTATCAGGTCAAGTCTGCTTA
```

Figure 3.13 [A]. Partial nucleotide sequence of CS-PCR product of TR 5802 showing ORF and the putative translated product (* indicate stop codon).

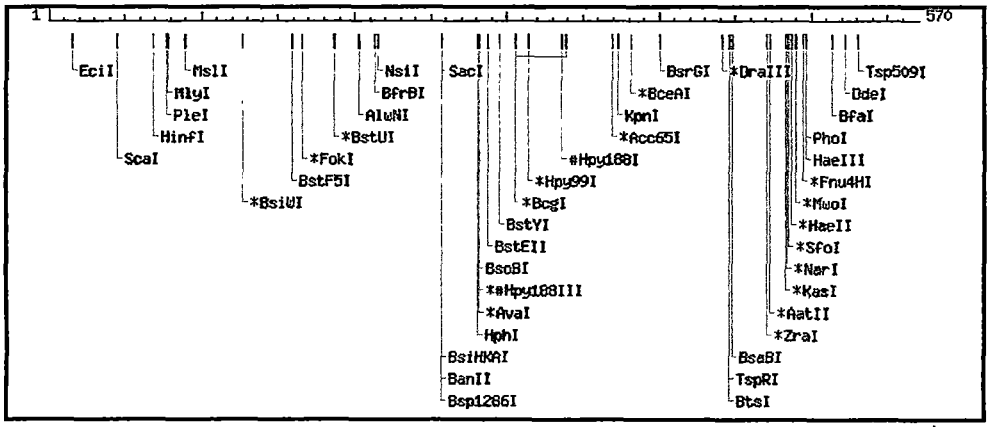


Figure 3.13 [B]. Restriction site analysis of the partial sequence generated from cloned CS-PCR product of TR 5802. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
GTTNGCCGTGCGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAG 60

TCGCCCTAAAACAAAGTTGGGCGAACCCGGAGCCTCATTAATTGTTAGCCGTTAAAATTA 120

AGCCCTTTACCAAACCAATACTTATTATGAAAAACACAATACACAGCATCGTGACCAACA 180
                          M  K  N  T  I  H  S  I  V  T  N  S
GCAACGATTCCGTCACACTGCGCCTCATGACTGAGCATGACCTTGCGATGCTCTATGAGT 240
  N  D  S  V  T  L  R  L  M  T  E  H  D  L  A  M  L  Y  E  W
GGCTAAATCGATCTCATATCGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTG 300
  L  N  R  S  H  I  V  E  W  W  G  G  E  E  A  R  P  T  L  A
CTGACGTACAGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACA 360
  D  V  Q  E  Q  Y  L  P  S  V  L  A  Q  E  S  V  T  P  Y  I
TTGCAATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGGAAGCG 420
  A  M  L  N  G  E  P  I  G  Y  A  Q  S  Y  V  A  L  G  S  G
GGGACGGNNTGTGGGAAGAAGATACCGATCCAGGAGTACGCGGAATAGACCAGTTTATGG 480
  D  G  X  W  E  E  D  T  D  P  G  V  R  G  I  D  Q  F  M  A
CGAATGCATCACAACTGGGCAAAGGCTTGGGAACCAAGTTGGTTCGAGCTCTGGTTGAGT 540
  N  A  S  Q  L  G  K  G  L  G  T  K  L  V  R  A  L  V  E  L
TGCTGTTCAATGATCCCGAGGTCACCAAGATCCAAACGGACCCGTCGCCGAGCAACTTGC 600
  L  F  N  D  P  E  V  T  K  I  Q  T  D  P  S  P  S  N  L  R
GAGCGATCCGATGCTACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAG 660
  A  I  R  C  Y  E  K  A  G  F  E  R  Q  G  T  V  T  T  P  D
ATGGTCCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAGTGATG 720
  G  P  A  V  Y  M  V  Q  T  R  Q  A  F  E  R  T  R  S  D  A
CCTAACCCTTCCATCGAGGGGGACGTCCAAGGGCTGGCGCCCTTGGCCGCCCCTCATGTC 780
  *(3'end of ORF)
AAACGTTAGATGCAGTAAGCACATAATTGTA                               811
```

Figure 3.14 [A]. Complete nucleotide sequence of CS-PCR product of TR 59 showing ORF and the putative translated product (* indicate stop codon).

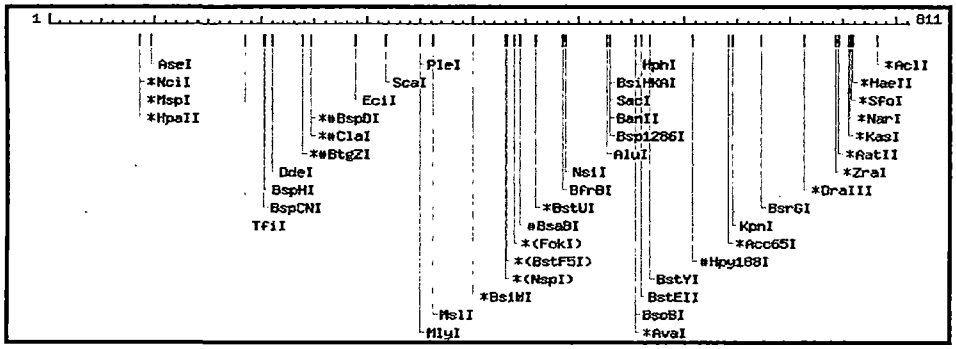
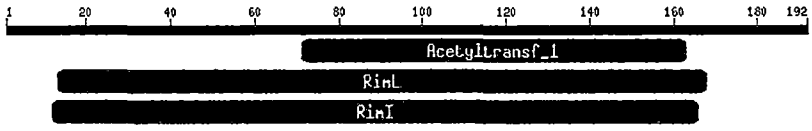


Figure 3.14 [B]. Restriction site analysis of the complete nucleotide sequence generated from cloned CS-PCR product of TR 59. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
          ....,....1....,....2....,....3....,....4....,....5....,....6
AA        MKNTIHSIVTNSNDSVTLRLMTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLP
PHD_sec       EEEEE      EEEEEE      HHHHHHHH                  HHHHHHHHHH

          ....,....7....,....8....,....9....,....10.1.,....11.1.,....12.1
AA        SVLAQESVTPYIAMLNGEPIGYAQSYVALGSGDGXWEEDTDPGVRGIDQFMANASQLGKGLGT
PHD_sec   HH       EEEEEEE    EEEEEEEEEEE              EEEEEEEEE        HHH

          .,....13.1.,....14.1.,....15.1.,....16.1.,....17.1.,....18.1
AA        KLVRALVELLFNDPEVTKIQTDPSPSNLRAIRCYEKAGFERQGTVTTPDGPAVYMVQTRQ
PHD_sec   HHHHHH     EEEEEE     HHHHHHHHH   EEE EEE     EEEEEE  HHHH

          .,....19.
AA        SDA
PHD_sec   HHH
```

Figure 3.14 [C]. Predicted secondary structure of the putative translated product of ORF of TR 59 amplicon

Result of conserve domain search for the putative translated product obtained from ORF of TR 59



gnl|CDD|25558 pfam00583, Acetyltransf_1, Acetyltransferase (GNAT) family. This family contains proteins with N-acetyltransferase functions..

CD-Length = 82 residues, 100.0% aligned
Score = 58.0 bits (140), Expect = 7e-10

```
Query:  71   YIAMLNGEPIGYAQSYVALGSGDGXWEED--TDPGVRGIDQFMANASQLGKGLGTKLVRA  128
Sbjct:  1    LVAEEDGELVGFASLRPIDEEGNVAEIEGLAVDPEYRG-----------KGIGTALLEA  48

Query:  129  LVELLFNDPEVTKIQTDPSPSNLRAIRCYEKAGFE  163
Sbjct:  49   LLEYAR-ELGLKRIELEVLEDNEAAIALYEKLGFK  82
```

gnl|CDD|11381 COG1670, RimL, Acetyltransferases, including N-acetylases of ribosomal proteins [Translation, ribosomal structure and biogenesis].

CD-Length = 187 residues, 81.8% aligned
Score = 69.2 bits (168), Expect = 3e-13

```
Query:  13   NDSVTLRLMTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLPSVLAQESVTPYI  72
Sbjct:  7    TLRLLLREVDLEDLELLAEWANDPEVMLFWWLPPPLTPPTSDEE--LLRLLAEAWEDLGG  64

Query:  73   AMLNGEPIGYAQSYVALGSGDGXWEEDTDPGVRGIDQFMANASQLGKGLGTKLVRALVEL  132
Sbjct:  65   GAFAIELKATGDGELIGVIGLSDIDRAANGDLAEIGYWLDPEYW-GKGYATEALRALLDY  123

Query:  133  LFNDPEVTKIQTDPSPSNLRAIRCYEKAGFERQGTV  168
Sbjct:  124  AFEELGLHRIEATVDPENEASIRVYEKLGFRLEGEL  159
```

gnl|CDD|10329 COG0456, RimI, Acetyltransferases [General function prediction only].

CD-Length = 177 residues, 82.5% aligned
Score = 47.3 bits (111), Expect = 1e-06

```
Query:  12   SNDSVTLRLMTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLPSVLAQESVTPY  71
Sbjct:  8    SEDKVTIREAINKDLLDVALAALEARTFDIRLPWSREYFEKDLTQAPELLLVAETG--GL  65

Query:  72   IAMLNGEPIGYAQSYVALGSGDGXWEED-----TDPGVRGIDQFMANASQLGKGLGTKLV  126
Sbjct:  66   DGLLDGKVVGFLLVRVVDGRPSADHEGHIYNLAVDPEYRG-----------RGIGRALL  113

Query:  127  RALVELLFNDPEVTKIQTDPSPSNLRAIRCYEKAGFERQG  166
Sbjct:  114  DEALERLRERGLADKIVLEVRESNEAAIGLYRKLGFEVVK  153
```

```
GCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGC 60

CCTAAAACAAAGTTGGGCGAACCCGGAGCCTCATTAATTGTTAGCCGTTAAAATTAAGCC 120

CTTTACCAAACCAATACTTATTATGAAAAACACAATACACAGCATCGTGACCAACAGCAA 180
                      M  K  N  T  I  H  S  I  V  T  N  S  N
CGATTCCGTCACACTGCGCCTCATGACTGAGCATGACCTTGCGATGCTCTATGAGTGGCT 240
 D  S  V  T  L  R  L  M  T  E  H  D  L  A  M  L  Y  E  W  L
AAATCGATCTCATATCGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGA 300
 N  R  S  H  I  V  E  W  W  G  G  E  E  A  R  P  T  L  A  D
CGTACAGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGC 360
 V  Q  E  Q  Y  L  P  S  V  L  A  Q  E  S  V  T  P  Y  I  A
AATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGGAAGCGGGGA 420
 M  L  N  G  E  P  I  G  Y  A  Q  S  Y  V  A  L  G  S  G  D
CGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAATAGACCAGTTACTGGCGAA 480
 G  W  W  E  E  E  T  D  P  G  V  R  G  I  D  Q  L  L  A  N
TGCATCACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCT 540
 A  S  Q  L  G  K  G  L  G  T  K  L  V  R  A  L  V  E  L  L
GTTCAATGATCCCGAGGTCACCA                                      563
 F  N  D  P  E  V  T
```

Figure 3.15 [A]. Partial nucleotide sequence of CS-PCR product of TR 63 showing ORF and the putative translated product.

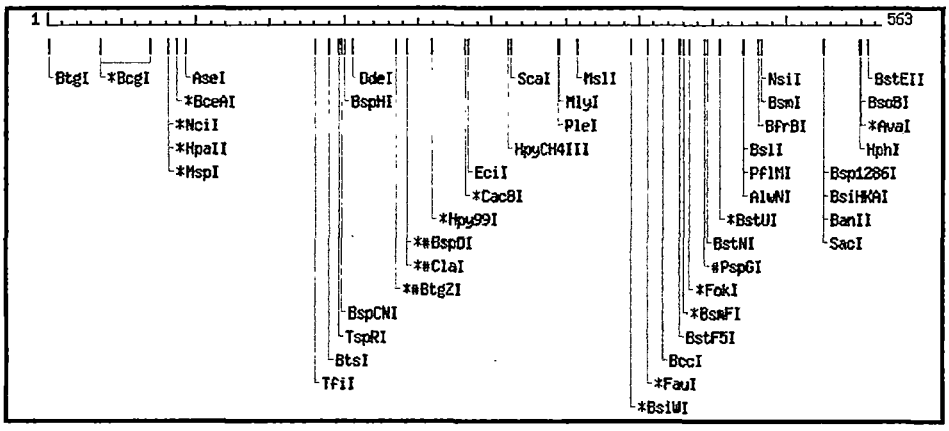


Figure 3.15 [B]. Restriction site analysis of the partial sequence generated from cloned CS-PCR product of TR 63. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
NCGCAGCAGGGCAGTCGCCCTAAAAACAAAGTTAACCCGGAACCAAAATTATGAAAGTAT 60
                                                      M  K  V  S
CATTAATGGCTGCAAAAGCGAAAAACGGAGTGATTGGTTGCGGTCCACACATACCCTGGT 120
  L  M  A  A  K  A  K  N  G  V  I  G  C  G  P  H  I  P  W  S
CCGCGAAAGGAGAGCAGCTACTCTTTAAAGCCTTGACGTACAACCAGTGGCTTTTGGTGG 180
  A  K  G  E  Q  L  L  F  K  A  L  T  Y  N  Q  W  L  L  V  G
GCCGCAAGACGTTCGAATCTATGGGAGCACTCCCTAATAGGAAATACGCGGTCGTTACTC 240
  R  K  T  F  E  S  M  G  A  L  P  N  R  K  Y  A  V  V  T  R
GCTCAGCCTGGACGGCCGATAATGACAACGTAATAGTATTCCCGTCGATCGAAGAGGCCA 300
  S  A  W  T  A  D  N  D  N  V  I  V  F  P  S  I  E  E  A  M
TGTACGGGCTGGCTGAACTCACCGATCACGTTATAGTGTCTGGTGGCGGGGAGATTTACA 360
  Y  G  L  A  E  L  T  D  H  V  I  V  S  G  G  G  E  I  Y  R
GAGAAACATTGCCCATGGCCTCTACGCTCCATATATCGACGATTGATATTGAGCCGGAAG 420
  E  T  L  P  M  A  S  T  L  H  I  S  T  I  D  I  E  P  E  G
GAGATGTTTTCTTTCCGAATATTCCCAATACCTTCGAAGTTGTTTTTGAGCAACACTTTA 480
  D  V  F  F  P  N  I  P  N  T  F  E  V  V  F  E  Q  H  F  S
GCTCAAACATTAACTATTGCTATCAAATTTGGCAAAAGGGTTAACAAAGCTATGCAATTG 540
  S  N  I  N  Y  C  Y  Q  I  W  Q  K  G  *(3'end of ORF)
ACGGTAAAAAGCTTCGTTCGCTTCGCTTGCTACGCTTCTTACCGCAATTGATAACGGCGT 600

TAGATGCACTAAGCACATAAT                                         621
```

Figure 3.16 [A]. Partial nucleotide sequence of CS-PCR product of TR 85 showing ORF and the putative translated product (* indicate stop codon).

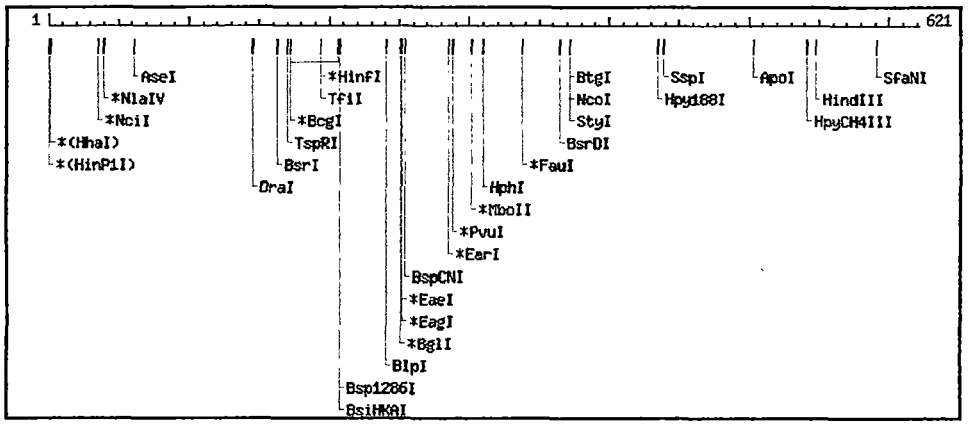


Figure 3.16 [B]. Restriction site analysis of the partial sequence generated from cloned CS-PCR product of TR 85. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
GTTNGACGTGTGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAG 60

TCGCCCTAAAACAAAGTTAACCTCTGAGGAAGAATTGTGAAACTATCACTAATGGTAGCT 120
                                        V  K  L  S  L  M  V  A
ATATCGAAGAATGGAGTTATCGGGAATGGCCCTGATATTCCATGGAGTGCCAAAGGTGAA 180
I  S  K  N  G  V  I  G  N  G  P  D  I  P  W  S  A  K  G  E
CAGCTCCTGTTTAAAGCTATTACCTATAACCAATGGCTGTTGGTTGGACGCAAGACTTTT 240
Q  L  L  F  K  A  I  T  Y  N  Q  W  L  L  V  G  R  K  T  F
GAATCAATGGGAGCATTACCCAACCGAAAGTATGCGGTCGTAACACGTTCAAGTTTTACA 300
E  S  M  G  A  L  P  N  R  K  Y  A  V  V  T  R  S  S  F  T
TCTGACAATGAGAACGTATTGATCTTTCCATCAATTAAAGATGCTTTAACCAACCTAAAG 360
S  D  N  E  N  V  L  I  F  P  S  I  K  D  A  L  T  N  L  K
AAAATAACGGATCATGTCATTGTTTCAGGTGGTGGGGAGATATACAAAAGCCTGATCGAT 420
K  I  T  D  H  V  I  V  S  G  G  G  E  I  Y  K  S  L  I  D
CAAGTAGATACACTACATATATCTACAATAGACATCGAGCCGGAAGGTGATGTTTACTTT 480
Q  V  D  T  L  H  I  S  T  I  D  I  E  P  E  G  D  V  Y  F
CCTGAAATCCCCAGCAATTTAGGCCAGTTTTACCAAGACTTCGCCTCTAACATAAATTAT 540
P  E  I  P  S  N  L  G  Q  F  Y  Q  D  F  A  S  N  I  N  Y
AGTTACAAATCTGGCAAAGGGTTACAAGTGGCAGCAACGGATCGCAACCTGTCACGCCTT 600
S  Y  K  S  G  K  G  L  Q  V  A  A  T  D  R  N  L  S  R  L
TGTACCAAAGCCGCGCCAGGTTGCGATCCGCTGTGCAGCGTTAGGCTACATGAAATCGTA 660
C  T  K  A  A  P  G  C  D  P  L  C  S  V  R  L  H  E  I  V
CATTACGAAGCGAATGCACCATGGATAGGAAGAATGAATGCCCAAACCCAAAGTGTGGGA 720
H  Y  E  A  N  A  P  W  I  G  R  M  N  A  Q  T  Q  S  V  G
                     M  D  R  K  N  E  C  P  N  P  K  C  G  K
AGGAAACTCCTGCCTGGCAATCGAGCGGCATGAGCGACAGTTGCCCGCATTTTTTCTGTG 780
R  K  L  L  P  G  N  R  A  A  *(end of ORF 1)
   E  T  P  A  W  Q  S  S  G  M  S  D  S  C  P  H  F  F  C  D
ATACTTGCTCGAATGTAATCCATAGAGAGCAGGACCATGCATTACTGTATGAAAATGAAA 840
   T  C  S  N  V  I  H  R  E  Q  D  H  A  L  L  Y  E  N  E  I
TCAATCAAGAGCTCTTGGATCGAATAGCAGCAACTCTTCCAGATTGCCCTTGCGGGGGTA 900
   N  Q  E  L  L  D  R  I  A  A  T  L  P  D  C  P  C  G  G  R
GGTTTGTTCCTGGTGCAAACCCAAAGTGTCCGAGTTGCAAGACCGAGTACGTGCACCAAT 960
   F  V  P  G  A  N  P  K  C  P  S  C  K  T  E  Y  V  H  Q  W
GGGATGCAGTGAAAAGGTTGAATGTACCTTTTATGCCAATCTTGGATGGTTCCTGCTTGA 1020
   D  A  V  K  R  L  N  V  P  F  M  P  I  L  D  G  S  C  L  I
TTCGAGATAGGCTGTATTCGTATGAAGTATGCATTGGTTCTAAACCAAAATACTGGTGGC 1080
   R  D  R  L  Y  S  Y  E  V  C  I  G  S  K  P  K  Y  W  W  R
GTTTGTTCACAAATGCCTTAACAAGTTTAGGCAAGGGACGCTCCTGACGTCGCGCCCCTG 1140
   L  F  T  N  A  L  T  S  L  G  K  G  R  S  *  (end of ORF 2)
GTAACACATATTGTNAGATATGCCATATGGTCCAGCA                         1177
```

Figure 3.17 [A]. Complete nucleotide sequence of CS-PCR product of TR 90 showing two ORFs and two putative translated products [the putative DHFR protein and hypothetical protein]. The translated sequence of dfrA1 comprises nucleotides 97 to 753. (* indicate stop codons).

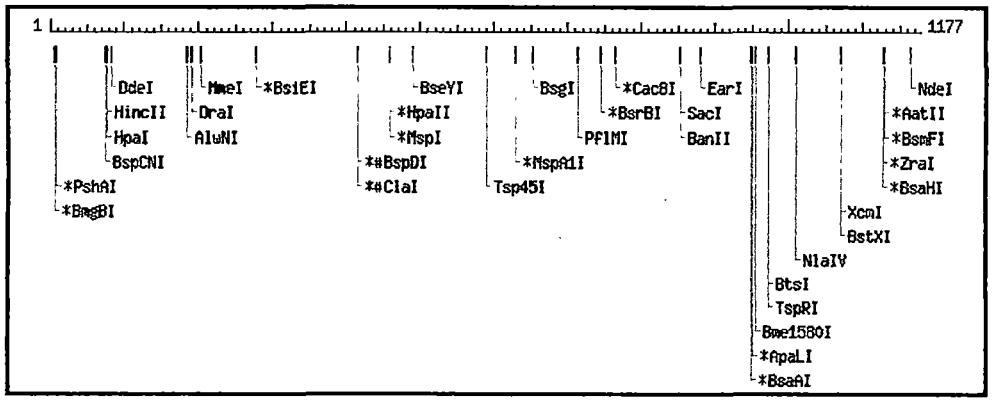
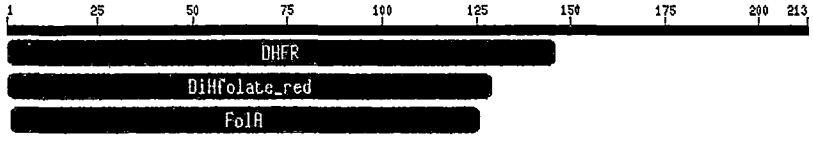


Figure 3.17 [B]. Restriction site analysis of the complete nucleotide sequence generated from cloned CS-PCR product of TR 90. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
         ...,....1...,....2...,....3...,....4...,....5...,....6
AA       MVAISKNGVIGNGPDIPWSAKGEQLLFKAITYNQWLLVGRKTFESMGALPNRKYAVVTRS
PHD_sec   EEEEE              HHHHHHHHHH    EEEEE              EEEEEEE

         ....,....7...,....8...,....9...,....10...,....11...,....12
AA       SFTSDNENVLIFPSIKDALTNLKKITDHVIVSGGGEIYKSLIDQVDTLHISTIDIEPEGDV
PHD_sec          EEEEE HHHHHHHHHH   EEEEE  HHHHHHHHHH EEEEEEEE

         ...,....13...,....14...,....15...,....16...,....17...,....18
AA       YFPEIPSNLGQFYQDFASNINYSYKSGKGLQVAATDRNLSRLCTKAAPGCDPLCSVRLHE
PHD_sec            EEEEE       EEEE   EEEE     HHHHEEE          EEEEEE

         ...,....19...,....20...,....21...,....22
AA       IVHYEANAPWIGRMNAQTQSVGRKLLPGNRAA
PHD_sec  EEEEEE  HHHH      HHH
```

Figure 3.17 [C]. Predicted secondary structure of the putative translated product of ORF 1 of TR 90 amplicon

Result of conserve domain search for the putative translated product obtained from ORF 1 of TR 90




```
gnl|CDD|27775 cd00209, DHFR, Dihydrofolate reductase (DHFR).
              CD-Length = 158 residues,  94.9% aligned
              Score =  152 bits (385), Expect = 3e-38
Query:  1     MVAISKNGVIGNGPDIPWSAKGEQLLFKAITYNQWLLVGRKTFESMG--ALPNRKYAVVT  58
Sbjct:  4     IVAVDENGVIGKDNKLPWHLPEDLKHFKKTTTGNPVIMGRKTFESIPRRPLPGRTNIVLS  63

Query:  59    RSSFTSDNENVLIFPSIKDALTNLKKITDHVIVSGGGEIYKSLIDQVDTLHISTIDIEPE  118
Sbjct:  64    RQLDYQDAEGVEVVHSLEEALELAENTVEEIFVIGGAEIYKQALPYADRLYLTRIHAEFE  123

Query:  119  GDVYFPEIPSNLGQ--FYQDFASNINYSYK  146
Sbjct:  124   GDTFFPEIDESEWELVSEEEVFEEDGYSYT  153


gnl|CDD|22940 pfam00186, DiHfolate_red, Dihydrofolate reductase..
              CD-Length = 173 residues,  81.5% aligned
              Score =  133 bits (335), Expect = 2e-32
Query:  1     MVAISKNGVIGNGPDIPWSAKGEQLLFKAITYNQW---LLVGRKTFESMGA----LPNRK  53
Sbjct:  5     IVAVDKNGGIGKDGDLPWRLPNDLKYFKAVTTGTPRNAVIMGRKTWESIPEKFRPLPGRL  64

Query:  54    YAVVTRS-SFTSDNENVLIFPSIKDALTNLKKIT----DHVIVSGGGEIYKSLIDQVDTL  108
Sbjct:  65    NIVLSRSEDYDAQGDNVVVSSSIEAALDLLAEPPEASIERVFVIGGAQLYAAALPLADRL  124

Query:  109  HISTIDIEPEGDVYFPEIPSN  129
Sbjct:  125  YLTRIDGEFEGDTFFPEIDAD  145


gnl|CDD|10137 COG0262, FolA, Dihydrofolate reductase [Coenzyme metabolism].
              CD-Length = 167 residues,  79.6% aligned
              Score =  108 bits (272), Expect = 4e-25
Query:  2     VAISKNGVIGNGPDIPWSAKGEQLLFKAITYNQWLLVGRKTFESMGA----LPNRKYAVV  57
Sbjct:  6     VAVSLDGVIGRDNSLPWHLPEDLAHFKATTLGKPVIMGRKTYESLPGEWRPLPGRKNIVL  65

Query:  58    TRSSFTSDNENVLIFPSIKDALTNLKKITD-HVIVSGGGEIYKSLIDQ--VDTLHISTID  114
Sbjct:  66    SRNPDLKTEGGVEVVDSIEEALLLLKEEGEDIFIIGGGELYRQFLPAGLADELILTIIP  125

Query:  115  IE-PEGDVYFPEI  126
Sbjct:  126  VLLGEGDTLFPEG  138
```

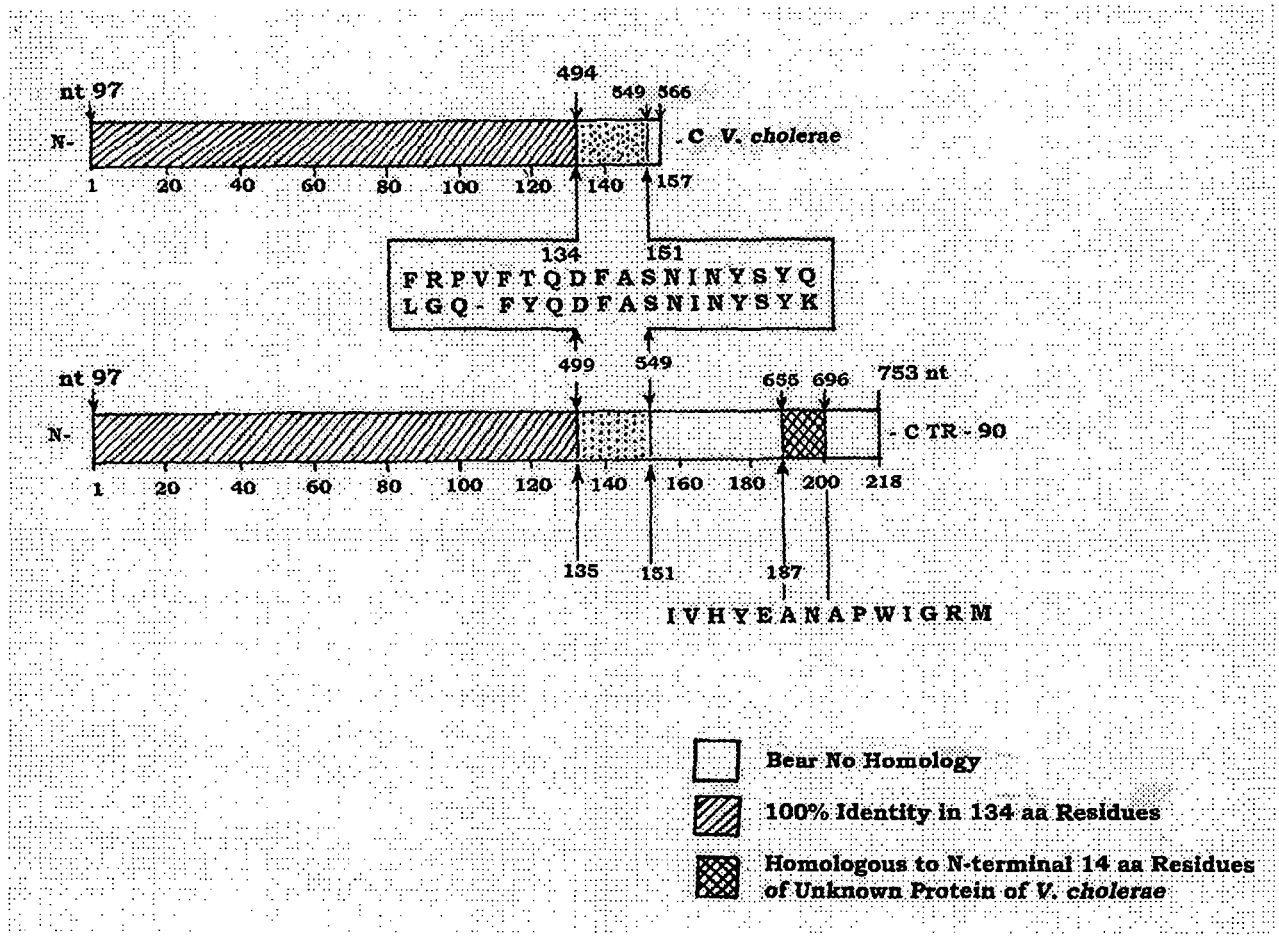


Figure 18A. Comparative analysis of putative peptide sequences of dihydrofolate reductase type I of *V. cholerae* and *Morganella* sp. TR 90.

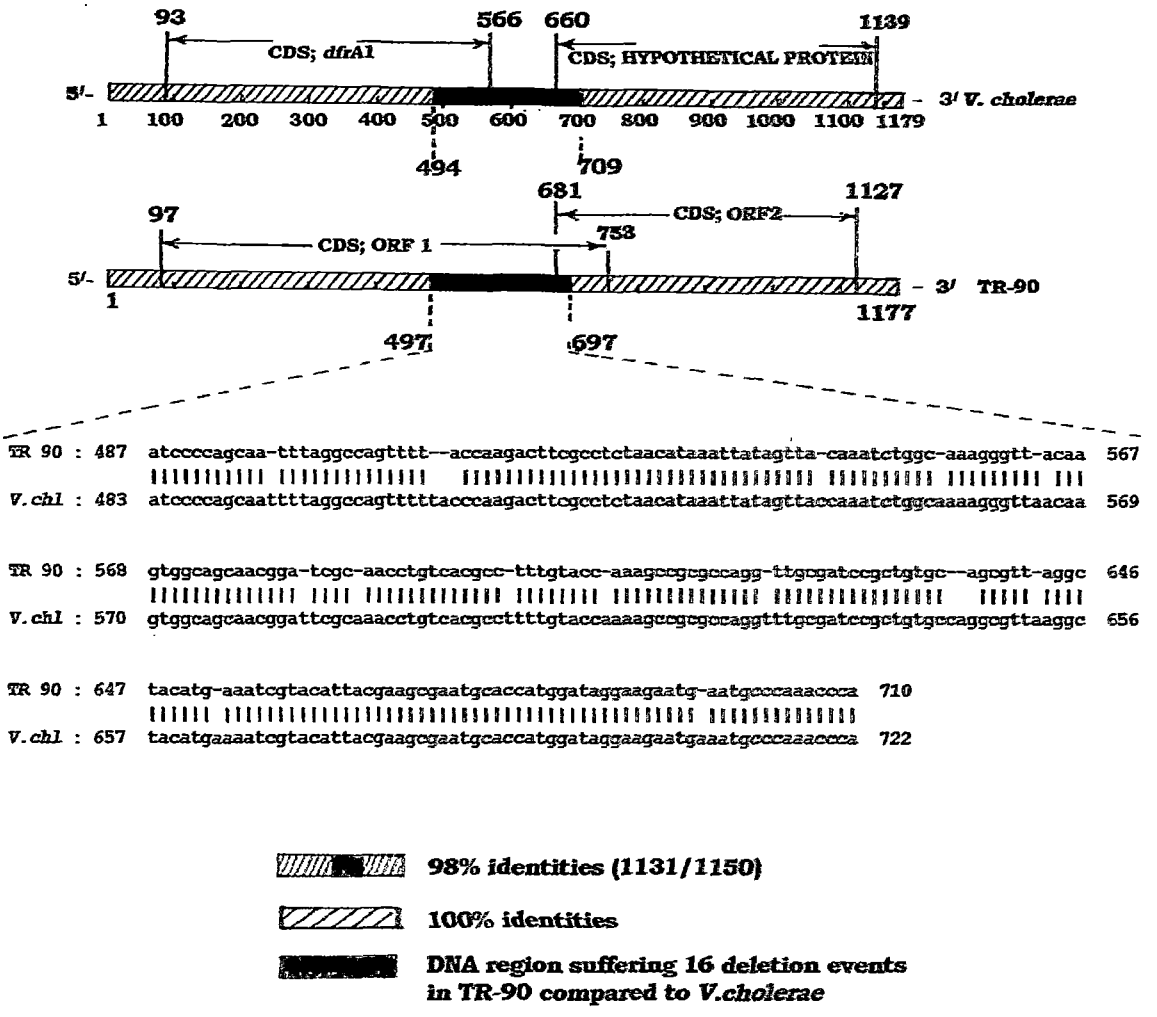


Figure 18B. Probable genesis of dfrA1 ORF in TR 90
A comparative analysis of *dfrA1* sequences of *V. cholerae* and *Morganella* sp. TR 90 showing mutations intensive DNA regions

```
GTTACGACGGCGTCGATGTGTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCA 60

GTCGCCCTAAAACAAAGTTAGCCATTACGGGGGTTGAATTGAAAATTTCATTGATTTCTG 120

CAACGTCAGAAAATGGCGTAATCGGTAATGGCCCTGATATCCCATGGTCAGCAAAAGGTG 180

AGCAGTTACTCTTTAAAGCGCTCACATATAATCAGTGGCTCCTTGTGGGAAGGAAACATT 240

TGACTCTATGGGTGTTCTTCCAAATCGAAAATATGCAGTAGTGTCGAGGAAAGGAATTTC 300
      M  G  V  L  P  N  R  K  Y  A  V  V  S  R  K  G  I  S
AAGCTCAAATGAAAATGTATTAGTCTTTCCTTCAATAGAAATCGCTTTGCAAGAACTATC 360
 S  S  N  E  N  V  L  V  F  P  S  I  E  I  A  L  Q  E  L  S
GAAAATTACAGATCATTTATATGTCTCTGGTGGCGGTCAAATCTACAATAGTCTTATTGA 420
 K  I  T  D  H  L  Y  V  S  G  G  G  Q  I  Y  N  S  L  I  E
AAAAGCAGATATAATTCATTTGTCTACTGTTCACGTTGAGGTTGAAGGTGATATCAATTT 480
 K  A  D  I  I  H  L  S  T  V  H  V  E  V  E  G  D  I  N  F
TCCTAAAATTCCAGAGAATTTCAATTTGGTTTTTGAGCAGTTTTTTTTGTCTAATATAAA 540
 P  K  I  P  E  N  F  N  L  V  F  E  Q  F  F  L  S  N  I  N
TTACACATATCAGATTTGGAAAAAAGGCTAACAAGTCGTTCCAGCACCAGTCGCTGCGCT 600
 Y  T  Y  Q  I  W  K  K  G  *
CCTTGGACAGTTTTTAAGTCGCGGTTTTATGGTTTTGCTGCGCAAAAGTATTCCATAAAA 660

CCACAACTTAAAAACTGCCGCTNAACTCGGCGTTAGATGCACTAAACACATAATTGGC   718
```

Figure 3.19 [A]. Complete nucleotide sequence of CS-PCR product of TR 95 showing ORF and the putative translated product (* indicate stop codon).

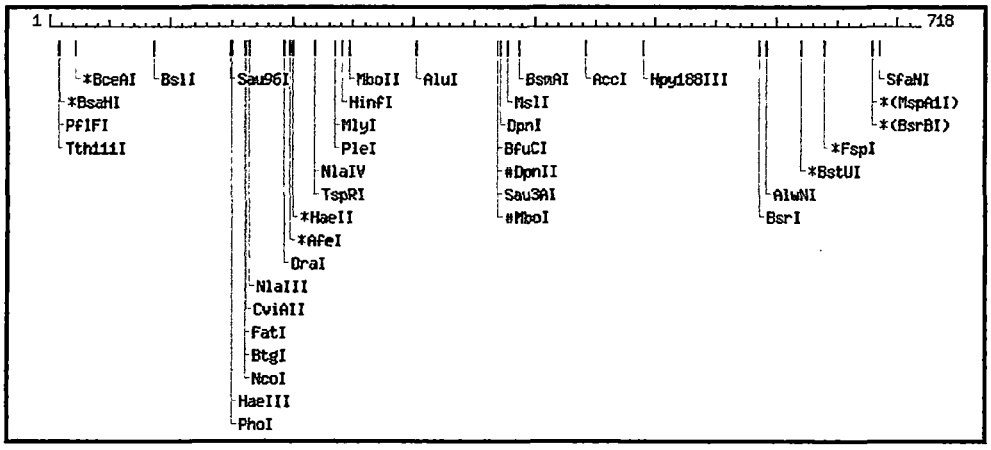
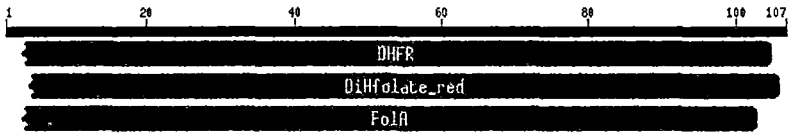


Figure 3.19 [B]. Restriction site analysis of the complete nucleotide sequence generated from cloned CS-PCR product of TR 95. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

```
        ....,....1....,....2....,....3....,....4....,....5....,....6
AA      MGVLPNRKYAVVSRKGISSSNENVLVFPSIEIALQELSKITDHLYVSGGGQIYNSLIEKA
PHD_sec         EEEEEE           EEEE  HHHHHHHHH    EEEEE  HHHHHHHH

        ....,....7....,....8....,....9....,....10.1.,..
AA      DIIHLSTVHVEVEGDINFPKIPENFNLVFEQFFLSNINYTYQIWKKG
PHD_sec  EEEEEEEEEE              EEEEEEEEE  EEEEEEEEE
```

Figure 3.19 [C]. Predicted secondary structure of the putative translated product of ORF of TR 95 amplicon

Result of conserve domain search for the putative translated product obtained from ORF of TR 95



gnl|CDD|27775 cd00209, DHFR, Dihydrofolate reductase (DHFR).
CD-Length = 158 residues, only 67.1% aligned
Score = 87.1 bits (216), Expect = 4e-19

```
Query:  3    VLPNRKYAVVSRKGISSSNENVLVFPSIEIALQELSKITDHLYVSGGGQIYNSLIEKADI  62
Sbjct:  53   PLPGRTNIVLSRQLDYQDAEGVEVVHSLEEALELAENTVEEIFVIGGAEIYKQALPYADR  112

Query:  63   IHLSTVHVEVEGDINFPKI-PENFNLVF--EQFFLSNINYTYQIWK  105
Sbjct:  113  LYLTRIHAEFEGDTFFPEIDESEWELVSEEEVFEEDGYSYTFETYE  158
```

gnl|CDD|22940 pfam00186, DiHfolate_red, Dihydrofolate reductase..
CD-Length = 173 residues, only 65.9% aligned
Score = 96.5 bits (240), Expect = 6e-22

```
Query:  4    LPNRKYAVVSRKGISSSNE-NVLVFPSIEIALQELSKIT----DHLYVSGGGQIYNSLIE  58
Sbjct:  60   LPGRLNIVLSRSEDYDAQGDNVVVSSSIEAALDLLAEPPEASIERVFVIGGAQLYAAALP  119

Query:  59   KADIIHLSTVHVEVEGDINFPKIP-ENFNLVFEQFFL-----SNINYTYQIWKK  106
Sbjct:  120  LADRLYLTRIDGEFEGDTFFPEIDADDWELVSSSEGVEEDKDNGYEYTFETWVK  173
```

gnl|CDD|10137 COG0262, FolA, Dihydrofolate reductase [Coenzyme metabolism].
CD-Length = 167 residues, only 63.5% aligned
Score = 60.7 bits (147), Expect = 4e-11

```
Query:  3    VLPNRKYAVVSRKGISSSNENVLVFPSIEIALQELSKITD-HLYVSGGGQIYNSLIE--K  59
Sbjct:  56   PLPGRKNIVLSRNPDLKTEGGVEVVDSIEEALLLLKEEGEDIFIIGGGELYRQFLPAGL  115

Query:  60   ADIIHLSTVHVEV-EGDINFPKI-PENFNLVFEQFFLSNINYTYQI  103
Sbjct:  116  ADELILTIIPVLLGEGDTLFPEGDPADWELVSSEDADEKGGYFYTF  161
```

```
GACCTTTGAAACTTCGGCTTCCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCAC 60
      E  T  S  A  S  P  G  E  S  E  I  L  R  A  V  E  V  T
CATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATT 120
 I  V  V  H  D  D  I  I  P  W  R  Y  P  A  K  R  E  L  Q  F
TGGAGAATGGCTGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACAT 180
 G  E  W  L  R  N  D  I  L  A  G  I  F  E  P  A  T  I  D  I
TGATCTGGCTATCTTGCTGTCAAAAGCAAGAGGACATAGCGTTGCCTTGGTAGGTCCAGC 240
 D  L  A  I  L  L  S  K  A  R  G  H  S  V  A  L  V  G  P  A
GGCGGAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAAC 300
 A  E  E  L  F  D  P  V  P  E  Q  D  L  F  E  A  L  N  E  T
CTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTAC 360
 L  T  L  W  N  S  P  P  D  W  A  G  D  E  R  N  V  V  L  T
GTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGCTGC 420
 L  S  R  I  W  Y  S  A  V  T  G  K  I  A  P  K  D  V  A  A
CGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACA 480
 D  W  A  M  E  R  L  P  A  Q  Y  Q  P  V  I  L  E  A  R  Q
GGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATT 540
 A  Y  L  G  Q  E  E  D  R  L  A  S  R  A  D  Q  L  E  E  F
TGTTCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCTAACAATTCG 600
 V  H  Y  V  K  G  E  I  T  K  V  V  G  K  *
TTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTAAAGCGTTAGATGCACTAAGCACAT 660

AAGTGCACCAGCC                                                  673
```

Figure 3.20 [A]. Partial nucleotide sequence of CS-PCR product of TR 97 showing ORF and the putative translated product (* indicate stop codon).

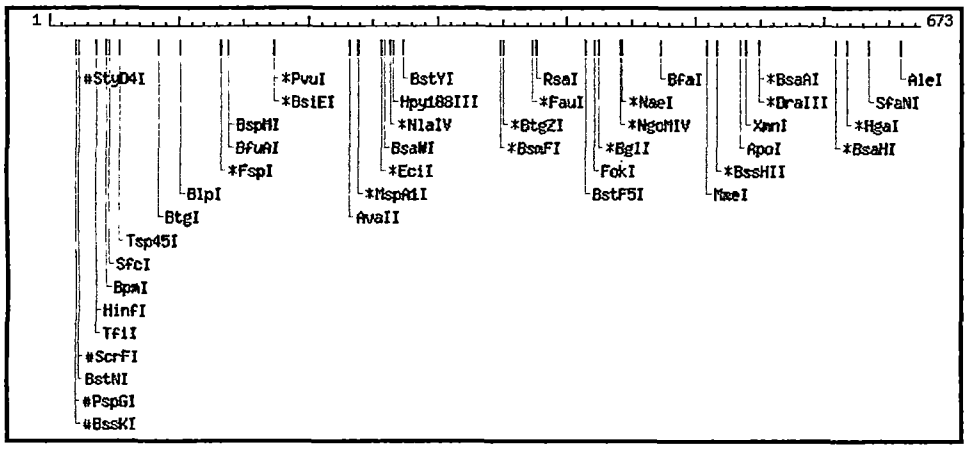


Figure 3.20 [B]. Restriction site analysis of the partial sequence generated from cloned CS-PCR product of TR 97. #, cleavage affected by CpG methylation; and *, cleavage affected by other methylations

Box 3.1. Mutational events due to replication slippage

490 520

1. ***V.cholerae***: 5′ G**CAA|T|TTTA**GGC**CAGTTTT|T|A|**CCC*AAGACTT* 3′

TR 90: 5′ G**CAATTTA**GGC**CAG**T**TTTA**CC*AAGACTT* 3′

494 521

540 571

2. ***V.cholerae***: 5′ A**GTTA|C|CAAA**TCTGG**C|A|AAA**GG**GTT|A|ACAA**GT 3′

TR 90: 5′ A**GTTACAAA**TCTGG**CAAA**GG**GTTACAA**GT 3′[direct repeat]

541 569

581 593

3. ***V.cholerae***: 5′ GGA|T|TCGC|A|AACC 3′

TR 90: 5′ GGATCGCAACC 3′[Quasipalindrome]

579 589

601 619

4. ***V.cholerae***: 5′ C**C|T|TTTG**TAC**C|A|AAAG**CCG 3′

TR 90: 5′ C**CTTTG**TAC**CAAAG**CCG 3′[inverted repeat]

597 613

640 657

5. ***V.cholerae***: 5′ GTGC|C|A|**g**GCGTT|A|AGGCT 3′

TR 90: 5′ GTGC**a**GCGTTAGGCT 3′[Quasipalindrome]

633 647

659 671

6. ***V.cholerae***: 5′ CATG|A|AAATCGTA 3′

TR 90: 5′ CATGAAATCG 3′

649 660

703 712

7. ***V.cholerae***: 5′ GAATG|A|AATG 3′

TR 90: 5′ GAATGAATG 3′[direct repeat]

692 700

3.4 Discussion

River waters are the main receptacle of urban effluents that contain high levels of antibiotic resistant bacteria. We found that culturable antibiotic resistant bacteria were widespread in non-concentrated water samples from the sampling stations of river Torsa (Chapter 1). It has been known for quite some time that cultivable bacteria represent only a small portion of the vast number of bacteria present in the environment and the number that can be enumerated is higher than can be cultivated. Usually, bacteria from a sample are cultivated first. Nutrient typical for known (pathogenic) bacteria or groups of bacteria with similar nutrient requirements are used for this purpose. That is, bacteria, which are able to grow on the nutrient used, are selected and then isolated. In the second step, these isolates are classified according to their susceptibility to a certain antibiotic or a mixture of antibiotics to detect resistant bacteria or multi-resistant ones. For this purpose, antibiotic concentrations up to the milligram per liter range are used. These concentrations are orders of magnitude higher than the environmental ones. The bacteria identified as resistant are investigated further by monitoring the genetic material encoding the resistance found. It has already been accepted that the input of bacteria already resistant following the use of antibiotics in human and veterinary medicine seems to be more important source of resistance genes in the environment. Therefore, more data are needed with respect to the input of already resistant bacteria into the environment for answering the questions related to the spread of resistance genes in nature. Integron gene sequences have been identified as a primary source of resistance genes within microbial populations.

In this study, the results have limitations and must be considered in light of the fact that many aquatic organisms are probably non-culturable. Another important consideration of the study was the exclusion of the gram-positive organisms. Although gram-positive organisms may be important as reservoir of resistance genes, only those antibiotic-resistant isolates shown to be gram-negative were used for further analysis. The ampicillin resistant isolates were predominantly gram-negative. The resistances of ampicillin resistant isolates to other antibiotics, that too on a rational basis, were performed to select MAR isolates for integron-assay.

Ampicillin-resistant MAR isolates were selected for the detection of class 1 integrons. Antibiotic ampicillin has been found to be the most abused antibiotic of this region (Mukherjee *et al.* 2005). The data on the incidence and recovery of antibiotic resistant bateria from the waters of Torsa, all through the year, revealed maximum frequency of ampicillin-resistant (which have been available therapeutically for a long time) bacteria. Earlier reports have shown that about 90% of the integron-positive bacteria were found resistant to ampicillin (White *et al.* 2001). On the basis of all these observations, it was thought that fishing integrons in ampicillin-resistant isolates would be more effective.

Twelve different antibiotics were used for selecting the MAR isolates. Earlier studies have shown that integrons were significantly associated with resistance to certain antibiotics including gentamicin, kanamycin, streptomycin, tobramycin, sulfafurazole, ampicillin, chloramphenicol, and tetracycline. The association of older antibiotics, ampicillin, chloramphenicol and

tetracycline with the presence of an integron was also likely to be the genetic linkage between integrons and conjugative plasmids and transposons. Amikacin and netilmicin were selected with a rationale that genes conferring resistance to newer antibiotics (modern aminoglycosides such as amikacin and netilmicin) are already part of gene cassettes. Antibiotics like cefotaxim, cephalexin and nitrofurantoin, in earlier studies also, revealed insignificant association with integron carriage.

In this study, 100 gram-negative MAR bacteria from river Torsa were examined and found that 40% contained detectable class 1 integron structure. The proportion of strains in this collection of antibiotic resistant bacteria carrying integrons is comparable to that of other studies. For example, 43-75% of antibiotic-resistant clinical bacteria contained class 1 integrons [Levesque *et al.* 1995, Jones *et al.* 1997, Martinez-Freijo *et al.* 1998, Chang *et al.* 2000, Jones *et al.* 2003], while non-selected gram-negative bacteria from an estuarine environment revealed the presence of *int1* 1 gene among 3.6% of the isolates [Rosser and Young 1999]. A study conducted on Rio Grande River (which separates the United States from Mexico) showed that 10% of the *E. coli* isolates were MAR and 13% of MAR contained class 1 integron sequences [Roe *et al.* 2003]. These results, together with those obtained in the present study, indicate that class 1 integrons are widespread in clinical as well as in environmental samples.

The MAR index of the 100 isolates under study ranged from 0.41-1.0. The rationality behind the criterion of selecting MAR isolates with such MAR indices for detecting the class 1 integrons lay in the fact that calculated MAR index exhibited by 82-100% of the class 1 integron bearing isolates was in the range of 0.4-0.9 [Guerra *et al.* 2000, Mazel *et al.* 2000, Thungapathra *et al.* 2002].

The selected 100 gram-negative MAR isolates were primarily differentiated into Pseudomonads and enteric bacteria by oxidase and glucose fermantation tests. 19 out of 100 isolates were oxidase positive and could not ferment glucose. This study did not find any significant difference in the incidence of integrons among the members of enterobacteriaceae (32 out of 81 MAR) and non-enterobacterial members (8 out of 19 MAR). This is similar to the observations of Rosser and Young [1999] who found no significant difference in the incidence of integrons among the coliforms, *Pseudomonas* spp. and *Vibrio* spp. The presence of class 1 integrons in six *Pseudomonas* spp. and two *Acinetobacter* spp. is significant in the context of gene transfer and dissemination of resistance gene cassettes in the environment. An earlier study on two European rivers expressed clinical concern over the development of drug resistance in mesophilic *Aeromonas* spp. [Goni-Urizza *et al.* 2000]. Among the enteric members, the genus *Citrobacter* represented highest proportion of the integron-positive isolates (8 of 40). All these isolates have been characterized as different strains depending on various biochemical characteristics. This study also revealed the presence of class 1 integron in one *Yersinia* sp. TR 63. Presence of class 1 integrons in *Yersinia enterocolitica* was reported for the first time by Soto *et al.* (2003). This is an enteroinvasive bacterium, mainly associated with enterocolitis and less frequently with wide

variety of clinical and immunologic manifestations. Although in this study the copiotrophs were cultured from river water samples, and were deemed to be environmental samples, it is possible that some of the strains, particularly those identified as coliforms, may have originated from human and animal wastes as fluvial waters receive human and wastewater discharges. The physico-chemical and bacteriological investigation on the Torsa River revealed that the ratio of fecal coliform to fecal streptococci ranged from 1.77 to 3.75 and the ratio of free ammonia to albuminoid ammonia ranged from 42 to 761 in the pre-monsoon months indicating pollution by human and agricultural wastes [Bhadra *et al.* 2003].

The observations made from this study, revealed a significant association between integron carriage and higher MAR index. This confirms the results of the earlier studies where a significant relationship between multiresistance and presence of integrons was found (Martinez Freijo *et al.* 1998, Schmitz *et al.* 2001, Leverstein van Hall *et al.* 2003).

Under the PCR conditions used in this study, the size range of inserted gene cassettes varied between 718 and 3200 bp. The different sizes of the gene cassettes inserted between the CS regions of the isolates demonstrate the variable nature of these structures, presumably reflecting differences in the number and type of inserted gene cassettes similar to the observations made by earlier authors (Schmitz 2001). In the present study, integron positive bacteria having single amplicon were the most predominant, although a few (20%) contained two regions of inserted DNA of different sizes (Table 3.3). Yielding two amplicons of different sizes with primers 5′ CS and 3′ CS (Table 3.3) have also been noted by earlier authors where they have confirmed by sequence analysis the co-existence of two distinct integrons carrying different gene cassettes in such isolates [Chang *et al.* 2000, Peters *et al.* 2001, Leverstein van Hall *et al.* 2002]. Additionally, many inserted regions of DNA, indistinguishable with respect to size, were detected in different isolates, as well as from the different strains of the same isolate, which is suggestive of horizontal gene transfer. PCR aimed at determining the proximity of the inserted gene cassettes to an integrase gene demonstrated some sequence heterogeneity among the 5′ CS regions in some of the isolates under study. Five isolates, TR 18, TR 40, TR 68, TR 82 and TR 99, gave a product with the 5′ CS and 3′ CS primers but no product with Int_2F and 3′ CS primer set. Martinez-Freijo *et al.* also made similar type of observations. They found that ten of the seventy integron-positive isolates gave a product with the 5′ CS and 3′ CS primers but no amplification product with Int_2F and 3′ CS primers. In addition, seven isolates gave a product with the 5′ CS and 3′ CS primers but no product with Int_2F and 3′ CS primer set. Several studies have shown the variable nature of the 3′ CS region of an integron with respect to size and genetic structure (Rosser and Young 1999). This study did not attempt to investigate this.

All the isolates positive for class 1 integrons were resistant to multiple antibiotics. However, as the data showed, the presence of class 1 integrons would account for the resistance to only a few drugs. Similar observations have been previously addressed which led to the obvious conclusion that there exist other determinants responsible for mediating

multi resistance in many of the isolates (Thungapathra *et al.* 2002, Chang *et al.* 2000). In this study *dfr* gene cassettes were most frequently found in the variable region of integrons and was often the only cassette present (Table. 3.4). The *dfrA1, dfrA5, dfrA7, dfrA12* and *dfrA17* gene cassettes were found among the isolates included in this study. Taxonomic identification of these isolates revealed that not a single genus but many different genera were involved in the carriage of these gene cassettes (Table 3.3). The *dfrA17* gene cassette was reported for the first time in an *Escherichia coli* isolate EC107 isolated from a clinical urine specimen in Taiwan (Chang *et al.* 2000). All of these five different *dfr* cassettes (*dfrA1, dfrA5, dfrA7, dfrA12* and *dfrA17*) were identified among the urinary *E. coli* isolates from the last two decades in Korea (Yu *et al.* 2004). According to the results of their study, the *E. coli* isolates that carried *dfrA17* associated with class 1 integrons were found to be phylogenetically unrelated, indicating that *dfrA17* was widely distributed in the different clones of *E.coli*. The prevalence of *dfrA17* was mainly due to the horizontal transfer of class 1 integrons through conjugative plasmids. The *dfrA17* gene cassette characterized in this study was found in *Serratia* sp. and did not produce 100% sequence identity with that of the previously described one from *E.coli*. The observation of this study also revealed the possibility of the spread of *dfrA17* gene cassette through conjugal transfer. Thungapathra *et al.* (2002) reported the presence of *dfrA1, dfrA5, dfrA12* gene cassettes among *Vibrio cholerae* isolates belonging to non-O1 and non-O139 serogroups isolated during 1997 to 1998 in Calcutta, India.

Both adenyl transferase (*aadA1* and *aadA6*) and acetyl transferase gene cassettes (*aac-6'-Ib*) were found to be present at roughly the same frequency. These cassettes have also been reported in various bacterial genera from different regions of the world.

The sequence analysis of the amplicon from *Morganella* sp. TR 90 enabled to characterize a novel ORF of 218 amino acids of which the first 152 amino acids bear 95% identities with the normal 157 amino acid long *dfrA1* protein. The sequence alignment data of *Morganella* sp. TR 90 and *V. cholerae* class 1 integron gene cassettes (AJ698460 vs AF455254) has helped to identify sequence contexts that may be important for promoting specific types of *in vivo* mutations in the gene cassettes leading to an extended version of DFR A1 protein(Figure 18.A) Accumulation and fixation of multiple mutations resulting in sequence divergence can ultimately lead to the emergence of a novel protein. Exquisite and insightful sequence analysis of mutation in genes e (lysozyme), *rII, ac,* and others have shown that many frameshift mutations, base substitutions, deletions or duplication can be explained by STREISINGERs slipped mispairing model (Streisinger *et al.* 1966) and its variations (Drake and Ripley 1994). Slipped mispairing that is potentially mutagenic can occur between simple or complicated repeats, in imperfect palindromes, or between nearly homologous sequences located at considerable distances, i.e, called ectopic sequences (Ripley 1990). It has been demonstrated directly that misaligned bases in potential stem-loop structures with no direct repeats are also hot spots of frameshift mutations (Ripley 1990). Earlier

authors have suggested that bulged mispairing of any single-stranded DNA with any complementary sequence, regardless of the reasons for single strandedness, can generate mispaired heteroduplex and thus be mutagenic (Drake and Ripley 1994).

Several studies have addressed the role of integrons in the spread of antibiotic resistance genes by lateral gene transfer (Leverstein van-Hall *et al.* 2002, Chen *et al.* 2004, Aubert *et al.* 2004). The spread of resistance genes is greatly enhanced following the movement of conjugative plasmids containing integrons among different bacterial species. In this study, class 1 integron borne *dfrA5, dfrA12* and *dfrA17* gene cassettes was found to be located on the conjugative plasmids of some MAR bacterial isolates. These isolates belonged to the genus *Escherichia, Citrobacter* and *Serratia* respectively. The earlier authors have also confirmed the location of class 1 integrons on conjugative plasmids by the similar PCR assay (Chen *et al.* 2004). The horizontal transfer of such plasmids enabled the rapid spread of the gene cassettes among a wide variety of bacterial species (Girlich *et al.* 2001, White *et al.* 2001, Fluit *et al.* 2004). It has also been shown that horizontal transfer of integron-carrying elements play a dominant role in the development of multiresistance by Enterobacteriaceae independent of species or isolate origin (Leverstein van-Hall *et al.* 2003). The diversity of species carrying integrons means that stage suitable for the storage, acquisition, rearrangement and expression of gene cassettes may be prevalent in nature. The same cassettes can spread in different bacterial species, in different human or animal hosts and in different areas of the globe. Such observations emphasize the apprehension that human, animal and environmental microbial ecosystems are inextricably entangled, with antibiotic resistance readily surpassing ecological boundaries and thinning out widely.

3.5. Conclusion

The results of this study revealed that integron structures were prevalent in the gram-negative, multiple-antibiotic-resistant copiotrophic bacterial population of the Torsa river. A similar study from a South African river has been reported recently [Biyela *et al.* 2004]. The present study indicated that River Torsa of India is contaminated with class 1 integron bearing copiotrophic bacterial population and therefore it may act as a reservoir as well as a medium for the spread of bacterial antibiotic resistance genes in the environment. The river water isolates carrying class 1 integron borne gene cassettes in their conjugative plasmids is a significant observation in the context of gene transfer and the dissemination of resistance genes in the environment. The predominance of selected aminoglycoside and trimethoprim resistance gene cassettes may, however, indicate that some gene cassettes are more stably incorporated within the integron structures than others. This study has also demonstrated that integron typing can be a useful tool for studying the dissemination of resistance genes among gram-negative bacteria. The accumulation of resistance genes by integrons is one explanation for the emergences of multiple resistant strains of Enterobacteriaceae and pseudomonads. 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. Since many integrons possess more than one antibiotic resistance-conferring gene cassette and are often located on genetic elements that carry the other resistance determinants, selection for one antimicrobial resistance determinant selects for many. The study of integrons and their associated gene cassettes could provide information about antibiotics, which should be used most carefully to prevent further accumulation of resistance. Further studies are needed, however, to determine whether antibiotic policies or other measures can halt or lower the amount of horizontal transfer in different environmental settings.

3.6 Summary of chapter 3

The presence of class 1 integrons, in multiple- antibiotic- resistant (MAR), gram-negative, copiotrophic bacteria of River Torsa was detected by using a polymerase chain reaction (PCR) based screening method. Of 100 isolates that were resistant to at least five of the twelve antibiotics tested, 40 carried class 1 integrons, with inserted DNA regions of 0.7 to 3.2 kb. Carriage of integrons in strains of higher MAR index was found to be statistically significant. DNA sequencing was used to identify the genetic content of the integron-variable regions. In addition to the identification of gene cassettes, *dfrA1, dfrA5, dfrA7, dfrA17* and a variant of *dfrA12* for trimethoprim; *aac-(6')-Ib* for amikacin and tobramycin; and *aadA1* and *aadA6* for streptomycin and spectinomycin resistance, a novel ORF, predicted from sequence of *Morganella* sp. TR 90, bearing homology with *Vibrio cholerae dfrA1* gene cassette, has been characterized. The residence of class 1 integrons on conjugative R plasmids was confirmed in six integron positive isolates. Gene cassettes identified from the transconjugants were found to be 100% identical in sequence context with the *dfrA5, dfrA12* and *dfrA17* cassettes identified previously from the respective donor isolates. All of these cassettes conferred resistance to trimethoprim. The results of the study has revealed that the waters of river Torsa in West Bengal of India, could eventually become a major reservoir for antibiotic resistant microbes due to dissemination of antibiotic resistance genes carried by class 1 integrons through conjugal transfer.

3.7 References

Adrian, P.V., Plesis, M.D., Klugman, K.P. and Amyes, S.G.B. 1998. New Trimethoprim-resistant Dihydrofolate reductase cassette, *dfr* XV inserted in a class I integron. *Antimicrob. Agents. Chemother.* **42** : 2221-2224.

Ahmed, A. M., T. Nakagawa., E. Arakawa., T. Ramamurthy., S. Shinoda, and T. Shimamoto. 2004. New aminoglycoside acetyltransferase gene, *aac(3')-Id*, in a class 1 integron from a multiresistant strain of *Vibrio fluvalis* isolated from an infant aged 6 months. J. Antimicrob.Chemother. **53** : 947-951.

Altschul, S. F., T. L. Madden., A. A. Schaffer., J. Zhang., Z. Zhang., W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new gwnwration of protein database search programs. Nucleic. Acid. Res. **25** : 3389-3402.

Amita., S. Roy Chowdhury., M. Thungapathra., T. Ramamurthy., G. B. Nair, and A. Ghosh. 2003. Class I integrons and SXT elements in E1 Tor strains isolated before and after 1992 *Vibrio cholerae* O139 outbreak, Calcutta, India. Emerg. Infect. Dis. **9** : 500-502.

Arakawa, Y., M. Murakami., K. Suzuki., H. Ito., R. Wacharotayankun., S. Ohsuka and N. Kato. 1995. A novel integron like element carrying the metallo-beta-lactamase gene bla_{IMP}. Antimicrob. Agents Chemother. **39** : 1612-1615.

Aubert, D., D. Girlich., T. Naas., S. Nagarajan, and P. Nordmann. 2004. Functional and structural characterization of the genetic environment of the extended-spectrum β-lactamase bla_{VEB-1} gene from a *Pseudomonas aeruginosa* isolate obtained in India. Antimicrob. Agents. Chemother. **48** : 3284-3290.

Bairoch, A., P. Bucher, and K. Hofmann. 1997. The PROSITE database, its status in 1997. Nucleic. Acid. Res. **25** : 217-221.

Bass, L., C. A. Liebert., M. D. Lee., A. O. Summers., D. G. White., S. G. Thayer and J. J. Maurer. 1999. Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. Antimicrob. Agents. Chemother. **43** : 2925-2929.

Bhadra, B., S. Mukherjee., R. Chakraborty, and A. K. Nanda. 2003. Physicochemical and bacteriological investigation on the river Torsa of North Bengal. J. Environ. Biol. **24** : 125-133.

Bissonnette, L., S. Champetier., J. P. Buisson, and P. H. Roy. 1991. Characterization of the nonenzymatic chloramphenicol resistance (*cmlA*) gene of the In4 integron of *Tn1696*: similarity of the product to transmembrane transport proteins. J. Bacteriol. **173** : 4493-4502.

Biyela, P. T., J. Lin, and C. C. Bezuidenhout. 2004. The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes, Water. Sci. Technol. **50** : 45-50.

Brenner, D. J. in: Noel R. Kreig (Ed.), *Bergey's manual of systematic bacteriology*, 1984, pp. 409-516 (Vol. I.)

Birnboim, H. C, and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic. Acid. Res. **7**: 1513-1523.

Bunny, K. L., R. M. Hall, and H. W. Stokes. 1995. New mobile gene cassettes containing an aminoglycoside resistance gene, *aacA7*, and a chloramphenicol resistance gene, *catB3*, in an integron in pBWH301. Antimicrob. Agents. Chemother. **39** : 686-693.

Carattoli, A., L. Villa., C. Pezzella., E. Bordi, and P. Visca. 2001. Expanding drug resistance through integron acquisition by IncFI plasmids of *Salmonella enterica* Typhimurium. Emerg. Infect. Dis. **7**: 444-447.

Chen, S., S. Zhao., D. G. White., C. M. Shroeder., R. Lu., H. Yang., P. F. McDermott., S. Ayers, and J. Meng. 2004. Characterization of multiple-antimicrobial-resistant *Salmonella* serovars isolated from retail meats. Appl. Environ. Microbiol. **70** : 1-7.

Chang, C. Y., L. L. Chang., Y. H. Chang., T. M. Lee, and S. F. Chang. 2000. Characterisation of drug resistance gene cassettes associated with class 1 integrons in clinical isolates of *Escherichia coli* from Taiwan, ROC. J. Med. Microbiol. **49** : 1097-1102.

Collis, C. M., G. Grammaticopoulos., J. Briton., H. W. Stokes, and R. M. Hall. 1993. Site specific insertion of gene cassettes into integrons. Mol. Microbiol. **9** : 41-52.

Collis, C. M, and R. M. Hall. 1992. Gene cassettes from the insert regions of integrons are excised as a covalently closed circles. Mol. Microbiol. **6** : 2875-2885.

Collis, C. M, and R. M. Hall. 1995. Expression of antibiotic resistance genes in the integrated

cassettes of integrons. Antimicrob. Agents. Chemother. **39** : 155-162.

Correia, M., F. Boavida., F. Grosso., M. J. Salgado., L. M. Lito., J. M. Cristino., S. Mendo, and A. Duarte. 2003. Molecular characterization of a class 3 integron in *Klebsiella pneumoniae*. Antimicrob. Agents. Chemother. **47** : 2838-2843.

Davis, L. G., L. D. Dibner, and J. F. Battery. 1986. Basic Methods in Molecular Biology. Elsevier Science Publishing Co., Inc., ISBN 0-444-01082-3, Amsterdam, The Netherlands.

Doublet, B., D. Boyd., M. R. Mulvey., and A. Cloeckaert. 2005. The *Salmonella* genomic island 1 is an integrative mobilizable element. Mol. Microbiol. **55** : 1911-1924.

Drake, J. W, and L. S. Ripley. 1994. Mutagenesis, pp. 98-124 in Molecular Biology of Bacteriophage T4, edited by J. D. Karam, J. W. Drake, K. N. Kreuzer, G. Mosig, D. H. Hall, F. A. Eiserling, L. W. Black, E. K. Spicer, E. Kutter, K. Carlson and E. S. Miller. ASM Press, Washington, DC.

Fluit, A. C, and F. -J. Schmitz. 2004. Resistance integrons and super integrons. Clin Microbiol Infect. **10** : 272-288.

Fluit, A. C, and F. -J. Schmitz. 1999. Class 1 integrons, gene cassettes, mobility, and epidemiology. Eur. J. Clin. Microbiol. Infect. Dis. **18** : 761-770.

Gebreyes, W. A, and C. Altier. 2002. Molecular characterization of multi-drug resistant Salmonella enterica subsp. Enterica serovar Typhimurium isolates from swine. J. Clin. Microbiol. **40** : 2813-2822.

Girlich, D., L. Poirel., A. Leelaporn., A. Karim, and C. Tribuddharat. 2001. Molecular epidemiology of the integron-located VEB-1 extended spectrum beta-lactamase in nosocomial enterobacterial isolates in Bangkok, Thailand. J. Clin. Microbiol. **39** : 175-182.

Goldstein, C., M. D. Lee., S. Sanchez., C. Hudson., B. Phillips., B. Register., M. Grady., C. Liebert., A. O. Summers., D. G. White, and J.J. Maurer. 2001. Incidence of class1 and class 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob. Agents. Chemother. **45** : 723-726.

Goni-Urizza, M., L. Pineau., M. Capdepuy., C. Roques., P. Caumette, and C. Quentin. 2000. Antimicrobial resistance of mesophilic *Aeromonas spp*. isolated from two European rivers, J. Antimicrob. Chemother. **46** : 297-301.

Gonzalez, G., K. Sossa., H. Bello., M. Dominguez., S. Mella, and R. Zemelman. 1998. Presence of integrons in isolates of different biotypes of *Acinetobacter baumanii* from Chilean hospitals. FEMS. Microbiol. Lett. **161**: 125-128.

Guerineau, F., L. Brooks, and P. Mullineaux. 1990. Expression of the sulfonamide resistance gene from plasmid R46. Plasmid. **23** : 34-41.

Guerra, B., S. Soto., S. Cal, and M. C. Mendoza. 2000. Antimicrobial resistance and spread of class 1 integrons among *Salmonella* serotypes. Antimicrob. Agents. Chemother. **44** : 2166-2169.

Guex, N, and M. C. Peitsch. 1997. SWISS-MODEL and the Swiss-PdbViewer: An environment for comperative protein modeling. Electrophoresis. **18** : 2714-2723.

Gupta, S. P. 2001. Statistical Methods, Sultan Chand and Sons, Educational Publishers, New Delhi. pp. 953-1003.

Hall, R. M, and C. M. Collis. 1995. Mobile gene cassettes and integrons: capture and spread by site- specific recombination. Mol Microbiol. **15** : 593-600.

Hall, R. M. 1997. Mobile gene cassettes and integrons: moving antibiotic resistance genes in gram-negative bacteria. Ciba. Found. Symp. **207** : 192-202.

Hall, R. M, and C. M. Collis. 1998. Antibiotic resistance in gram negative bacteria: the role of gene cassettes and integrons. Drug. Resist. Updates. **1**:109-119.

Hall, R. M, and H. W. Stokes. 1993. Integrons: novel DNA elements which capture genes by site-specific recombination. Genetica. **90** :115-132.

Hanson, K., O. Skold, and L. Sindstrom. 1997. Non palindromic attI sites of integrons are capable of site-specific recombination with one another and with secondary targets. Mol. Microbiol. **26** : 441-453.

Heir, E., B-A. Lindstedt., T. M. Leegaard., E. Gjernes, and G. K. Kapperud. 2004. Prevalence and characterization of integrons in blood culture Enterobacteriaceae and gastrointestinal *Escherichia coli* in Norway and reporting of a novel class 1 integron-located lincosamide resistance gene. Annals of Clinical Microbiology and Antimicrobials. **3** : 12.

Jones, L. A., C. J. McIver., W. D. Rawlinson, and P. A. White. 2003. Polymerase chain reaction screening for integrons can be used to complement resistance surveillance programs. Commun Dis Intell. **27** : S103-S110.

Jones, M. E., E. Peters., A. M. Weeraink., A. Fluit, and J. Verhoef. 1997. Widespread occurrence of integrons causing multiple antibiotic resistances in bacteria. Lancet. **349** : 1742-1743.

Kang, H. Y., Y. S. Jeong., J. Y. Oh., S. H. Tae., C. H. Choi., D. C. Moon., W. K. Lee., Y. C. Lee., S. Y. Seol., D. T. Cho, and J. C. Lee. 2005. Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. J. Antimicrob. Chemother. **55** : 639-644.

Kazama, H., H. Hamashima., M. Sasatsu, and T. Arai. 1998. Distribution of the antiseptic-resistance gene qacEΔ1 in gram-positive bacteria. FEMS. Microbiol. Lett. **165**: 295-299.

Krumperman, P. H. 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high risk sources of fecal contamination of foods. Appl. Environ. Microbiol. **46** : 165-170.

L'Abee-Lund, T. M, and H. Sorum. 2001. Class 1 integrons mediate antibiotic resistance in the fish pathogen *Aeromonas salmonicida* worldwide. Microb. Drug. Resist. **7** : 263-272.

Laraki, N., M. Galleni., I. Tham., M.L. Riccio., G. Amicosante., J. Frere, and G. M. Rossolini. 1999. Structure of In31, a bla –containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. Antimicrob. Agents. Chemother. **43** : 890-901.

Leverstein–van Hall, M. A., A. T. A. Box., H. E. M. Blok., A. Paauw., A. C. Fluit, and J. Verhoef. 2002. Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant enterobacteriaceae in a clinical setting. J. Infect. Dis. **186** : 49-56.

Leverstein-van Hall, M. A., H. E. M. Blok., A. R. Donders., A. Paauw., A. C. Fluit, and J. Verhoef. 2003. Multidrug resistance among enterobacteriaceae is strongly associated with the presence of integrons and is independent of species and isolate origin. J. Infect. Dis. **187** : 251-259.

Levesque, C., L. Piche., C. Larose, and P. H. Roy. 1995. PCR Mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents. Chemother. **39** : 185-91.

Lindstedt, B. A., E. Heir., I. Nygard, and G. Kapperud. 2003. Characterization of class 1 integrons in clinical strains of *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Enteritidis from Norwegian hospitals. J. Med. Microbiol. **52** : 141-149.

Martinez, E, and F. de la Cruz. 1990. Genetic elements involved in *Tn21* site specific integration, a novel mechanism for the dissemination of antibiotic resistance genes. EMBO J. 9 : 1275-1281.

Marchler-Bauer, A, and S. H. Bryant. 2004. "CD-search : protein domain annotations on the fly". Nucleic. Acids. Res. **32** : W327-W331.

Martinez Freizo, P., A. C. Fluit., F. J. Schmitz., V. S. C. Grek., J. Verhoef, and M. E. Jones. 1998. Class I integrons in gram negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. J. Antimicrob. Chemother. **42** : 689-696.

Martinez Freizo, P., A. C. Fluit., F. J. Schmitz., J. Verhoef, and M. E. Jones. 1999. Many class I integrons comprise distinct stable structures occurring in different species of enterobacteriaceae isolated from widespread geographic regions in Europe. Antimicrob. Agents. Chemother. **43** : 686-689.

Mazel, D., B. Dychinco., V. A. Webb, and J. Davies. 2000. Antibiotic resistance in ECOR collection: integrons and identification of a novel *aad* gene. Antimicrob. Agents. Chemother. **44** : 1568-1574.

Morabito, S., R. Tozzoli., A. Caprioli., H. Karch, and A. Carattoli. 2002. Detection and characterization of class 1 integrons in enterohemorrhagic *Escherichia coli*. Microb. Drug. Resist. **8** : 85-91.

Mukherjee, S., B. Bhadra., R. Chakraborty., A. Gurung., S. Some, and R. Chakraborty. 2005. Unregulated use of antibiotics in Siliguri city vis-à-vis occurrence of MAR bacteria in community waste water and river Mahananda, and their potential for resistance gene transfer. J. Environ. Biol. **26:** 229-238.

Naas, T., Y. Mikami., T. Imai., L. Poirel, and P. Nordmann. 2001. Characterization of In53, a class 1 plasmid- and composite transposon-located integron of *Escherichia coli* which

characterizes an unusual array of gene cassettes. J. Bacteriol. **183** : 235-249.

Nandi, S., J. J. Maurer., C. Hofacre, and A. O. Summers. 2004. Gram positive bacteria are a major reservoir of class 1 antibiotic resistance integrons in poultry litter. PNAS. **101**: 7118-7122.

Neild, B. S., A. J. Holmes., M. R. Gillings., G. D. Reechia., B. C. Mabbutt, K. M. H. Nevalainen and H. W. Stokes. 2001. Recovery of new integron classes from environmental DNA. FEMS Microbiol. Lett. **195** : 59-65.

Nunes-Duby, S. E., H. J. Kwon., R. S. Tirumalai., T. Ellenberger, and A. Landy. 1998. Similarities and differences among 105 members of the Int family of site specific recombinases. Nucleic Acids Res. **26** : 391-406.

O'Halloran, F., B. Lucey., B. Cryan., T. Buckley, and S. Fanning. 2004. Molecular characterization of class 1 integrons from Irish thermophilic *Campylobacter* spp. J. Antimicrob. Chemother. **53** : 952-957.

Ouellette, M, and P. H. Roy. 1987. Homology of ORFs from Tn2603 and from R46 to site specific recombinases. Nucleic Acids Res. **15** : 10055.

Park, J. C., J. C. Lee., J. Y. Oh., Y. W. Jeong., J. W. Cho., H. S. Joo., W. K. Lee, and W. B. Lee. 2003. Antibiotic selective pressure for the maintenance of antibiotic resistant genes in coliform bacteria isolated from the aquatic environment. Water. Sci. Technol. **47** : 249-253.

Partridge, S. R., G. D. Reechia., C. Scaramuzzi., C. M. Collis., H. W. Stokes, and R. M. Hall. 2000. Definition of the *att1* site of class 1 integrons. Microbiology. **146** : 2855-2864.

Partridge, S. R., H. J. Brown., H. W. Stokes, and R. M. Hall. 2001. Transposons *Tn1696* and *Tn21* and their integrons In4 and In2 have independent origins. Antimicrob. Agents. Chemother. **45** : 1263-1270.

Partridge, S. R., G. D. Reechia., H. W. Stokes, and R. M. Hall. 2001. Family of class 1 integrons related to In4 from *Tn1696*. Antimicrob. Agents. Chemother. **45** : 3014-3020.

Partridge, S. R., H. J. Brown, and R. M. Hall. 2002. Characterization and movement of the class 1 integron known as *Tn2521* and *Tn1405*. Antimicrob. Agents. Chemother. **46** : 1288-1294.

Paulsen, I. T., T.G. Littlejohn., P. Randstrom., L. Sundstrom., O. Skold., G. Swedberg, and R. A. Skurray. 1993. The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob. Agents. Chemother. **37** : 761-768.

Peters, E. D. J., M. A. Leverstein–van Hall., A.T. A. Box., J. Verhoef., and A. C. Fluit. 2001. Novel gene cassettes and integrons, Antimicrob. Agents. Chemother. **45** : 2961-2964.

Ploy, M. C., F. Denis., P. Courvalin, and T. Lambert. 2000. Molecular characterization of integrons in *Acinetobacter baumannii*: description of a hybrid class 2 integron. Antimicrob. Agents. Chemother. **44** : 2684-2688.

Reechia, G. D., H. W. Stokes, and R. M. Hall. 1994. Characterisation of specific and secondary recombination sites recognized by the integron DNA integrase. Nucleic Acids. Res. **22** : 2071-2078.

Ripley, L. S. 1990. Frameshift mutations-determinants of specificity. Ann. Rev. Genet. **24** : 189-213.

Roe, M. T., E. Vega, and S. D. Pillai. 2003. Antimicrobial resistance markers of class 1 and class 2 integron bearing *Escherichia coli* from irrigation water and sediments, Emerg. Infect. Dis. **9** : 822-826.

Rosser, S. J, and H. K. Young. 1999. Identification and characterization of class 1 integrons in bacteria from an aquatic environment. J. Antimicrob. Chemother. **44** : 11-18.

Rowe-Magnus, D. A, and D. Mazel. 1999. Resistance gene capture. Current Opinion in Microbiology. **2** : 483-488.

Rowe-Magnus, D. A., A. M. Guerout., P. Ploncard., B. Dychinco., J. Davies, and D. Mazel. 2001. The evolutionary history of chromosomal super integrons provides an ancestry for multi-resistant integrons. Proc. Natl. Acad. Sci. USA. **98** : 652-657.

Rowe-Magnus, D. A, and D. Mazel. 2002. The role of integrons in antibiotic resistance gene capture. Int. J. Med. Microbiol. 292 : 1-11.

Sallen, B., A. Rajoharison., S. Desvarenne, and C. Mabilat. 1995. Molecular epidemiology of integron associated antibiotic resistance genes in clinical isolates of Enterobacteriaceae. Microb. Drug. Resist. 1: 195-202.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Chapter 4

Taxonomic characterization of class 1 integron positive isolates

and integron associated antibiotic resistance genes among motile aeromonads from a fish farming environment. Appl. Environ. Microbiol. **67** : 5675-5682.

Schmitz, F. J., P. Martinez-Freijo., S. Theis., A. C. Fluit., J. Verhoef., H. P. Heinz, and M. E. Jones. 1999. Prevalence of class 1 integrons and association with decreased antibiotic susceptibility in German gram-negative blood culture isolates. Clin. Microbiol. Infect. **5** : 496-498.

Schmitz, F. J., D. Hafner., R. Geisel., P. Follmann., C. Kirschke., J. Verhoef., K. Kohrer, and A. C. Fluit. 2001. Increased prevalence of class 1 integrons in *Escherichia coli*, *Klebsiella* species, and *Enterobacter* species isolates over a 7-year period in a German university hospital. J. Clin. Microbiol. **39** : 3724-3726.

Schnabel, E. L, and A. L. Jones. 1999. Distribution of tetracycline resistance genes and transposons among phylloplane bacteria in Michigan apple orchards. Appl. Environ. Microbiol. **65**: 4898-4907.

Schwede, T., J. Kopp., N. Guex, and M. C. Peitsch. 2003. SWISS-MODEL: an automated protein homology-modeling server. Nucleic. Acids. Res. **31** : 3381-3385.

Singh, R., C. M. Schroeder., J. Meng., D. G. White., P. F. McDermott., D. D. Wagner., H. Yang., S. Simjee., C. DebRoy., R. D. Walker, and S. Zhao. 2005. Identification of antimicrobial resistance and class 1 integrons in shiga toxin producing *Escherichia coli* recovered from humans and food animals. J. Antimicrob. Chemother. **56** : 216-219.

Soto, S. M., M. J. Lobato, and M. C. Mendoza. 2003. Class 1 integron borne gene cassettes in multidrug-resistant *Yersinia enterocolitica* strains of different phenotypic and genotypic types. Antimicrob. Agents. Chemother. **47** : 421-425.

Stokes, H. W, and R. M. Hall. 1984. Characterization of specific and secondary recombination sites recognized by integron DNA integrase. Nucleic. Acids. Res. 22 : 2071-2078.

Stokes, H. W, and R. M. Hall. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene integration functions: integrons. Mol. Microbiol. **3** :1669-1683.

Stokes, H. W, and R. M. Hall. 1991. Sequence analysis of the inducible chloramphenicol resistance determinant in the Tn1696 integron suggests regulation by translational attenuation. Plasmid. **26** : 10-19.

Stokes, H. W., D. B. Gorman., G. D. Reechia., M. Parsekhian, and R. M. Hall. 1997. Structure and function of 59-base element recombination sites associated with mobile gene cassettes. Mol. Microbiol. **26** : 731-745.

Streisinger, G., Y. Okada., J. Emrich., J. Newton, and A. Tsugita *et al.* 1966. Frameshift mutations and the genetic code. Cold spring Harbor Symp. Quant. Biol. **31**: 77-84.

Sunde, M, and H. Sorum. 2001. Self-transmissible multidrug resistance plasmids in *Escherichia coli* of the normal intestinal flora of healthy swine. Microb. Drug. Resist. **7**: 191-196.

Sundin, G. W. 2002. Distinct recent lineages of the *strA-strB* streptomycin- resistance genes in clinical and environmental bacteria. Curr. Microbiol. **45** : 63-69.

Sundstrom, L., P. Radstrom., G. Swedberg, and O. Skold. 1988. Site-specific recombination promotes linkage between trimethoprim and sulfonamide resistance genes. Sequence characterization of dhfrV and sulI and a recombination active locus of *Tn21.* Mol. Gen. Genet. **213** : 191-201.

Thungapathra, M., Amita, K. K. Sinha., S. Ray Chaudhuri., P. Garg., T. Ramamurthy., G. B. Nair, and A. Ghosh. 2002. Occurrence of antibiotic resistance gene cassettes *aac*(6')-*Ib*, *dfr A5*, *dfr A12*, and *ereA2* in class I integrons in Non-O1, Non-O139 *Vibrio cholerae* strains in India. Antimicrob. Agents. Chemother. **46** : 2948-2955.

Villa, L., P. Visca., F. Tosini., C. Pezzella, and A. Carattoli. 2002. Composite integron array generated by insertion of an ORF341-type integron within a Tn 21-like element. Microb. Drug. Resist. 8:1-8.

White, P. A., C. J. McIVER, and W. D. Rawlinson. 2001. Integrons and gene cassettes in the enterobacteriaceae. Antimicrob. Agents. Chemother. 45 : 2658-2661.

Yu, H. S., J. C. Lee., H. Y. Kang., Y. S. Jeong., E. Y. Lee., C. H. Choi., S. H. Tae., Y. C. Lee., S. Y. Seol, and D. T. Cho. 2004. Prevalence of *dfr* genes associated with integron and dissemination of *dfrA17* among urinary isolates of *Escherichia coli* in Korea. J. Antimicrob. Chemother. 53 : 445-450

4.1 Introduction

The ability to classify bacteria into the correct species and to distinguish different strains is of vital importance. The classical identification method is to use phenotypic traits such as morphology and metabolism, together with physiological characteristics, to group an unknown bacterium with other bacteria sharing the same traits. However, not all strains within a given species may be positive for a common trait and the same strain may exhibit biochemical variability. Moreover, small alterations in the realization of a test may give varying and false results. Consequently, techniques based on phenotypic tests might not exhibit an unambiguous identification of certain species.

The detailed biochemical characterizations of the integron-positive isolates have been presented in this chapter. The bacterial isolates were identified according to Bergey's Manual of Systematic Bacteriology following the principle of numerical taxonomy method of numerical taxonomy. Numerical taxonomy is defined as the grouping by numerical methods of taxonomic units into taxa on the basis of their character states. Information about the properties of organisms is converted into a form of suitable numerical analysis and then compared by computer. The resulting classification is based on general similarity as judged by comparison of many characteristics, each given equal weight. The process begins with a determination of the presence or absence of selected characters in the group of organism under study. A character usually is defined as an attribute about which a single statement can be made. After character analysis, an association coefficient, a function that measures the agreement between characters possessed by two organisms, is calculated for each pair of organisms in the group. The similarity coefficient (S_{SM}) is the proportion of characters that match regardless of whether the attribute is present or absent. Jaccard coefficient (S_j) is calculated by ignoring any characters that both organisms lack. Both coefficients increase linearly in value from 0.0 (no matches) to 1.0 (100% matches).

The calculation of association coefficients for two organisms

The similarity coefficient

(S_{SM}) = a+d/a+b+c+d

The Jaccard coefficient (S_j) = a/a+b+c

a = number of characters coded as present (1) for both organisms

b and c =numbers of characters differing (1,0 or 0,1) between the two organisms

d = number of characters absent (0) in both organisms

Total number of characters compared = a+b+c+d

4.1.1 Materials and methods

4.1.1.1 Bacterial isolates

Forty integron-positive isolates were used for biochemical characterization.

4.1.1.2 Media and culture condition

The integron positive bacterial isolates were maintained in Luria-Bertani agar medium supplemented with appropriate antibiotic. For biochemical tests, cultures were grown overnight in Luria broth at 37 ^{0}C and one loop-full culture/ 1% inoculum were added in respective media. Culture media used for the experiments were supplied by HIMEDIA, India Ltd. (unless otherwise mentioned).

4.1.1.3 Biochemical tests

The following biochemical tests were conducted for the identification and characterization of the isolates.

I. **Gram Reaction** : Gram reaction was performed following protocols stated by Cappuccino and Sherman (1996). A smear of the culture was made on a glass slide, air-dried and was heat-fixed. The smear was stained with crystal violet for one minute, washed with tap water and stained with gram's iodine for one minute followed by washing and decolorization with 95% ethanol. It was washed and counter stained with safranin for 45 seconds, washed with tap water, air dried and finally examined under compound light microscope with oil immersion objective lens. Gram-negativity of the isolates was also tested on McConkey Agar plate (HIMEDIA).

II. **Indole test** : Cells were grown in 5ml tryptophan broth (g/l; NaCl, 5.0; $MgSO_4.7H_2O$, 0.2; $NH_4H_2PO_4$, 1.0; K_2HPO_4; L-tryptophan, 10.0, pH 7) and were incubated at 35 ^{0}C for 24h. 0.2 ml of the test reagent (p-dimethylaminobenzaldehyde, 0.5gm; Isoamyl alcohol, 0.5 ml; Conc. HCl, 2.5 ml) was then carefully added into the full-grown culture and the tubes were allowed to stand for 10min after a gentle shaking. A dark red colored ring on the surface was regarded as positive test.

III. **Methyl Red test** : The organisms were grown in 10 ml of glucose peptone broth for 2-3 days at 30 ^{0}C. The medum contained (g/l)- peptone, 5.0; KH_2PO_4, 5.0; glucose 10.0 and the pH of the medium were adjusted to 7.4. Few drops of methyl red reagent (5 mg dissolved in 30 ml of 95% ethanol and diluted to 50 ml with water) were added to the growing culture. Methyl red positive reaction was indicated by bright red coloration and negative reaction was indicated by yellow color of the culture medium.

IV. **Voges-Proskauer test** : One loop full of overnight grown test culture was inoculated in 5ml of the sterilized buffered glucose broth (g/l, Protease peptone, 7.0; Glucose, 5.0; K_2HPO_4, 5.0; pH 7.0) and was incubated at 35 ^{0}C for 48h. One ml aliquot of the full-grown culture were taken in a separate test tube and 0.6ml of α-naptha solution (α- napthol, 5.0gm dissolved in 100ml ethanol) and 0.2ml 7(N) KOH were sequentially added and mixed well by shaking. Development of pink to crimson color at the surface within 5min considered as positive test.

V. **Catalase test** : The organisms were grown on Tryptic Soy Agar (TSA) plates for 24- 28 h at 35 ^{0}C to get isolated colonies. A drop of H_2O_2 was put over a colony. As a negative control H_2O_2 was always dropped on any part of the plate devoid of any bacterial growth. Catalase positive character was indicated by effervescence of gas bubbles from the colony surface, immediately after the addition of H_2O_2.

VI. **Oxidase test** : The test was performed by adding bacterial culture on a strip of filter paper which was initially impregnated with 1% (w/v) aqueous solution of N-N-dimethyl-p-phenylenediamine. Development of

pink color within 30 seconds indicated the oxidase positive tests.

VII. **Citrate utilization test** : The Simon's Citrate medium was used for this test. The composition of the Simon's Citrate medium is as follows (g/l); NaCl, 5.0; $MgSO_4.7H_2O$, 0.2; $NH_4H_2PO_4$, 1.0; K_2HPO_4, 1.0; Na-Citrate, 5.0; bromothymol blue, 0.02; Yeast Extract, 0.05 agar powder 20; pH 6.8. Blue coloration of the media after 24- 48 h of inoculation was recorded as positive reaction.

VIII. **Urease test** : Urease activity was tested with a medium having following composition (g/l): part A: peptone, 1.0; glucose, 1.0; NaCl, 5.0; KH_2PO_4, 2.0; phelol red, 0.12; agar, 20.0; pH 8.0; and part B (g/l): urea 40.0. After filter sterilization part A and part B were mixed together aseptically and were cooled to approximately 40–50 ^{0}C, and petri-plates were poured with the medium. In each plate, a loopful of overnight-grown culture was streaked and incubated at 30 ^{0}C for 1–2 days. Development of deep red color surrounding the bacterial growth was indicative of positive test.

IX. **Lysine Decarboxylase test :**Moeller Decarboxylase broth [(g/l) Peptic digest of animal tissue, 5.0; beef extract, 5.0; Dextrose, 0.5; bromo cresol purple, 0.01; pyridoxal, 0.005; cresol red, 0.005; pH 6.0] supplemented with 1% L-lysine was used for this experiment. 50 µl of overnight grown culture(s) were added in 5 ml broth and incubated at 35 ^{0}C for 18 –20 h. Change of color from yellow to red was recorded as positive test.

X. **Ornithine Decarboxylase test:** Moeller Decarboxylase broth [(g/l) Peptic digest of animal tissue, 5.0; beef extract, 5.0; Dextrose, 0.5; bromo cresol purple, 0.01; pyridoxal, 0.005; cresol red, 0.005; pH 6.0] supplemented with 1% L-Ornithine was used for this experiment. 50 µl of overnight grown culture(s) were added in 5 ml broth and incubated at 35 ^{0}C for 18–20 h. Change of color from yellow to red was recorded as positive test.

XI. **Nitrate reduction test :** The enzyme nitrate reductase is a molybdenum-containing membrane bound enzyme, which reduces nitrate to nitrite in presence of an electron donor. Nitrate reduction and denitrification (as a function of dissimilatory reduction of nitrate denoting the presence of the enzyme nitrate reductase) was examined in the medium having following composition (g/l); KNO_3, 1.0; peptone, 5.0; pH 7.5. 5 ml volume of medium were dispensed in each tube and sterilized by autoclaving at 15 psi for 15 min. Each culture tube was inoculated with a single isolate and incubated at 30 ^{0}C for 24 h. Formation of nitrite in the culture medium was tested by adding 1.0 ml test reagent (0.8g of sulfanilic acid dissolved in 100 ml of 5(N) acetic acid and 0.5 g of α – napthalamine dissolved in 100 ml acetic acid, mixed immediately before use). Development of red color indicates a nitrate reductase positive test. To monitor the production of gas

by nitrate respiration, inverted Durham tubes were placed in culture medium.

XIII. **Gelatin hydrolysis test :** Gelatin hydrolysis was examined in a medium with components (g/l); gelatin, 20.0; nutrient broth, 1.0. Melted medium of 6 ml aliquots were dispensed into different culture tubes, plugged with cotton and sterilized by autoclaving at 15 psi for 15 min. each tube was inoculated with freshly grown culture by stabbing, and incubated for 2 –3 days at 35 ^{0}C. The culture tubes, after growth of the organisms, were kept at 4 ^{0}C along with an un-inoculated (control) tube. The tubes that did not solidify even at 4 ^{0}C indicated the liquefaction of gelatin and the isolates were recorded as gelatinase positive.

XIV. **Starch Hydrolysis :** For this test organisms were streaked on Starch agar plates [(g/l); NA, 23; soluble starch, 5.0; pH 7.2] and incubated at 37 ^{0}C for 1 –2 days. After the growth of the culture the petri-plates were flooded with Lugol's iodine solution. Appearance of colorless zone surrounding the bacterial growth indicated the hydrolysis of starch leaving the other part dark blue in color due to the reaction of starch with iodine.

XV. **Casein hydrolysis :** Milk Agar plates were streaked with overnight grown loop-full culture(s) and caseinase producing bacteria showed a clear zone along the growth.

XVI. **Lipase test :** One loop full of overnight grown culture was streaked on Tributyrin agar plate (1.0 ml Tributyrin added in 100 ml sterile Tributyrin agar base) and was incubated at 37 ^{0}C. Isolates showed opaque zone around the growth area was considered as lipase positive.

XVII. **Fermentation of sugars :** Fermentation of sugars was tested using 'Phenol Red Broth base' medium [(g/l); protease peptone 10.0; beef extract, 1.0; Sodium chloride, 5.0; phenol red 0.018; pH 7.4] containing different sugars at a concentration of 1% (w/v). An aliquot of 50 µl overnight grown culture was inoculated in 5 ml of test media and incubated at 35 ^{0}C for 24 –48 h. Change in the color of the media from red to yellow was recorded as positive test.

XVIII. **Utilization of organic acid salts** To assay the utilization of organic acid salts as a carbon source, inorganic M70 media (Veron, 1975) was used. To prepare 1L of M70 medium, 10 ml of 'Metal-70' solution [(mg/l): H_3PO4, 1960; $FeSO_4$. $7H_2O$, 55.6; $ZnSO_4$. $7H_2O$, 28.7; $MnSO_4$. $4H_2O$, 22.3; $CuSO_4$. 5 H_2O, 2.5; $Co(NO_3)$. $6H_2O$; H_3BO_4, pH 6.2] was added in to 490 ml of 'P-Ca-Mg solution' [(mg/l); $CaCl_2$. $2H_2O$, 14.7; $MgSO_4$. $7H_2O$, 123; KH_2PO_4, 680; K_2HPO_4, 2610, pH 7.2], the resultant mix was filter- sterilized and added in 500 ml autoclaved 'Nitrogen Base' solution [(g/l); NaCl, 10; NH_4SO_4, 1]. The substrate was filter-sterilized and added to the sterile M70 media at a concentration of 10 % (w/v). To the 10 ml of media 100 µl of growing cultures were added separately and growth was observed after 2 –6 days after incubation at 30 ^{0}C.

4.3 Results and Discussions

The results of detailed biochemical characterization of all the integron-positive isolates have been presented in the Tables 4.1, 4.2, 4.3 and 4.4.

Thirty two integron-positive isolates were identified as different members of the family Enterobacteriaceae. Isolate TR 02 and TR 53 yielded a maximum similarity coefficient (S_{SM} : $\overline{0.95}$ and $\overline{0.91}$) to *Escherichia coli*. A maximum similarity coefficient (S_{SM} : 0.87) to *Enterobacter cloacae* was produced by isolate TR $\overline{04}$. Nine isolates, TR 06, TR 10, TR 11, TR 12, TR 13, TR 14, TR 15, TR 17 and TR 91 yielded a maximum similarity coefficient to *Citrobacter freundii* (S_{SM} value ranged from 0.83 to 0.91). To *Kluyvera ascorbata,* maximum similarity coefficient of 0.83 and 0.91 were produced by TR 37 and TR 97. Five isolates, TR 39, TR 40, TR 48, TR 78 and TR 82 yielded maximum similarity coefficient of 0.87, 0.83, 0.87, 0.83 and 0.79 to *Serratia plymuthica, Serratia ficara, Serratia fonticola, Serratia ficara* and *Serratia odorifera* respectively. TR 56, TR 76 and TR 77 exhibited a maximum similarity coefficient to *Proteus mirabilis* (S_{SM} values ranged from 0.79 to 0.83). To *Yersinia enterocolitica*, S_{SM} value of 0.79 was produced by TR 63. Five isolates were identified as representatives of the genus *Providencia*. Of the five isolates, TR 18 and TR 81 produced S_{SM} of 0.79 and 0.83 to *Providencia rettgeri*, whereas, TR 58, TR 68 and TR 73 yielded a maximum similarity coefficient to *Providencia stuartii* (S_{SM} : 0.95, 0.87 and 0.91). Two isolates - TR 79 and TR 95, yielded maximum S_{SM} (0.79) to *Salmonella* sp. Isolate TR 85 yielded a maximum similarity coefficient to *Enterobacter sakazakii* (S_{SM} 0.83) while maximum similarity coefficient to *Morganella morganii* (S_{SM} 0.83) was produced by the isolate TR 90.

TR 20, TR 27, TR 49, TR 52, TR 59, TR 62, TR 92 and TR 99 did not bear any significant resemblance with the enteric members. After performing the tests specific for the identification of enteric members, some more tests- lipase test, casein hydrolysis, starch hydrolysis, utilization of different organic salts- were performed. Six of the isolates (TR 27, TR 52, TR 59, TR 62, TR 92 and TR 99) were further identified as *Pseudomonas* sp. and the remaining two (TR 20 and TR 49) were identified as *Acinetobacter* sp.

Table 4.1. Biochemical characterization of integron-positive isolates

	TR 02	TR 04	TR 06	TR 10	TR 11	TR 12	TR 13	TR 14	TR 15	TR 17
Indole	+	-	-	-	-	-	-	-	-	-
Methyl Red	+	-	+	+	+	+	+	+	+	-
Voges Proskauer	-	+	-	-	-	-	-	-	-	+
Citrate, Simmons	-	+	+	+	+	+	-	+	+	+
Urease	-	+	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	-	-	-	-	-	-	-	-	+
Ornithine decarboxylase	+	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+
Gelatin Liquefaction	-	-	-	-	-	-	-	-	-	-
D-Glucose, acid production	+	+	+	+	+	+	+	+	+	+
D-Glucose, gas production	+	+	+	+	+	+	+	+	+	-
Lactose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+
Dulcitol	+	-	+	+	+	+	+	+	+	+
Salicin	-	+	+	+	+	+	+	+	+	+
D- Adonitol	+	+	+	+	+	+	+	+	+	+
Myo-Inositol	-	+	+	+	+	+	+	+	+	+
D- Sorbitol	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	-	+	+	+	+	+	+	+	-
Raffinose	+	+	+	+	+	+	+	+	+	+
L-Rhamnose	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	-	+
Nitrate reduction	-	-	-	-	-	-	-	-	+	-
Oxidase,Kovacs	-	-	-	-	-	-	-	-	-	-
D-Mannose	+	+	+	+	+	+	+	+	+	+

Table 4.2. Biochemical characterization of integron positive isolates

	TR 18	TR 20	TR 27	TR 37	TR 39	TR 40	TR 48	TR 49	TR 52	TR 53
Indole	-	-	-	+	-	-	+	-	-	+
Methyl Red	+	-	-	+	+	+	+	-	-	+
Voges Proskauer	-	-	-	-	-	-	-	-	-	-
Citrate, Simmons	+	+	+	+	+	+	+	+	+	+
Urease	+	-	+	-	-	-	-	-	-	-
Lysine decarboxylase	-	-	-	+	-	-	+	+	-	+
Ornithine decarboxylase	-	-	+	+	-	-	-	-	-	+
Motility	+	+	+	+	+	+	+	+	+	+
Gelatin Liquefaction	-	+	+	-	-	-	-	-	+	-
D-Glucose, acid production	+	-	+	+	+	+	+	-	-	+
D-Glucose, gas production	-	-	+	-	+	+	+	-	-	+
Lactose	-	-	-	-	+	-	+	-	-	+
Sucrose	-	-	-	+	+	+	+	+	-	+
D-Mannitol	+	-	+	+	+	+	+	-	-	+
Dulcitol	-	-	-	+	-	-	+	-	-	+
Salicin	-	-	-	-	+	+	-	-	-	-
D- Adonitol	-	-	-	-	+	+	-	-	-	-
Myo-Inositol	+	-	-	-	+	+	+	-	-	-
D- Sorbitol	-	-	+	+	+	+	+	-	-	+
L-Arabinose	-	-	-	-	-	-	+	-	-	-
Raffinose	-	-	-	+	+	+	+	-	-	+
L-Rhamnose	-	+	+	+	+	+	+	-	-	+
Maltose	+	-	-	+	+	+	+	+	-	+
D-Xylose	+	-	-	+	+	+	+	-	-	+
Trehalose	-	-	-	+	+	+	+	-	-	+
Cellobiose	+	-	-	+	+	+	+	-	-	-
Melibiose	-	-	-	-	+	+	+	-	-	+
Nitrate reduction	-	-	-	-	-	-	-	-	-	-
Oxidase,Kovacs	-	-	-	-	-	-	-	-	-	-
D-Mannose	+	-	-	+	+	+	+	-	-	+

Table 4.3. Biochemical characterization of integron positive isolates

	TR 56	TR 58	TR 59	TR 62	TR 63	TR 68	TR 73	TR 76	TR 77	TR 78
Indole	-	+	-	-	+	-	+	-	-	-
Methyl Red	-	+	+	-	+	+	+	-	+	+
Voges Proskauer	-	-	-	-	-	-	-	-	-	-
Citrate, Simmons	+	+	+	+	+	+	+	+	+	+
Urease	+	-	-	-	-	-	-	+	-	-
Lysine decarboxylase	-	-	-	+	-	-	-	-	+	-
Ornithine decarboxylase	+	-	-	+	-	-	-	+	-	-
Motility	+	+	+	+	+	+	+	+	+	+
Gelatin Liquefaction	-	-	-	+	-	-	+	+	+	-
D-Glucose, acid production	+	+	-	-	+	+	+	+	+	+
D-Glucose, gas production	+	-	-	-	-	-	-	+	-	-
Lactose	-	-	-	-	-	-	-	-	-	-
Sucrose	-	+	+	-	+	-	+	+	+	+
D-Mannitol	-	-	-	-	+	-	-	-	+	+
Dulcitol	+	-	+	-	-	-	-	-	-	+
Salicin	-	+	+	-	+	-	+	+	+	+
D- Adonitol	-	+	+	-	+	+	+	+	+	+
Myo-Inositol	-	+	+	+	+	+	+	-	+	+
D- Sorbitol	-	-	+	-	+	+	-	-	+	+
L-Arabinose	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	+	-
L-Rhamnose	+	-	+	-	-	-	-	-	+	+
Maltose	-	-	-	-	-	-	-	+	+	+
D-Xylose	+	-	+	-	+	-	-	+	+	+
Trehalose	+	+	+	-	+	+	+	+	+	+
Cellobiose	-	-	+	-	+	-	-	+	+	-
Melibiose	-	+	+	-	+	-	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-	-	-
Oxidase,Kovacs	-	-	-	-	-	-	-	-	-	-
D-Mannose	+	+	+	-	+	+	+	+	+	-

Table 4.4. Biochemical characterization of integron positive isolates

	TR79	TR81	TR82	TR85	TR90	TR91	TR92	TR95	TR97	TR99
Indole	-	-	-	-	+	-	-	+	+	-
Methyl Red	+	+	+	-	+	+	+	+	+	-
Voges Proskauer	-	-	+	+	-	-	-	-	-	-
Citrate, Simmons	+	+	+	+	+	+	+	+	+	+
Urease	-	+	+	-	+	-	+	-	-	-
Lysine decarboxylase	-	-	+	+	-	-	-	+	+	+
Ornithine decarboxylase	-	-	-	-	+	-	+	-	+	+
Motility	+	+	+	+	+	+	+	+	+	+
Gelatin Liquefaction	-	-	+	-	-	-	+	-	-	+
D-Glucose, acid production	+	+	+	+	+	+	+	+	+	+
D-Glucose, gas production	-	-	-	+	+	+	+	-	+	+
Lactose	-	-	+	+	-	+	-	+	+	-
Sucrose	+	-	+	+	+	+	+	+	+	-
D-Mannitol	+	+	+	+	-	+	-	+	+	-
Dulcitol	+	+	+	-	-	+	-	-	-	-
Salicin	+	+	+	+	+	+	+	+	+	-
D- Adonitol	+	+	+	+	+	+	+	+	-	+
Myo-Inositol	+	+	+	+	-	+	-	-	+	-
D-Sorbitol	+	+	+	-	-	+	-	+	+	-
L-Arabinose	-	-	-	+	-	-	-	-	-	-
Raffinose	-	-	+	+	-	+	-	-	+	-
L-Rhamnose	+	+	+	+	-	+	-	+	+	-
Maltose	+	-	+	+	+	+	+	+	+	-
D-Xylose	+	-	+	+	+	+	-	+	+	-
Trehalose	+	+	+	+	-	+	+	+	+	+
Cellobiose	-	-	+	+	+	+	-	-	+	+
Melibiose	+	-	+	+	+	+	-	+	+	+
Nitrate reduction	-	+	+	-	+	-	-	-	-	-
Oxidase,Kovacs	-	-	-	-	-	-	-	-	-	-
D-Mannose	-	+	+	-	+	-	-	-	-	-

4.2 Phylogenetic analysis of the isolate TR 90 [isolate possessing novel ORF encoding dihydrofolate reductase for trimethoprim resistance] using 16S rRNA gene sequence

Techniques based on nucleic acids such as DNA-DNA hybridization, RFLP analysis and different techniques using PCR and sequencing have been developed to improve identification of bacteria. The nucleic acid sequence particularly is of large practical value since it contains more evolutionary information than the traditionally used phenotypic traits and because it is precisely defined and relatively simple to determine. Two important properties of a nucleotide sequence to be used for bacterial identification are as follows: it must be universal in its distribution and it must contain sufficient sequence variations. The most commonly used molecule is 16S rRNA since it is generally accepted that the sequence of 16S rRNA can be used to distinguish genera and well resolved species. Beside functional constancy, ubiquitous distribution and size (1.5 KB), genes encoding for 16S rRNA exhibited both evolutionary conserved locus as well as highly variable region. For these reasons, comparison of 16S rRNA gene sequences of the organisms could be used to calculate evolutionary distance between organisms (Woese *et al.* 1990). The 16S rRNA gene of bacteria can be amplified and sequenced by using specific primers, designed from conserved regions. To identify the systematic position of the isolates, the 16S rRNA gene was sequenced and the sequence was used to generate phylogenetic trees with nearest relatives.

4.2.1 Material and Methods

4.2.1.1 Bacterial isolate

Isolate TR 90 was selected for phylogenetic analysis using 16S rRNA gene sequence.

4.2.1.2 Isolation of total genomic DNA

Total cellular DNA of TR 90 was prepared by the method as proposed by Silhavy *et al.* (1984). A fresh colony of the respective culture was inoculated in 70ml Luria broth (LB) and incubated at 35^{0}C for 18-20 hr. Cells were harvested through centrifugation (10000 rpm for 10 min at 4 ^{0}C washed with Tris.HCl-EDTA (TE, 50mM: 50mM, pH 8.0) and finally re-suspended in 6 ml of TE (1:1) and were kept at -20 ^{0}C. Frozen cells were thawed followed by the addition of lysozyme (30 mg dissolved in 1 ml TE) and were kept in ice bath for 30 min. Then SDS solution [125 mg dissolved in 1ml of TE (50:50)] was added into it and was incubated at 40 ^{0}C for 30 min. Equal volume of phenol (equilibrated in 50 mM Tris.HCl, pH 8.0) was added and placed in a shaker (100- 125 rpm) at 37 ^{0}C for 15 min. The whole concoction was centrifuged at 12000 rpm for 15 min at 10 ^{0}C and the aqueous phase of it was collected. To this aqueous phase, equal volume of chloroform (Chloroform: Isoamyl alcohol, 24:1) was added. Tubes were inverted several times to facilitate proper mixing, centrifuged and aqueous phase was collected in a beaker. To this, 1/10th volume of 3M sodium acetate (pH 4.5) was added followed by addition of double volume of dehydrated ethanol (chilled). Then DNA was spooled with sterile bent glass rod and air-dried. The dried DNA sample was re-suspended in appropriate amount of TE (50mM: 1mM, pH 8.0) and treated with RNAase (RNAaseA dissolved in 10 mM Tris.Hcl, pH 7.5 with 15 mM NaCl, heated at 100 ^{0}C for

15 min) for 30 min followed by extraction with phenol and reprecipitation with ethanol. Finally the DNA was suspended in TE (10mM: 1mM, pH 8.0). For estimation of DNA, 10 µl of the DNA sample was diluted with 990 µl TE in a 1ml quartz cuvette and absorption was measured at 260 nm. The concentration of DNA was calculated considering that A_{260nm} of 1.0 is equivalent to 50 µg of double-stranded DNA (Towner, 1991).

4.2.1.3 Amplification, cloning and sequencing of 16S rRNA gene of the strains

PCR amplification was performed using 'PCR Amplification Kit' (GENEI, India), in 50µl reaction volume, following instructions provided by supplier. Each 50 µl PCR mix contain; 3 µl of 10mM dNTP mix, 5 µl of 10X buffer containing 15 mM $MgCl_2$, 12.5 pmol of each forward (f) and reverse (r) primers [27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500r (5'-AGAAAGGAGGTGATCCAGGC-3') (Gerhardt *et al.* 1994) corresponding to the *Escherichia coli* numbering system] (Brousius *et al.* 1978)], 20 ng target DNA and 3U *Taq* DNA Polymerase. The PCR was done in a GemAmp PCR system (Applied Biosystems). PCR cycling parameters included an initial denaturation at 94 ^{0}C for 3 min; followed by 30 cycles of denaturation at 94 ^{0}C for 30 sec, annealing at 58 ^{0}C for 30 sec and amplification at 72^{0}C for 1 min and final extension at 72 ^{0}C for 5 min. The 1.5 KB amplicon was purified using PCR purification kit (KT 62, GENEI, India), cloned into pGEM®T–easy vector system II (Promega, USA) and transformed in *E. coli* XL1 Blue following techniques stated in section 4.3.2 of Chapter 4. Plasmid from the resulting clones, extracted by using 'alkaline lysis method' (Birnboim and Doly, 1979), was checked for the presence of 1.5 KB inserts from *Eco*RI digestions prior to sequencing. Sequencing was carried out with an ABI DNA sequenetor model 377a (Applied Biosystems) using Big-Dye Terminator kit (Applied Biosystems).

4.2.1.4 Phylogenetic analysis of the strains

The 16S rRNA gene sequence of the isolate was used as a query to search for homologous sequence in the nucleotide sequence databases by using BlastN program (Altschul *et al.* 1997). The 16S rDNA sequences showing high similarities were retrieved from the GenBank database and were aligned with 16S rRNA gene sequences of the isolate by using CLUSTAL W software (Thompson 1997) of the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw).

Distances were calculated according to Jukes and Cantor (1969) one-parameter, Kimura two-parameter (1980), Tajima and Nei (1987) three-parameter, and Tamura and Nei (1993) four-parameter methods when software package MEGA 3.0 (Kumar *et al.* 2004) was used for generation of phylogenetic trees. When PHYLIP v 3.6 software package (Felsenstein 2002) was used for the same purpose, distances were calculated by using Jukes and Cantor one-parameter and Kimura two-parameter methods.

Phylogenetic trees were inferred by using the neighbour-joining (Saitou and Nei, 1987) and parsimony (Felsenstein 1983) analysis methods. Bootstrap analysis was based on 100 re-samplings. For neighbour-joining and parsimony analysis

both MEGA 3.0 and PHYLIP 3.6c software packages were used; and for Maximum-likelihood analysis only PHYLIP 3.6c was utilized.

4.2.2 Results and discussion

The 1.5 kb 16S rDNA of the isolate TR 90 was amplified and checked in an agarose gel comparing with a molecular size marker (Figure 4.1). The PCR product was purified and cloned in pGEM T-easy vector prior to sequencing. The recombinant plasmid containing 1.5 kb PCR product of TR 90 was named as pMC90 (Figure 4.2).

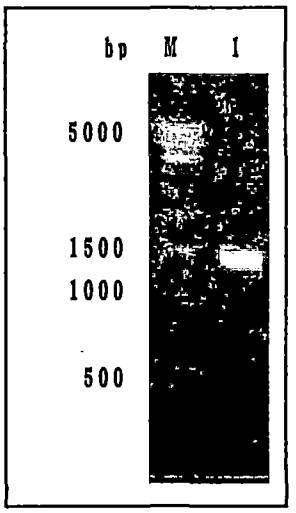


Figure 4.1. Agarose gel electrophoresis of the amplified 16S rRNA gene from isolate TR 90 (Lane 1)using primers 27f and 1500r (M, marker lane).

The 16S rRNA gene sequence (Figure 4.3) of the isolate was deposited in EMBL nucleotide database under accession no. AM156948. The sequence was used as a query to search for homologous sequence in the nucleotide sequence databases by running BlastN program. The consensus sequence, obtained using three replicate sequence reads, was compared with those in GenBank using the BLAST program. The 16S rRNA gene sequences of eleven different strains of *Morganella* showed 99% identity with the 16S rDNA sequence of TR 90, which substantiated the assumption taken from the biochemical tests enlisted in Table 4.4. For neighbor joining, KITSCH, and FITCH analysis, distances were calculated by using both Kimura-two parameter (Kimura 1980) and Jukes-Cantor one parameter (Jukes and Cantor 1969) model with DNADIST. The 16S rDNA sequence of *Plesiomonas shigelloides* (the most closely related species of the *Enterobacteriaceae* family) was used to root all the trees. To determine the confidence values for individual branches, 100 bootstrap replications were done for each generated tree using SEQBOOT and CONSENSE from the PHYLIP package. The phylogenetic tree obtained with Kimura's two-parameter model and the neighbour-joining method (Figure 4.4), showed that isolate TR 90 form a separate cluster with *Morganella species* and therefore undoubtedly a member of the genus *Morganella.* The same results were obtained when phylogenetic distances were calculated by using the Jukes-Cantor one-parameter, Tamura-Nei three-parameter and Tajima-Nei four-parameter models. The maximum-parsimony (Figure 4.5) analysis also showed the same result. Sequence similarity, based on pairwise sequence comparisons, was investigated by using complete 16S rDNA sequences.

4.3 Conclusion

Phylogenetic analysis based on 16S rDNA gene sequence clearly indicated that isolate TR 90 is a species of *Morganella* with which it shared 99% identity. In the phylogenetic trees obtained by neighbour-joining and maximum parsimony methods, TR 90 branched in a cluster with other strains of *Morganella*. The isolate showed some differences in biochemical properties with the *Morganella* type strain. Therefore further exploration of genomic DNA relatedness as well as chemotaxonomic features are required for the identification of proper taxonomic position of this isolate.

4.4 Summary of Chapter 4

Taxonomic characterizations of forty integron-positive isolates were done following principles of numerical taxonomy. Thirty-two isolates were identified as members of the family Enterobactericeae. Six of the isolates were identified as *Pseudomonas* spp. While two isolates were identified as representatives of the genus *Acinetobacter*. Phylogenetic analysis of the isolate TR 90, using 16S rRNA gene sequence, identified it as one of the species of *Morganella*.

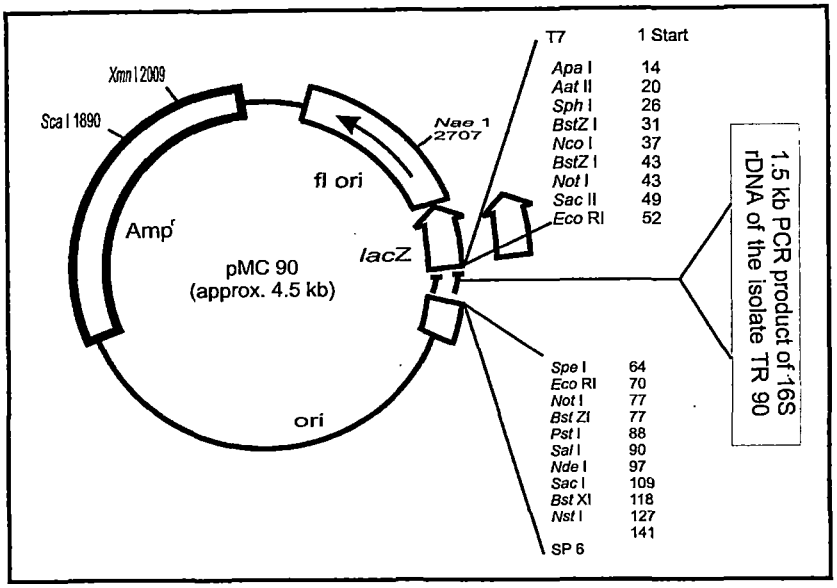


Figure 4.2. Construction of recombinant plasmid pMC 90 (not in scale). *Amp*^{r}, gene for ampicillin resistance; *ori*, origin of replication; *lacZ*, gene for the synthesis of β-galactosidase.

```
   1  ggaattcgat tagagtttga tcctggctca gattgaacgc tggcggcagg cctaacacat
  61  gcaagtcggg cggtaacagg gggaagcttg cttctctgct gacgagcggc ggacgggtga
 121  gtaatgtatg gggatctgcc tgatggcggg ggataactac tggaaacggt agctaatacc
 181  gcataatgtc ttcggaccaa agcgggggac ctcagggcct cgcgccatca gatgaaccca
 241  tatgggatta gctagtaggt gaggtaacgg cttacctagg cgacgatccc tagctggtct
 301  gagaggatga tcagccacac tgggactgag atacggccca gactcctacg ggtggcagca
 361  gtggggaata ttgcacaatg ggcgcaagcc tgatgcagcc atgccgcgtg tatgaagaag
 421  gccttcgggt tgtaaagtac tttcagtcgg gaggaaggtg gtaaggttaa taaccttatc
 481  aattgacgtt accgacagaa gaagcaccgg ctaactccgt gccagcagcc gcggtaatac
 541  ggagggtgca agcgttaatc ggaattactg ggcgtaaagc gcacgcaggc ggttgattga
 601  gtcagatgtg aaatccccgg gcttaacccg ggaattgcat ctgatactgg tcagctagag
 661  tcttgtagag gggggtagaa ttccatgtgt agcggtgaaa tgcgtagaga tgtggaggaa
 721  taccggtggc gaaggcggcc ccctggacaa agactgacgc tcaggtgcga aagcgtgggg
 781  agcaaacagg attagatacc ctggtagtcc acgctgtaaa cgatgtcgac ttggaggttg
 841  tgcccttgag gcgtggcttc cggagctaac gcgttaagtc gaccgcctgg ggagtacggc
 901  cgcaaggtta aaactcaaat gaattgacgg gggcccgcac aagcggtgga gcatgtggtt
 961  taattcgatg caacgcgaag aaccttacct actcttgaca tccagagaac ttagcagaga
1021  tgctttggtg ccttcgggaa ctctgagaca ggtgctgcat ggctgtcgtc agctcgtgtt
1081  gtgaaatgtt gggttaagtc ccgcaacgag cgcaaccctt atcctttgtt gccagcgcgt
1141  gatggcggga actcaaagga gactgccggt gataaaccgg aggaaggtgg ggatgacgtc
1201  aagtcatcat ggcccttatg agtagggcta cacacgtgct acaatggcgt atacaaaggg
1261  aagcgacccc gcgagggcaa gcggaactca taaagtacgt cgtagtccgg attggagtct
1321  gcaactcgac tccatgaagt cggaatcgct agtaatcgta gatcagaatg ctacggtgaa
1381  tacgttcccg ggccttgtac acaccgcccg tcacaccatg ggagtgggtt gcaaaagaag
1441  taggtagctt aacctccggg agggcgctta ccactttgtg attcatgact ggggtgaagt
1501  cgtaacaagg taaccgtaaa tcactagtga attcgcggcc gcctgcag
```

Figure 4.3. 16S rRNA gene sequence of the isolate TR 90 (EMBL Accession no. AM156948)

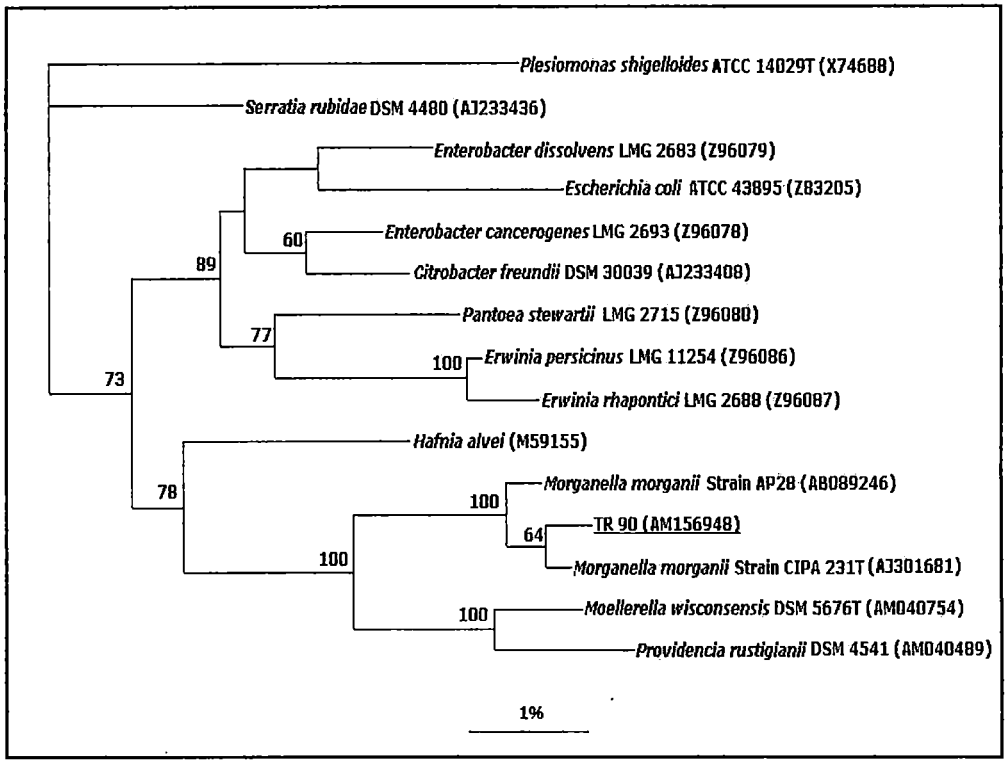


Figure 4.4. Phylogenetic tree derived by neighbor-joining method showing systematic position of TR 90 within the members of the family Enterobacteriaceae. The evolutionary distances were calculated by using Kimura two-parameter model. Bootstrap values for 100 replications (only values above 60 are given) are shown at the branch nodes. The bar represents one substitution per 100 nucleotides.

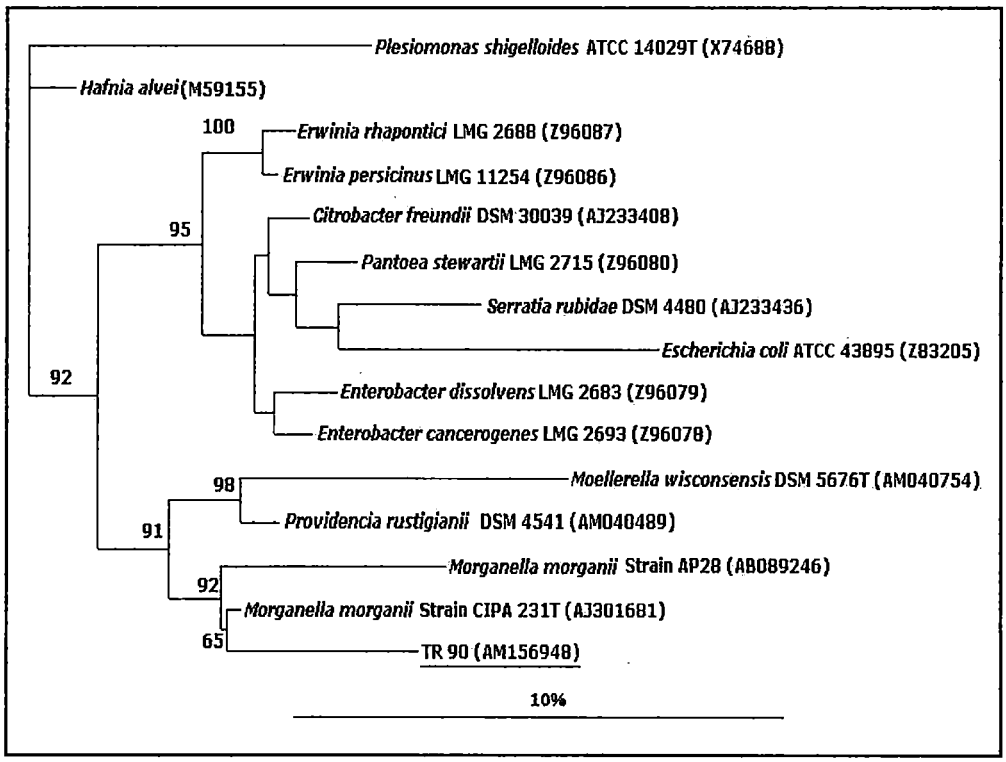


Figure 4.5. Phylogenetic tree derived by maximum-parsimony method showing systematic position of TR 90 within the members of the family Enterobacteriaceae. The bootstrap values were calculated using SEQBOOT program for 100 replications (only values above 60 are given). The bar represents 10 substitutions per 100 nucleotides.

4.5 References

Altschul, S. F., T. L. Madden., A. A. Schaffer., J. Zhang., Z. Zhang., W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new gwnwration of protein database search programs. Nucleic. Acid. Res. **25** : 3389-3402.

Birnboim, H. C, and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic. Acid. Res. **7**: 1513-1523.

Brosius, J., M. L. Palmer., P. J. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli. Proc. Natl. Acad. Sci. USA. **75** : 4801-4805.

Cappucino, J. G, and N. Sherman. Microbiology : Alaboratory manual. Fourth Edition. ISBN 0-8053-0573-4.

Feselstein, J. 1983. Parsimony in systematics: biological and statistical issues. Ann. Rev. Ecol. Syst. **14** : 313-333.

Feselstein, J. 2002. PHYLIP (Phylogeny Inference Package) version 3.6c. Department of genetics, University of Washington, Seattle, USA.

Gerhardt, P., R. G. E. Murray., W. A. Wood, and N. R. Kreig (editors) (1994). Methods for General and Molecular Bacteriology. Washington, DC: American Society for Microbiology.

Jukes, T. H, and C. R. Cantor. 1969. Evolution of protein molecules. In mammalian protein metabolism, pp. 21-132. Edited by H. N. Munro. New York: Academic Press.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comperative studies of nucleotide sequences. J. Mol. Evol. **16** : 111-120.

Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics. **5**: 150-163.

Saitou, N, and M. Nei. 1987. The neighbor-joining method : a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4** : 406-425.

Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory Press.

Tajima, F, and M. Nei. 1987. Estimation of evolutionary distance between nucleotide sequences. Mol. Biol. Evol. **1**: 269-285.

Tamura, K, and M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondria DNA in humans and chimpanzees. Mol. Biol. Evol. **10** : 512-526.

Thompson, J. D., T. J. Gibson., F. Plewniak., F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL _ X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic. Acids. Res. **25** : 4876-4882.

General Discussion and Summary

General Discussion and Summary

Whatever the reasons for antibiotic resistance, such traits are apparently found in many bacteria and in different environments. Any body of water that receives human waste products can be studied for its content of antibiotic resistant bacteria. Earlier studies have reported the occurrence of high frequencies of antibiotic resistant organisms within the SPC populations (Armstrong *et al.* 1982). Antibiotic resistance of the native bacterial population other than those of fecal origin was also an important consideration for the precise assessment of the environmental pool of bacterial antibiotic resistance. Several groups have considered the whole bacterial populations, gram-negative bacteria, heterotrophic bacteria or viable bacteria and dealt with global antibiotic resistance (the frequency of cells able to grow on antibiotic supplemented media). Incidences of antibiotic resistant bacteria in fresh water sources have been reported in certain parts of the world (Kelch and Lee 1978, Niemi *et al.* 1983, French *et al.* 1987, Young 1993, Ogan *et al.* 1993, Ash *et al.* 2002, Roe *et al.* 2003). Earlier studies have shown that rivers in Australia, United States of America and South Africa have become major reservoirs for antibiotic resistant microbes (Boon *et al.* 1999, Ash *et al.* 2002, Lin *et al.* 2004). As river water serves as a major source of water for direct or indirect consumption by humans as well as animals, the presence of antibiotic resistant bacteria in river waterways offers cause for concern.

In the present study, an intensive bacteriological investigation was made of the incidence and abundance of antibiotic resistant copiotrophic bacteria in water samples of the river Torsa. Water samples were collected from three sampling stations, Hashimara (SS I), Falakata (SS II) and Coochbehar (SS III) respectively (shown in Figure 1.1). It was found that the culturable antibiotic-resistant copiotrophic bacteria were widespread in water samples from three different sampling sites located on river Torsa. A wide range of variation in the incidence of recovered bacterial population was recorded. Time series analysis using the SPSS package for determining the trends of occurrences of five different antibiotic resistant bacterial fractions of the culturable copiotrophs showed different trends. Wilcoxon signed pair matched ranks test and ANOVA analysis with two way classified data (with and without replication) were performed to score whether there exists any significant variations in occurrence between the antibiotic resistant bacterial populations with respect to site and season. It was evident from this analysis that percent occurrence of ampicillin and chloramphenicol differed significantly from other antibiotic resistant populations.

This investigation has also documented the occurrence of multiple-antibiotic-resistant (MAR) copiotrophic bacterial isolates in river water of Torsa. Replica plating technique was used to determine the Antibiotic-Resistance- Pattern(s) (ARPs) [against five different antibiotics] of the antibiotic-resistant isolates (that were recovered on plates containing single antibiotic). It was found that more than 90% of the antibiotic-resistant bacteria

obtained from each sampling station were resistant to two or more antibiotics (MAR bacteria). The percent occurrence of population exhibiting resistance to only one antibiotic (singly-resistant) was in the range of 5.04-9.73%. Lin *et al.* (2004) studied the antibiotic resistance profiles of the enteric bacteria isolated from the Mhlathuze catchment and found that 94.7% of these isolates were resistant to at least one class of antibiotic while 75.2% were multi resistant. However, Park *et al.* (2003) showed that 53.6% of coliform isolates of an aquatic environment were resistant to one or more antibiotics tested. An examination of MAR bacteria isolated from three different sampling sites revealed striking differences in types and frequencies. There was no clear pattern of antibiotic resistance in the antibiotic resistant bacterial population isolated from water samples of the river Torsa, collected in different sampling months spanning a period from January 2000 to December 2001. Significant negative correlation was scored between the singly and quadruply resistant; and doubly and quadruply resistant population of SS I. No significant correlation was scored between the antibiotic resistant populations recovered from water samples of SS II. Significant negative correlation between doubly and triply resistant; and singly and quadruply resistant groups was scored among the recovered antibiotic resistant population of the SS III.

Yearlong study on the Torsa river water revealed the occurrence of antibiotic-resistant copiotrophic bacteria. Analysis of the antimicrobial resistance patterns of the isolates revealed their multi-drug resistant nature. A recent study on the river Mahananda, in the northern part of West Bengal, India, has shown that the river is highly contaminated with antibiotic resistant bacteria. It was found that a large proportion of these resistant organisms carried conjugative plasmids with antibiotic resistant traits (Mukherjee *et al.* 2005). For copiotrophic bacterial isolates exhibiting distinct antibiotic resistance patterns, plasmid contents were analyzed and the transferability of the resistance determinants were examined. The bacterial isolates studied for plasmid content and transmissibility of the R plasmids, were recovered from the water samples collected from the sampling station III. Authors of earlier studies have reported that urban effluents contain high levels of antibiotic residues and antibiotic resistant bacteria belonging to the human and animal commensal flora, mainly Enterobacteriaceae (Halling-Sorensen *et al.* 1998, Bhattacherjee *et al.* 1988). It was shown in a previous study that the urban discharge resulted in the increase of resistant strains of riverine autochthonous and allochthonous bacteria (Goni-Urizza *et al.* 2000). The average BOD values calculated from the water samples collected round the year from each sampling site, revealed that water samples from SS III had more BOD compared to the other sites (Bhadra *et al.* 2005). Biological oxygen demand (BOD) is an expression of estimating the organic waste load of water. Therefore, the higher BOD is an indicative of high organic pollution of the water. With higher BOD values for the water samples of SS III, it was expected that the frequency of bacteria exhibiting MAR in the bacterial assemblages would be greater among the population isolated from the site with the putatively high human impact.

One hundred MAR isolates, examined for their plasmid content and conjugal ability,

were chosen from the pool of 4254 antibiotic-resistant bacteria isolated from SS III. The isolates were ampicillin-resistant and exhibited a MAR index ranging from 0.40-1.0 (tested against twelve different antibiotics). All the 100 isolates under study were also resistant to nitrofurantoin. Twenty nine were resistant to amikacin, 73 were resistant to cefotaxim, 96 were resistant to cephalexin, 76 were resistant to chloramphenicol, 57 were resistant to gentamicin, 70 were resistant to kanamycin, 43 were resistant to netilmicin, 53 were resistant to streptomycin, 89 were resistant to tetracycline and 49 were resistant to tobramycin. Sixty-four different MAR combinations were recorded among them. The number of occurrences of different antibiotics in ARPs of the MAR isolates were as follows: Amikacin in 16, ampicillin in 64, cefotaxim in 43, cephalexin in 60, chloramphenicol in 43, gentamicin in 36, kanamycin in 43, netilmicin in 24, nitrofurantoin in 64, streptomycin in 32, tetracycline in 55 and tobramycin in 31. It was hinted by earlier authors (Hsu *et al.* 1992) that differences in percentage of bacterial resistance to various antibiotics may reflect the history of antibiotic application and hence there is a possibility of using bacterial drug resistance as an indicator of antibiotic pollution. Plasmids were detected in seventy-seven isolates. No correlation was scored between the number of antibiotic resistance markers and the numbers of plasmid bands detected. The loss of antibiotic efficacy through the emergence and transfer of bacterial antibiotic resistance is an increasing reality (Kruse and Sorum 1994, Salyers 1997). The rapid dissemination of antibiotic resistance genes in bacterial populations can be partly attributed to plasmid mediated horizontal transfer. With the passage of antibiotic resistance genes from resistant to formerly sensitive bacteria, maintenance of antibiotic resistance in pathogenic native bacteria could provide a reservoir for antibiotic resistance genes. These antibiotic resistant bacteria are significant environmental contaminants. The results of earlier works suggested that antibiotic resistant bacteria survive better than the sensitive bacteria in surface waters (Kelch and Lee 1978). It was observed that R factor mediated antibiotic resistance increased the survival ability of those antibiotic resistant bacteria. It was apparent from the present study that copiotrophic bacterial isolates harbored R plasmid DNA. Some of the donor isolates, TR 01, TR 02, TR 100, exhibited very high rate of conjugal transfer frequency. The donor isolate TR 02 transferred its antibiotic resistance determinants to *E. coli* DH5α with a frequency of 1.44×10^{-3}. In an earlier study, R plasmid transfer frequencies were estimated by conjugation of drug resistant *E. coli* strains isolated from river water with *E. coli* DH5α recipient marked with chromosomal resistance to nalidixic acid. In another study, conjugation between rifampicin resistant *E. coli* K12 and MRE (multidrug resistant Enterobacteriaceae) clinical strains resulted in the transfer of complete resistance patterns at frequencies ranging from 10^{-4} to 10^{-2} (Leverstein van Hall *et al.* 2002). A fairly high rate of transfer (10^{-3}) of an antibiotic resistance plasmid from an *E. coli* donor to *Yersinia pestis* was observed in the flea midgut (Hinnebusch *et al.* 2002). The abundance of antibiotic resistant strains in an environmental setting where bacteria presumably do not come into contact with antibiotics, suggests that resistance genes can also be stably maintained in the absence of antibiotic selection.

Characterization of naturally occurring antibiotic resistance plasmids and understanding their self-transmissible nature enables to address the long term problem of increasing prevalence of antibiotic resistance and strategies aimed against conjugative gene transfer. The construction of gene banks for naturally occurring resistance plasmids (R plasmids) has tremendous biotechnological potential. Novel gene cassettes characterized from the naturally occurring R plasmids like antibiotic resistance genes against newer antibiotics and antimicrobial agents, may help us in designing and construction of novel vectors.

Furthermore, the bacteria identified as resistant to multiple antibiotics were investigated for the presence of antibiotic-resistance gene cassettes in their genetic material. Integron gene sequences had been identified as a primary source of resistance genes within microbial populations. Although gram-positive organisms may be important as reservoir of resistance genes, only those antibiotic-resistant isolates shown to be gram-negative were used for further analysis. The ampicillin-resistant isolates were selected for integron-assay. Earlier reports have shown that presence of class 1 integrons were most frequent among ampicillin-resistant MAR isolates (White *et al.* 2001). Antibiotic ampicillin has been found to be the most abused antibiotic in this region of study (Mukherjee *et al.* 2005). Forty, out of 100 MAR isolates (40%), were found to carry detectable class 1 integron structures. The proportion of strains, in this collection of antibiotic resistant bacteria, carrying integrons has been found comparable to that of other studies. It was reported earlier that 43-75% of antibiotic-resistant clinical bacteria contained class 1 integrons [Levesque *et al.* 1995, Jones *et al.* 1997, Martinez-Freijo *et al.* 1998, Chang *et al.* 2000, Jones *et al.* 2003], while non-selected gram-negative bacteria from an estuarine environment revealed the presence of *int1* 1 gene among 3.6% of the isolates [Rosser and Young 1999]. A study conducted on Rio Grande River (which separates the United States from Mexico) showed that 10% of the *E. coli* isolates were MAR and 13% of MAR contained class 1 integron sequences [Roe *et al.* 2003]. It was mentioned that the MAR index of the 100 isolates assayed for presence of class 1 integrons ranged from 0.41-1.0. The rationality behind the selection criterion lay in the fact that the calculated MAR indices exhibited by 82-100% of the class 1 integron bearing isolates in different studies were in the range of 0.4-0.9 [Guerra *et al.* 2000, Mazel *et al.* 2000, Thungapathra *et al.* 2002].

The presence of class 1 integrons in six *Pseudomonas* spp. and two *Acinetobacter* spp. is significant in the context of gene transfer and dissemination of resistance gene cassettes in the environment. A significant association between integron carriage and higher MAR index was observed. Similar results were noted in earlier studies where significant relationship between multiresistance and presence of integrons was found (Martinez Freijo *et al.* 1998, Schmitz *et al.* 2001, Leverstein van Hall *et al.* 2003). Yielding two amplicons of different sizes with primers 5′ CS and 3′ CS (Table 3.3) have also been noted by earlier authors where they have confirmed by sequence analysis the co-existence of two distinct integrons carrying different gene cassettes in such isolates [Chang *et al.* 2000, Peters *et al.* 2001, Leverstein van Hall *et al.* 2002]. All

the isolates, positive for class 1 integrons, were resistant to multiple antibiotics. Characterization of gene cassettes revealed a significant association between the nature of the gene cassettes and the corresponding antibiotic resistance phenotype of the isolate. The *dfrA1, dfrA5, dfrA7, dfrA12* and *dfrA17* gene cassettes encoding trimethoprim resistance were found among the isolates included in this study. However, *dfrA1* gene cassette obtained from TR 90 was an extended version of the normal *dfrA1*. Analysis of the partial nucleotide sequence of the CS-PCR product of TR 17 revealed the presence of an unpunctuated ORF of 154 amino acids of which only 120 amino acids from the N-terminal end bears 100% identity with the typical dfrA12 protein sequence. A stretch of 34 amino acids from the C-terminal end did not match with existing sequences of the protein database. The polypepetide sequence of the dfrA17 protein obtained from TR 48 was 23 amino acids shorter than the normal dfrA17 and 30 amino acid residues from the C-terminal end of the typical dfrA17 was not present in the translated product obtained from partial sequencing of TR 48 amplicon. Aminoglycoside adenyltransferase (*aadA1 and aadA6*) and aminoglycoside acetyltransferase (*aac-6'-Ib*) gene cassettes were also detected (Table 3.4).

Several studies have addressed the role of integrons in the spread of antibiotic resistance genes by lateral gene transfer (Leverstein van-Hall *et al.* 2002, Chen *et al.* 2004, Aubert *et al.* 2004). The spread of resistance genes is greatly enhanced following the movement of conjugative plasmids containing integrons among different bacterial species. In this study, class 1 integron borne *dfrA5, dfrA12* and *dfrA17* gene cassettes was found to be located on the conjugative plasmids of some MAR bacterial isolates. The horizontal transfer of such plasmids enabled the rapid spread of the gene cassettes among a wide variety of bacterial species (Girlich *et al.* 2001, White *et al.* 2001, Fluit *et al.* 2004).

Taxonomic characterization of the class 1 integron bearing isolates was deemed necessary. Thirty-two integron-positive isolates belonged to the family Enterobacteriaceae, of which two were tentatively identified as *Escherichia* spp., two as *Kluyvera* spp., three as *Proteus* spp., five as *Serratia* spp., nine as *Citrobacter* spp., five as *Providencia* spp., two as *Salmonella* sp., one as *Morganella* sp., one as *Yersinia* sp., and two as Enterobacter spp. Eight integron-positive isolates did not bear any similarity with the members of the family Enterobacteriaceae. Among them, six isolates were tentatively identified as *Pseudomonas* spp. and two were the representatives of the genus *Acinetobacter*. Phylogenetic analysis of the isolate TR 90 bearing a novel ORF, showing partial homology with *dfrA1,* was performed. On the basis of 16S rRNA gene sequence similarity, strain TR 90 was shown to belong to the γ-*Proteobacteria* and to be related to *Morganella morganii* (99%).

Exploring antibiotic resistance and its various causes requires a multidisciplinary and cross-sectoral approach. National initiatives are not enough to reduce the risks and to effectively prevent microorganisms from becoming resistant to antibiotics used in different fields. The rates of antibiotic resistance for specific organisms are often significantly different from country to country and debate has

centered on potential implications of recognized diversities in antimicrobial utilization both in terms of volume and routes of administration (Morris *et al.* 2002). As the problem of antibiotic resistance is a global one, coordinated global surveillance programs are needed to track the changing patterns of antimicrobial resistance. The ecological nature of the problem has to be identified with an aim to recognize the nature of the resistance story.

References

Armstrong, J. L., J. J. Calomiris, and R. J. Seidler. 1982. Selection of antibiotic-resistant standard plate count bacteria during water treatment. Appl. Environ. Microbiol. **44**: 308-316.

Ash, R. J., B. Mauck, and M. Morgan. 2002. Antibiotic resistance of gram-negative bacteria in rivers, United States. Emerg. Infect. Dis. **8** : 713-716.

Aubert, D., D. Girlich., T. Naas., S. Nagarajan, and P. Nordmann. 2004. Functional and structural characterization of the genetic environment of the extended-spectrum β-lactamase bla_{VEB-1} gene from a *Pseudomonas aeruginosa* isolate obtained in India. Antimicrob. Agents. Chemother. **48** : 3284-3290.

Bhadra, B., S. Das., R. Chakraborty and A. K. Nanda. 2005. Investigation of some basic water quality parameter of the North Bengal Terai river Kaljani- a tributary of river Torsa, and comparison their of with the main stream. J. Environ. Biol. **26** : 277-286.

Bhattacherjee, J. W., S. P. Pathak, and A. Gaur. 1988. Antibiotic resistance and metal tolerance of coliform bacteria isolated from Gomati river water at Lucknow city. J. Gen. Appl. Microbiol. **34** : 391-399.

Boon, P. I, and M. Cattanach. 1999. Antibiotic resistance of native and fecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, south-eastern Australia. Lett. Appl. Microbiol. **28**: 164-168.

Chang, C. Y., L. L. Chang., Y. H. Chang., T. M. Lee, and S. F. Chang. 2000. Characterisation of drug resistance gene cassettes associated with class 1 integrons in clinical isolates of *Escherichia coli* from Taiwan, ROC. J. Med. Microbiol. **49** : 1097-1102.

Chen, S., S. Zhao., D. G. White., C. M. Shroeder., R. Lu., H. Yang., P. F. McDermott., S. Ayers, and J. Meng. 2004. Characterization of multiple-antimicrobial-resistant *Salmonella* serovars isolated from retail meats. Appl. Environ. Microbiol. **70** : 1-7.

Fluit, A. C, and F. -J. Schmitz. 2004. Resistance integrons and super integrons. Clin Microbiol Infect. **10** : 272-288.

French, G. L., J. Ling., K. I. Chow, and K. K. Mark. 1987. Occurrence of multiple antibiotic resistance and R plasmid in gram negative bacteria isolated from fecally contaminated fresh water streams. Epidemiol. Infect. **98**: 285-299.

Girlich, D., L. Poirel., A. Leelaporn., A. Karim, and C. Tribuddharat. 2001. Molecular epidemiology of the integron-located VEB-1 extended spectrum beta-lactamase in *nosocomial enterobacterial isolates in* Bangkok, Thailand. J. Clin. Microbiol. **39** : 175-182.

Goni-Urizza, M., M. Capdepuy., C. Arpin., N. Raymond., P. Caumette, and C. Quentin. 2000. Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and *Aeromonas* spp. Appl. Environ. Microbiol. **66**: 125-132.

Guerra, B., S. Soto., S. Cal, and M. C. Mendoza. 2000. Antimicrobial resistance and spread of class 1 integrons among *Salmonella* serotypes. Antimicrob. Agents. Chemother. **44** : 2166-2169.

Halling-Sorensen, B., S. Nors Nielsen., P. F. Lanzky., F. Ingerslev., H. C. Holtenlutzhoft, and S. E. Jorgensen. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment-a review. Chemosphere. **36** : 357-393.

Hinnebusch, B. J., M. L. Rosso., T. G. Schwan, and E. Carniel. 2002. High frequency conjugative transfer of antibiotic resistance genes to *Yersinia pestis* in the flea midgut. Mol. Microbiol. **46**: 349-354.

Hsu, C. H., S. C. Hwang, and J. K. Liu. 1992. Succession of bacterial drug resistance as an indicator of antibiotic application in aquaculture. J. Fish. Soc. Taiwan. **19** : 55-64.

Jones, L. A., C. J. McIver., W. D. Rawlinson, and P. A. White. 2003. Polymerase chain reaction screening for integrons can be used to complement resistance surveillance programs. Commun Dis Intell. **27** : S103–S110.

Jones, M. E., E. Peters., A. M. Weeraink., A. Fluit, and J. Verhoef. 1997. Widespread occurrence of integrons causing multiple antibiotic resistances in bacteria. Lancet. **349** : 1742-1743.

Kelch, W. J, and J. S. Lee. 1978. Antibiotic resistance patterns of gram negative bacteria

isolated from environmental sources. Appl. Environ. Microbiol. **36** : 450-456.

Kruse, H, and H. Sorum. 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl. Environ. Microbiol. **60** : 4015-4021.

Leverstein-van Hall, M. A., H. E. Blok., A. R. Donders., A. Paauw., A. C. Fluit, and J. Verhoef. 2003. Multidrug resistance among enterobacteriaceae is strongly associated with the presence of integrons and is independent of species and isolate origin. J. Infect. Dis. **187** : 251-259.

Leverstein–van Hall, M. A., A. T. A. Box., H. E. M. Blok., A. Paauw., A. C. Fluit, and J. Verhoef. 2002. Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant Enterobacteriaceae in a clinical setting. J. Infect. Dis. **186** : 49-56.

Levesque, C., L. Piche., C. Larose, and P. H. Roy. 1995. PCR Mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents. Chemother. **39** : 185-91.

Lin. J., P. T. Biyela, and T. Puckree. 2004. Antibiotic resistance profiles of environmental isolates from Mhlathuze River, KwaZulu- Natal (RSA). Water SA. **30** : 23-28.

Martinez Freizo, P., A. C. Fluit., F. J. Schmitz., V. S. C. Grek., J. Verhoef, and M. E. Jones. 1998. Class I integrons in gram negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. J. Antimicrob. Chemother. **42** : 689-696.

Mazel, D., B. Dychinco., V. A. Webb, and J. Davies. 2000. Antibiotic resistance in ECOR collection: integrons and identification of a novel *aad* gene. Antimicrob. Agents. Chemother. **44** : 1568-1574.

Morris, A. K, and R. G. Masterton. 2002. Antibiotic resistance surveillance : action for international studies. J. Antimicrb. Chemother. **49** : 7-10.

Mukherjee, S., B. Bhadra., R. Chakraborty., A. Gurung., S. Some, and R. Chakraborty. 2005. Unregulated use of antibiotics in Siliguri city vis-à-vis occurrence of MAR bacteria in community waste water and river Mahananda, and their potential for resistance gene transfer. J. Environ. Biol. **26**: 229-238.

Niemi, M., M. Sibakov, and S. Niemala. 1983. Antibiotic resistance among different species of fecal coliforms isolated from water samples. Appl. Environ. Microbiol. **45** : 79-83.

Ogan, M.T, and D. E. Nwiika. 1993. Studies on the ecology of aquatic bacteria of the Lower Niger Delta: multiple antibiotic resistance among the standard plate count organisms. J. Appl. Bacteriol. **74** : 595-602.

Park, J. C., J. C. Lee., J. Y. Oh., Y. W. Jeong., J. W. Cho., H. S. Joo., W. K. Lee, and W. B. Lee. 2003. Antibiotic selective pressure for the maintenance of antibiotic resistant genes in coliform bacteria isolated from the aquatic environment. Water. Sci. Technol. **47**: 249-253.

Peters, E. D. J., M. A. Leverstein–van Hall., A.T. A. Box., J. Verhoef., and A. C. Fluit. 2001. Novel gene cassettes and integrons, Antimicrob. Agents. Chemother. **45** : 2961-2964.

Roe, M. T., E. Vega, and S. D. Pillai. 2003. Antimicrobial resistance markers of class 1 and class 2 integron bearing *Escherichia coli* from irrigation water and sediments. Emerg. Infect. Dis. **9** : 822-826.

Roe, M. T., E. Vega, and S. D. Pillai. 2003. Antimicrobial resistance markers of class 1 and class 2 integron bearing *Escherichia coli* from irrigation water and sediments, Emerg. Infect. Dis. **9** : 822-826.

Rosser, S. J, and H. K. Young. 1999. Identification and characterization of class 1 integrons in bacteria from an aquatic environment. J. Antimicrob. Chemother. **44** : 11-18.

Salyers, A. A, and C. F. Amiable-Cuevas. 1997. Why are antibiotic resistance genes so resistant to elimination? Antimicrob. Agents. Chemother. **41** : 2321-2325.

Schmitz, F. J., P. Martinez-Freijo., S. Theis., A. C. Fluit., J. Verhoef., H. P. Heinz, and M. E. Jones. 1999. Prevalence of class 1 integrons and association with decreased antibiotic susceptibility in German gram-negative blood culture isolates. Clin. Microbiol. Infect. **5** : 496-498.

Thungapathra, M., Amita, K. K. Sinha., S. Ray Chaudhuri., P. Garg., T. Ramamurthy., G. B. Nair, and A. Ghosh. 2002. Occurrence of antibiotic resistance gene cassettes *aac*(6')-*Ib*, *dfr A5*, *dfr A12*, and *ereA2* in class I integrons in Non-O1, Non-O139 *Vibrio cholerae* strains in India. Antimicrob. Agents. Chemother. **46** : 2948-2955.

White, P. A., C. J. McIVER, and W. D. Rawlinson. 2001. Integrons and gene cassettes in the enterobacteriaceae. Antimicrob. Agents. Chemother. 45 : 2658-2661.

Young, H. K. 1993 Antimicrobial resistance spread in the aquatic environments. Antimicrob. Agents. Chemother. **31**: 627-635.

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- Mukherjee, S, and R. Chakraborty. 2005. Incidence of class 1 integrons in multiple antibiotic resistant gram-negative copiotrophic bacteria from the river Torsa in India. Research in Microbiology. doi : 10.1016/j.resmic.2005.08.003.

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Nucleotide sequence deposited in GenBank/EMBL database

- Mukherjee, S and Chakraborty, R. (2004) *Escherichia* sp. TR 02 partial *dhfr* gene for dihydrofolate reductase type V. Accession no. **AJ620333.**
- Mukherjee, S and Chakraborty, R. (2004) *Pseudomonas* sp. TR 52 *aad*A gene for amino glycoside adenylyltransferase. Accession no. **AJ620334.**
- Mukherjee, S and Chakraborty, R. (2004) Gram negative bacterium TR 59 *aac-6'-Ib* gene for aminoglycoside 6'-N- acetyltransferase . Accession no. **AJ697861.**
- Mukherjee, S and Chakraborty , R . (2004) *Salmonella* sp.TR 95 class 1 integron dihydrofolate reductase like (*dfr*VII) gene , complete sequence . Accession no. **AY604170.**
- Mukherjee,S and Chakraborty , R . (2004) *Citrobacter* sp. TR 17 class 1 integron dihydrofolate reductase like (*dfr*XII) gene. Accession no. **AY604169.**
- Mukherjee, S and Chakraborty , R . (2004) *Kluyvera* sp. TR 97 partial *aad*A1 gene for aminoglycoside adenyltransferase . Accession no. **AJ698461.**
- Mukherjee, S and Chakraborty , R . (2004) *Morganella* sp. TR 90 mRNA for dihydrofolate reductase (*dhfr* gene), allele A1. Accession no. **AJ98460.**
- Mukherjee, S and Chakraborty , R . (2005) *Enterobacter* sp. TR 85 *dhfr*V gene for dihydrofolate reductase type V. Accession no. **AJ867252.**
- Mukherjee, S and Chakraborty , R . (2005) *Serratia* sp. TR 48 partial *dfr 17* gene for dihydrofolate reductase type XVII. Accession no. **AJ868226**
- Mukherjee, S and Chakraborty , R . (2005) *Serratia* sp. TR 40 *aadA1a* gene for aminoglycoside adenyltransferase AAD(3').Accession no. **AJ938160.**
- Mukherjee, S and Chakraborty , R . (2005) *Yersinia* sp. TR 63 partial *aac-6'-Ib* gene for aminoglycoside 6'-N-acetyltransferase. Accession no. **AJ937775.**
- Mukherjee, S and Chakraborty , R . (2005) *Providencia* sp. TR 58 partial *dfrAI* gene for dihydrofolate reductase type 1. Accession no. **AJ937774.**
- Mukherjee, S and Chakraborty , R . (2005) *Providencia* sp. TR 58 *aac-(6')-Ib* gene for aminoglycoside 6'-N- acetyltransferase. Accession no. **AJ938159.**
- Mukherjee, S and Chakraborty , R . (2005) *Morganella* sp. TR 90 16S rRNA gene, isolate TR 90. Accession no. **AM156948.**

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Physico-chemical and bacteriological investigation on the River Torsa of North Bengal.

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Abstract *: A few physico-chemical and bacteriological parameters on certain locations of the river Torsa was studied. The major characteristics of Torsa river water were high alkalinity, high concentration of free ammonia with respect to albuminoid ammonia and the presence of bacteria of fecal origin. Marked seasonal variations of the parameters were also observed.*

Key words *: Water analysis, Torsa River, Physico-chemical and bacteriological characteristics, Alkalinity, Free ammonia and albuminoid ammonia, Fecal pollution.*

Introduction

Torsa, an international river, intersects three countries, China (Tibet), Bhutan and India before entering Bangladesh. Torsa, which is also called as 'Amo-chu', is one of the principal rivers in Western Bhutan. The river flows rapidly and follows a confined valley between precipitous mountains. Even in the winter, 'Amo-chu' is a fierce, swift stream. Torsa cuts across in a southeasterly direction and passes by a market town of Phuntsholing on the Indo-Bhutan Border. In the west of Torsa river Baxa-dolomites form striking ridges, this can be seen, from Phuntsholing (Karan and Jenkins, 1967). As it leaves the foothills of Bhutan and enters the undulating Duars plain in the northern part of West Bengal, it widens into a braided channel that also drains the forest cover of Jaldapara wild life sanctuary. Water quality of this turbulent river, therefore, long remained unaltered being determined by its environment, climate, geologic, hydrologic, physiographic, biological and cultural backdrop. The principal water source of wild life, forest tribes and rural communities is often the river Torsa or some combination of well, pond and river water, depending on season and purposes for which water is used.

With the development of roadways connecting Bhutan and India and advent of modernization of Bhutan in 1950, there have been seen changes in urban and industrial development in and around Phuntsholing lying on the bank of Torsa and adjoining Duars plain in India (Karan and Jenkins, 1967). Indiscriminate dolomite mining in Bhutan hillocks for cement factories leading to large-scale seepage of dolomite in ground and surface water is a well-noted phenomenon (Dutta, 1998; Statesman News Service, 1998). On the other hand, the swelling rural population of this region does not have services of any kind whatsoever, either for potable water or for excreta disposal. The consequent consumption of the river water, which has now degraded, is making the inhabitants of this valley more prone to diseases and health problems. Such observations also put wild life health into question. Furthermore, with industrialization projected to increase many times over the present level, river water pollution will become an even greater concern.

Water quality monitoring of rivers of North Bengal has generally been overlooked for various reasons among which are resources and manpower constraint, institutional inertia, and public apathy due to lack of awareness. In the present investigation, Torsa River was undertaken to examine its water quality for evaluating the impact of various anthropogenic activities.

Materials and Methods

Reagents and other materials : All chemicals were of analytical grade. Deionized water, doubled distilled in glass stills, was used for analytical work. Sterilized sample water bottles were used for collecting water for bacteriological analysis.

Sampling and analysis : Water samples were collected separately for determination of physico-chemical and bacteriological characteristics from three sampling stations (SS-I, Hasimara; SS-II, Falakata; SS-III, Coochbihar) of river Torsa, which are shown in Fig.-1, in January, February, March, May, June, September, October, November and December spanning a period from September 1999 to May 2001. Temperature and pH were measured at the collection sites. The samples were preserved for other parameters in accordance with the Standard methods (APHA, 1989: Manivasakam, 1980). For bacteriological analysis, water samples collected in sterile sample bottles were transported to the laboratory in ice box and minimum elapsed time between collection and analysis in no case did exceed 30 h. Bacteriological analysis consisted of standard plate count, presumptive and confirmatory tests for coliforms and MPN of total coliform, fecal coliform and fecal streptococci (APHA, 1989).

Results and Discussion

Thirty-two samples, consisted of 11, 12 and 9 samples from Hasimara (SSI), Falakata (SSII) and Coochbihar (SSIII) respectively, were collected and studied on monthly basis (except April, July and August) between September 1999 and May 2001. Leaving the peak monsoon months of July and August, the months were grouped as pre-monsoon (March, May and June), post monsoon (September and October) and winter (November to February). The values of various characteristics were shown in Table 1.1 to 1.3.

Air temperature at all spots was recorded regularly throughout the study. The air temperature ranged from 20°C to 37°C. The minimum was recorded in February 2001 at SSII and maximum in October at SSI. The pattern of temperature fluctuation was more or less similar in all the three sampling stations. The temperature of water depends on the season and on the temperature of the ground with which it is in contact. The temperature of Torsa varied from 17.5° to 30°C. A maximum difference of 10-11°C between air and water temperature was observed in the month of October at all sampling stations. A minimum difference of 1°C between air and water temperature was observed at SSII in the month of February. Lower water temperatures were recorded in January and February at all stations. In general, the pH values of Torsa remained >8.0 in all months except the pre-monsoon months. We shall be discussing the implication of higher pH value of Torsa in conjunction with the total alkalinity value.

The conductivity of water depends upon the concentration of ions and its nutrient status and the variation in dissolved solid content is indicated by conductivity measurements. Torsa showed a range of 100 to 280μmhos/cm. The conductivity values of the post monsoon months nearly doubled the values obtained in pre-monsoon months at SSII and III. On the contrary, maximum conductivity value from SSI was obtained in January. A constant value of 120μmhos/cm was observed in pre-monsoon months at SSII. Minimum conductivity value was obtained in the month of June at all stations.

The suspended particles, soil, silt, decomposed or undecomposed organic matter, total dissolved solids as well as microscopic organisms etc. are the main source of turbidity in water, which always interferes with the penetration of light. The turbidity values ranged from 1.0 to 5.5 NTU in winter months. Turbidity in pre and post monsoon months was not studied as this parameter was lately introduced. A maximum value of 69 NTU was observed in May at SSII.

Total dissolved solids in Torsa were found to be low (32.0 to 46.9 mg/l) in March at all stations. The maximum value of 556mg/l was

observed in the month of May at SSI, while the highest values obtained from SSII and III were in the month of June.

The hardness of water reflects the nature of geologic formation with which the water is in contact. Total hardness value of Torsa ranged

Table – 1 : Average values of physico-chemical & microbiological features of water of Torsa River (1999-2001) at Hasimara (Sample Site-I)

Sl. No.	Physico-chemical characters	Pre-monsoon			Post-monsoon			Winter		
		Mar. 2000	May 2000 & 2001	June 2001	Sept. 1999	Oct. 2000	Nov. 2000	Dec. 2000	Jan. 2000 & 2001	Feb. 2001
01.	Air temperature (^{0}C)	27.0	27.6	29.0	32.6	37.0	26.0	26.0	23.0	27.5
02.	Water temperature (^{0}C)	18.5	22.3	25.0	25.5	26.0	21.0	20.5	19.0	20.0
03.	pH	7.1	7.4	8.0	8.5	8.6	8.6	8.3	8.1	8.7
04.	Conductivity (μ mhos/cm)	170	130	100	110	190	200	130	230	190
05.	Turbidity (NTU)	--	--	3.9	--	--	--	1.2	1.3	1.0
06.	TDS (mg/l)	46.9	556	101.3	--	--	--	225.6	206.7	88.0
07.	TSS (mg/l)	229	130.9	196.7	--	--	--	26.3	--	--
08.	Total hardness (mg/l)	--	65.2	36.7	--	74.0	86.0	102.0	38.4	42.4
09.	Ca-hardness (mg/l)	20.8	43.7	9.7	-	16.6	46.1	19.0	11.5	11.3
10.	Mg-hardness (mg/l)	-	21.5	27.0	-	57.4	40.0	83.0	26.9	31.1
11.	Alkalinity (mg/l)	-	-	117.1	-	-	-	13 4.4	-	115.0
12.	Chloride (mg/l)	-	-	6.5	-	-	-	-	5.8	9.9
13.	DO (mg/l)	8.1	7.5	6.7	7.5	7.5	8.2	8.1	8.5	8.2
14.	BOD (For 5 days at 20^0 C (mg/l)	0.75	1.13	0.7	0.2	1.26	0.8	1.3	1.3	0.8
15.	COD (mg/l)	2.0	-	1.5	6.4	6.6	4.8	0.8	2.4	3.2
16.	Free ammonia 'N' (mg/l)	17.0	17.3	15.2	21.7	8.8	36.2	15.5	18.2	61.2
17.	Albuminoid ammonia 'N' (mg/l)	0.31	0.32	0.02	0.51	0.24	1.2	0.49	2.1	3.3
18.	Nitrate 'N' (mg/l)	0.13	0.98	0.12	0.17	0.25	1.2	0.1	0.3	0.1
19.	Nitrite 'N' (mg/l)	0.006	0.008	0.003	0.002	0.007	0.004	0.001	0.004	0.007
20.	Dissolved phosphate (mg/l)	0.77	0.87	0.55	0.68	-	0.26	0.24	0.54	0.18
21.	Total phosphate (mg/l)	-	-	1.1	-	-	-	-	6.77	39.75
22.	Total phosphorus (mg/l)	-	-	0.35	-	-	-	-	2.18	12.8
Microbiological parameters										
01.	Heterotrophic count (CFU/ml)	-	$9x10^4$	$9x10^3$	$5x10^4$	$1x10^6$	$1x10^4$	-	$4.5x10^4$	-
02.	Total coliform (MPN/100 ml)	-	>1600	>1600	>1600	1100	>1600	>1600	100	-
03.	Fecal coliform (MPN/100ml)	-	1600	900	1600	-	900	1600	80	-
04.	Fecal streptococci (MPN/100ml)	-	11	240	70	-	50	70	-	-

- Not done

from 32 to 126 mg/l. This value range of the swift Torsa may be compared with mountainous Bhagirathi at Uttarkashi, Tehri and Deoprayag where the values ranged from 27 to 71 mg/l, being minimum in high flow seasons and maximum in lean seasons (Gautam, 1990). Interestingly, in the

month of October, November and December the total hardness, values of Torsa were characteristically high and on average double, the average value obtained in other months from all the sampling stations. Magnesium-hardness of Torsa ranged from 19.8 to 101.4 mg/l, which is

Table – 2 : Average values of physico-chemical & microbiological features of water of Torsa River (1999-2001) at Falakata (Sample Site-II)

Sl. No.	Physico-chemical characters	Pre-monsoon			Post-monsoon			Winter		
		Mar. 2000	May 2000 & 2001	June 2000 & 2001	Sept. 1999	Oct. 2000	Nov. 2000	Dec. 2000	Jan. 2000 & 2001	Feb. 2001
01.	Air temperature (^{0}C)	29.0	28.5	29.8	31.6	35.0	27.0	27.0	26.5	20.0
02.	Water temperature (^{0}C)	20.5	26.3	24.2	30.0	25.0	22.5	22.0	20.5	19.0
03.	PH	7.4	8.1	8.3	7.8	8.4	8.5	8.1	8.15	8.0
04.	Conductivity (μ mhos/cm)	120.0	120.0	120.0	264	220	270	190	165	190.0
05.	Turbidity (NTU)	-	69.0	-	-	-	-	1.5	1.1	1.1
06.	TDS (mg/l)	34.3	124.5	452.0	-	-	-	203.1	224.3	129.2
07.	TSS (mg/l)	249.0	124.5	129.4	-	-	-	23.1	--	--
08.	Total hardness (mg/l)	-	36.0	59.7	-	90.2	114.0	110.0	50.4	56.4
09.	Ca-hardness (mg/l)	14.4	15.5	29.8	-	19.5	55.7	23.5	13.4	14.9
10.	Mg-hardness (mg/l)	-	20.0	19.8	-	70.3	67.7	86.5	37.0	41.5
11.	Alkalinity (mg/l)	-	113.4	-	92	-	-	113.1	-	110
12.	Chloride (mg/l)	-	6.9	-	-	-	-	-	7.9	10.1
13.	DO (mg/l)	8.6	6.5	7.1	6.9	7.5	7.8	7.9	8.5	8.1
14.	BOD (For 5 days at 20^0 C (mg/l)	0.89	0.70	0.95	1.2	1.3	1.1	0.4	1.6	1.1
15.	COD (mg/l)	2.7	2.6	-	5.4	1.6	4.8	1.2	2.7	9.7
16.	Free ammonia 'N' (mg/l)	16.5	19.0	16.3	9.0	22.2	35.5	8.0	15.4	52.15
17.	Albuminoid ammonia 'N' (mg/l)	0.33	0.43	0.38	0.24	0.47	1.3	0.92	0.23	3.2
18.	Nitrate 'N' (mg/l)	0.20	0.13	1.67	0.29	0.22	1.9	0.17	0.44	0.09
19.	Nitrite 'N' (mg/l)	0.005	0.005	0.006	0.007	0.007	0.003	0.008	0.008	0.008
20.	Dissolved phosphate (mg/l)	0.77	0.30	0.21	-	0.87	0.42	0.30	0.72	0.208
21.	Total phosphate (mg/l)	-	3.78	-	-	-	-	-	4.47	3.52
22.	Total phosphorus (mg/l)	-	1.21	-	-	-	-	-	1.44	1.14
Microbiological parameters										
01.	Heterotrophic count (CFU/ml)	$5x10^4$	$5x10^5$	-	$2x10^6$	$4x10^4$	$3x10^4$	-	$1.9x10^2$	-
02.	Total coliform (MPN/100 ml)	1600	1600	-	1100	1600	500	240	130	-
03.	Fecal coliform (MPN/100ml)	1600	900	-	-	1600	300	50	80	-
04.	Fecal streptococci (MPN/100ml)	900	-	-	-	22	140	23	11	-

- Not Done

higher than the value reported for mountainous Bhagirathi (2-27 mg/l) (Gautam, 1990). In the month of October, November and December, there was a characteristic increment of Mg-

hardness in Torsa (Table 1.1 to 1.3). This observation may be correlated with the dolomite mining in the dry season and subsequent washout in the river water.

Total alkalinity value of Torsa ranged from 82.0 to 134.4 mg/l, which is significantly high compared to the range of 16-60 mg/l in mountainous Bhagirathi stream as observed by

Table – 3 : Average values of physico-chemical & microbiological features of water of Torsa River (1999-2001) at Cooch Behar (Sample Site-III).

Sl. No.	Physico-chemical characters	Pre-monsoon			Post-monsoon			Winter		
		Mar. 2000	May 2001	June 2001	Sept. 1999	Oct. 2000	Nov. 2000	Dec. 2000	Jan. 2001	Feb. 2001
01.	Air temperature (^{0}C)	28.0	31.0	28.5	31.0	35.0	28.0	25.5	23.5	25.0
02.	Water temperature (^{0}C)	19.5	27.5	24.0	29.0	25.0	23.0	20.0	17.5	20.0
03.	pH	7.8	7.8	8.16	8.1	8.4	8.3	8.0	8.16	8.1
04.	Conductivity (μ mhos/cm)	125	150	110	225	280	270	190	140	170
05.	Turbidity (NTU)	-	16	-	-	-	-	1.5	1.0	5.5
06.	TDS (mg/l)	32.0	102.5	380	--	--	--	197.3	-	129.6
07.	TSS (mg/l)	202	650	128.8	--	--	--	21.6	-	-
08.	Total hardness (mg/l)	-	39.17	56.1	-	124.1	130	126	63.6	51.1
09.	Ca-hardness (mg/l)	20.84	15.2	32.3	-	23.5	16.5	24.8	16.8	13.2
10.	Mg-hardness (mg/l)	-	23.9	23.8	-	100.5	63.5	101.4	46.8	37.8
11.	Alkalinity (mg/l)	-	82.0	-	-	-	-	100.4	-	100.2
12.	Chloride (mg/l)	-	7.6	-	-	-	-	-	7.9	11.1
13.	DO (mg/l)	8.6	7.1	7.5	7.1	7.5	7.8	8.3	8.7	8.1
14.	BOD (For 5 days at 20^0 C (mg/l)	1.09	1.9	0.67	1.6	1.4	1.4	1.9	1.6	1.3
15.	COD (mg/l)	4.7	2.9	-	5.4	8.0	-	2.8	2.5	9.8
16.	Free ammonia 'N' (mg/l)	88.3	19.03	15.7	8.7	22.3	23.7	10.6	17.07	50.28
17.	Albuminoid ammonia 'N' (mg/l)	0.41	0.03	0.37	0.29	0.7	1.0	0.92	4.8	3.1
18.	Nitrate 'N' (mg/l)	0.20	0.12	1.05	0.29	0.22	2.2	0.14	0.09	0.09
19.	Nitrite 'N' (mg/l)	0.004	0.005	0.001	0.006	0.007	0.002	0.008	0.006	0.008
20.	Dissolved phosphate (mg/l)	0.61	0.41	0.24	-	1.1	0.46	0.38	0.20	0.21
21.	Total phosphate (mg/l)	-	3.6	-	-	-	-	-	4.47	4.43
22.	Total phosphorus (mg/l)	-	1.17	-	-	-	-	-	1.44	1.43
Microbiological parameters										
01.	Heterotrophic count (CFU/ml)	-	$6.5x10^5$	$3x10^5$	$3x10^5$	$1.9x10^4$	$2.7x10^5$	-	$5.1x10^2$	-
02.	Total coliform (MPN/100 ml)	-	1600	-	-	900	1600	1600	117	-
03.	Fecal coliform (MPN/100ml)	-	1600	-	-	300	1600	1600	117	-
04.	Fecal streptococci (MPN/100ml)	-	80	-	-	22	22	80	-	-

- Not Done

Gautam (1990). Total alkalinity is a measure of bicarbonates, carbonates and hydrates. The alkalinity of Torsa was found higher than the hardness value. Alkalinity and pH are the factors in determining the amenability of the water to biological treatment (Manivasakam, 1980). It is explained that if the alkalinity is greater than hardness, it indicates the presence of basic salts-

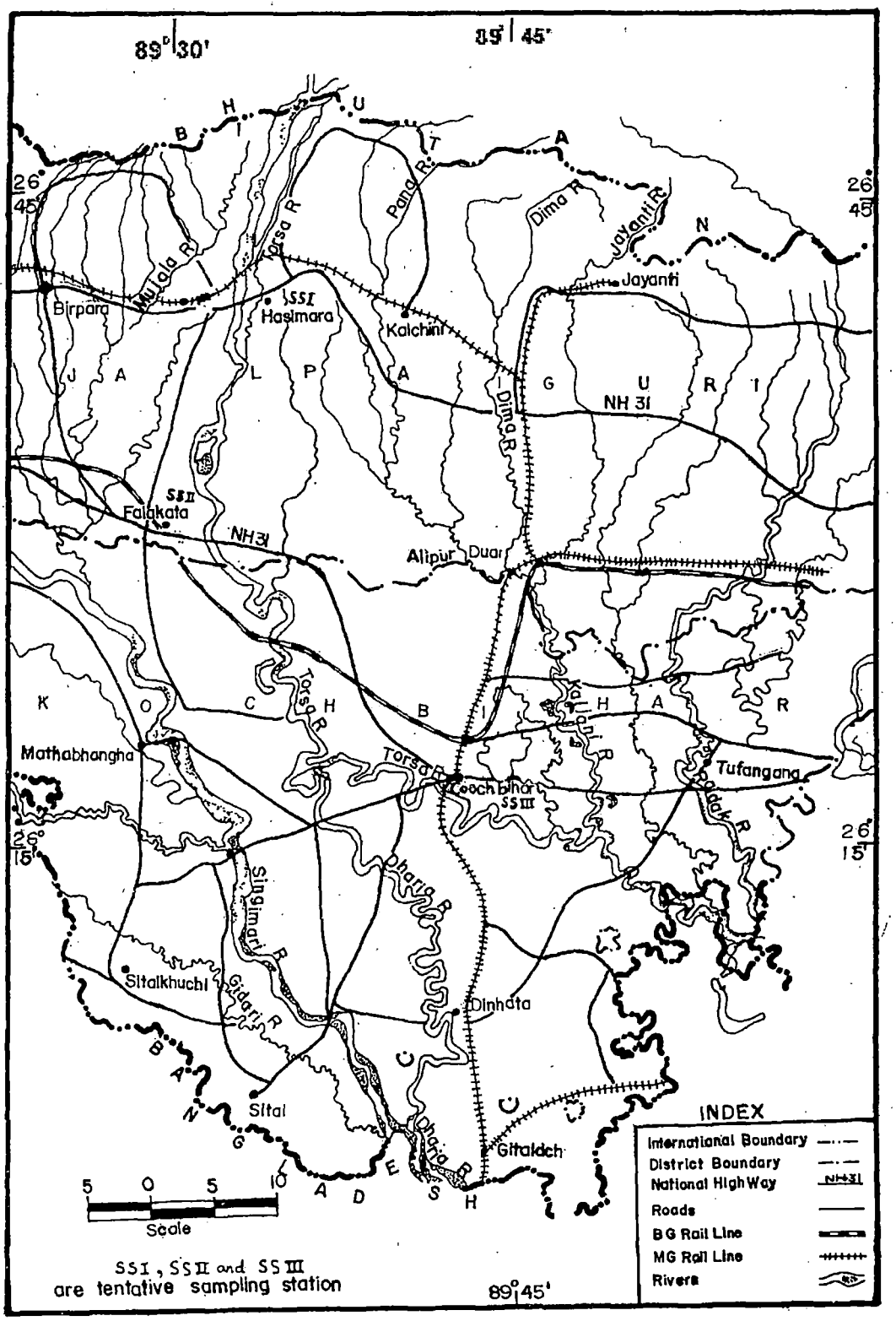

Fig. 1 : Major River system around Torsa Basin in North Bengal.

sodium and potassium in addition to those of calcium and magnesium.

Another parameter, that was introduced lately in our studies was chloride, which is the common anion found in water and sewage. Torsa contained chloride in the range of 5.8 to 11.1. mg/l. Chloride concentration was found relatively high in the month of February from all the stations. The concentration of chloride in Bhagirathi waters varied from 2.8 to 4.3 mg/l (Gautam, 1990).

During the period of investigation the minimum values of DO was recorded 6.5 and 6.7 mg/l at SSII (May) and SSI (June) respectively, while the maximum value of 8.7 mg/l was obtained in January at Sample site III. All the spots showed more or less similar pattern of variation in DO values having higher values during winter. Low temperature and aeration rate during winter was possibly responsible for increased amount of dissolved oxygen. Torrential nature of the river and its gradient may be held responsible for its average high value of DO throughout the course of this river. Bhagirathi at Uttarkashi, Tehri and Deoprayag yielded minimum DO value of 7.0 mg/l and maximum value of 10.9 mg/l (Gautam, 1990).

The BOD value of SSIII was in the range of 1.09 to 1.9 mg/l except for the month of June, which showed 0.67 mg/l. The BOD value fluctuated between 0.7 to 1.6 mg/l at SSII except December, which showed a value of 0.4 mg/l. For SSI the BOD values were in the range of 0.7 to 1.3 mg/l except September that showed 0.2 mg/l. BOD value of Torsa did not exceed 2.0 mg/l. The BOD values of mountainous Bhagirathi fluctuated from 1.5 to 6.9 mg/l (Gautam, 1990). Even within the narrow range, BOD values of Torsa were comparatively higher during winter months at all stations which may be due to low dilution capacity of river during winter. To explain the overall low BOD values in other months, it may be argued otherwise that increased temperature, high flow and increased sediment load reduced the BOD concentration during summer and post monsoon months in river water (Pyatkin and Krivoshein, 1980; Goltermann *et al.*, 1983).

Maximum COD value of 9.7 to 9.8 mg/l was recorded in the month of February from SSII and III. Maximum COD value from SSI was recorded in the post monsoon months (September and October). In case of mountainous Bhagirathi, the COD fluctuated between 5.25 to 11.53 mg/l (Gautam, 1990).

The free ammonia content was many times the content of albuminoid ammonia in the River Torsa. When interpreting the results, the free ammonia and albuminoid ammonia should be considered together, since the relative proportion is more important than the actual quantities. Hence, Torsa water, which contains more free ammonia than albuminoid ammonia, the water, may be suspected of polluted with sewage. The presence of more than traces of ammoniacal nitrogen in river water (which is used as drinking water source) is undesirable. Maximum value of 88.3 mg/l free ammonia was obtained in the month of March from SSIII. All the sampling stations showed higher level of free ammonia in the month of February (50 to 61 mg/l). The ratio of free ammonia to albuminoid ammonia was found much higher in the pre-monsoon months (range, 42 to 761) than the winter (range, 4 to 67). The lowest ratio of four was observed in January from SSIII. A rough guide with regard to the ammonia is that if the albuminoid ammonia content is 0.08 mg/l the free ammonia content should not exceed 0.05 mg/l. If it exceeds the limit, manurial pollution may be suspected (Manivasakam, 1980).

Nitrites are generally formed in water due to bacterial action on ammonia and organic nitrogen. Since nitrites are readily oxidized to nitrates, they are seldom present in significant concentration in surface waters. Nitrite was present in the range of 0.001 to 0.008 mg/l. On the other hand, nitrate content of Torsa River was in the range of 0.09 to 2.2 mg/l. The level of nitrates in mountainous Bhagirathi was observed between 0.001 and 0.99 mg/l (Gautam, 1990). The low level of nitrate as well as nitrite in

comparison to high level of ammonia nitrogen indicates that the nitrogenous organic matter is undergoing oxidation or nitrification and that the process is far from being complete.

Interestingly, the dissolved phosphate content of Torsa River did not exceed 1.0 mg/l while total phosphate and total phosphorus content reached its maximum value of 39.75 mg/l and 12.8 mg/l respectively in the month of February from SSI.

Fecal pollution is a major concern for many rivers of North Bengal where it can originate from human sources and non-human sources (our unpublished observation). Its impact can degrade water quality and restrict its use for drinking and recreational activities. River Torsa receive fecal pollution from a variety of sources, including humans, cattle and wild life. The fecal coliform *Escherichia coli* has been used as an indicator of human enteric pathogens for many years (Goldreich, 1966). However, it is now well established that *Escherichia coli* is not limited to humans but also exists in the intestines of many other warm-blooded animals (Orskov and Orskov, 1981). Consequently, its presence in water is not specific to human sources of pollution. It is, therefore, important to know whether fecal pollution originates from human or non-human source in order to properly assess the risk. There have been attempts to develop methods that differentiate the sources of fecal pollution. Initially, the ratio of fecal coliforms to fecal streptococci was proposed where a ratio of >4.0 would indicate human source pollution, whereas a ration of <0.7 would indicate non-human source pollution (Goldreich and Kenner, 1969). Ratios between 0.7 and 4.4 usually indicate wastes of mixed human and animal sources (APHA, 1989). In the present discussion, we would not like to consider those ratios where fecal streptococcus contents were found below 100/100 ml in order to minimize misinterpretation of ratios. The ratio of fecal coliform to fecal streptococci in Torsa ranged from 1.77 to 3.75. The maximum and minimum fecal coliform : fecal streptocci value was obtained in the month of June and March at SSI and SSII respectively. SSI (Hasimara), which is just the downstream of Phuntsholing and Jaigaon settlement, receives fecal matter of human origin more than SSII. Falakata (SSII) being located after Jaldapara Wild Life Sanctuary, indicates fecal pollution from wastes of mixed human and animal sources in the month of March and November. Total coliform and fecal coliform count was found highest and the least in the month of March and January respectively from all sampling stations.

As it is not possible to recover all viable bacteria in a water sample with a single procedure, we have attempted standard plate count to determine the density of aerobic and facultative anaerobic heterotrophic bacteria in Torsa water. Heterotrophic bacterial load was found maximum ($2x10^6$ CFU/ml) in the post monsoon month of September at SSII. SSI and III record its maximum load of $1x10^6$ and $3x10^5$ CFU/ml in the month of October and September respectively. Low heterotrophic count (1.9 to $5.1x10^2$ CFU/ml) was observed in January at SSII and III. It is also important to note that the water temperatures at the respective sampling station(s) was also at their highest in the respective month exhibiting highest heterotrophic bacterial load. It was also suggested by previous authors that higher water temperature induced the growth of bacteria, which resulted in increased metabolic activity, while low temperature reduced it (Pyatkin and Krivoshein, 1980). Another interesting observation was that when maximum heterotrophic bacterial load of water sample is at respective sampling stations (so far recorded), the free ammonia content detected was the least (See Table 1.1 to 1.3).

The major characteristics in chemical data are high pH, high alkalinity, high magnesium hardness and high free ammonia concentration and corresponding values of albuminoid ammonia concentration suggests high sewage pollution from catchment localities, hence sewage treatment planning in the catchment localities is suggested. High pH, alkalinity and magnesium hardness values indicate the soil erosion due to

excessive mining of dolomites in its catchment area. This study will, therefore, be useful in determining its suitability for different purposes in this region. It may also serve as the database for further studies on this river.

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References

APHA: Standard methods for examination of water and wastewater. 17th ed. APHA Washington D.C. 1197 pp. (1989).

Dutta, K.: Ominous portents. The Statesman. Suppl. NB plus. 27.11.98 (1998)

Gautam, A.: Ecology and pollution of mountain waters (A case study of Bhagirathi river). Ashish Publishing House. New Delhi. India. (1990).

Goldreich, E.E.: Sanitary significance of fecal coliform in the environment. Water Pollution Control Research Series, publication WP-20-3. Federal Water Pollution Control Administration, U.S. Department of the Interior, Cincinnati, Ohio. (1966).

Goldreich, E.E. and B.A. Kenner: Concepts of fecal steptococci in stream pollution. *J. Water Pollut. Control Fed.*, **41**, 336-352 (1969).

Goltermann, H.L., R.G. Sly and R.L. Thomas: Study of the relationship between water quality and sediments. Pub. Unesco. (1983).

Karan, P.P. and Jenkins: Bhutan: A physical and cultural geography. University of Kentucky Press, Lexington, 1967.

Manivasakam, N.: Physico-chemical examination of water, sewage and industrial effluents. Pragati Prakashan, Meerut. India (1980).

Orskov, F. and I. Orskov: *Enterobacteriaceae*, *In* Medical Microbiology and Infectious Diseases, (Ed. A.I. Broude.) The W.B. Saunders Co., Philadelphia, Pa. pp. 340-352. (1981).

Pyatkin, K.D. and Yu. S. Krivoshein: Microbiology (trans. Aksenova and V. Lisovskaya). Mir Publishers, Moscow. 133-135. (1980).

Statesman News Service: Bhutan ready for joint river project. The Statesman, dt. 13.12.98 (1998).

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Incidence of class 1 integrons in multiple antibiotic-resistant Gram-negative copiotrophic bacteria from the River Torsa in India

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Abstract

The presence of class 1 integrons in multiple-antibiotic-resistant (MAR) Gram-negative copiotrophic bacteria from the River Torsa in India was detected using a polymerase chain reaction (PCR)-based screening method. Among 100 isolates that were resistant to at least five of the twelve antibiotics tested, 40 carried class 1 integrons, with inserted DNA regions of 0.7–3.2 kb. Carriage of integrons in strains of higher MAR index was found to be statistically significant. DNA sequencing was used to identify the genetic content of the integron-variable regions. In addition to the identification of gene cassettes *dfrA1, dfrA5, dfrA7, dfrA17* and a variant of *dfrA12* for trimethoprim, *aac(6′)-Ib* for amikacin and tobramycin and *aadA1* and *aadA6* for streptomycin and spectinomycin resistance, a novel ORF predicted from a sequence of *Morganella* sp. TR 90 bearing homology with the *Vibrio cholerae dfrA1* gene cassette was characterized. To our knowledge, this is the first report of the incidence and abundance of class 1 integrons in copiotrophic river water bacteria from India.

Keywords: Antibiotics; MAR bacteria; Class 1 integrons; Gene cassettes; Copiotrophs; Ttrimethoprim resistance

1. Introduction

Integrons are considered to be well-organized vehicles for the transfer of resistance markers in unrelated bacterial populations [17,31]. Integron structures are naturally occurring gene expression systems that can potentially take into custody one or more gene cassettes and convert them into functionally expressed genes [16]. It is these gene cassettes that encode the resistance determinants to several antimicrobial agents [11]. The essential components of an integron include the integrase gene (*intI*), the attachment site (*attI*) and the promoter [9,11,31]. Based on the nature of the integrase, three classes of integrons, with clinical and epidemiological relevance for antibiotic resistance have been described [10,11]. Class 1, the best-characterized integrons, have been frequently reported in clinical [8,18,24,25,36–38] and environmental isolates [7,12,27]. Like the other classes of integrons, their 5′ CS contains the *intI1* and *attI* loci while their 3′ CS is specific and usually contains a truncated antiseptic resistance gene (*qacEΔ1*), a sulfonamide resistance gene (*sulI*) and an open reading frame (ORF5) of unknown function [10,23,31]. Several studies have addressed the prevalence of class 1 integrons in bacteria from different aquatic environments, including fish farms, estuarine environments, rivers, and irrigation water sources [5,28,30,32,33,35].

The Torsa is an international river which crosses through three countries, China (Tibet), Bhutan and India, before entering Bangladesh. Previous studies in our laboratory have shown that the river is contaminated with fecal coliform bacteria [4]. Antibiotic-resistant bacteria have been detected in waters of the Torsa throughout the year (data not shown). In this study, we examined 100 Gram-negative multiple-antibiotic-resistant (MAR) isolates from the river Torsa which resisted 5 or more antibiotics for the presence of class 1 integrons, employing a highly reproducible PCR strategy as described earlier by Levesque et al. [23]. Nucleotide sequence determination of 12 amplicons enabled us to characterize the nature of gene cassettes of 20 isolates out of a total of 40 integron-positive MAR strains. We also presented the description of a new ORF encoding DHFR for trimethoprim resistance.

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2. Materials and methods

2.1. Collection of samples

Water samples were collected from the river Torsa, spanning a period from January 2000 to December 2001. Sampling was done according to standard methodology [1]. Samples were brought to the laboratory on ice and were analyzed within 24 h of collection.

2.2. Antibiotic resistance determination

Antibiotic resistance was determined by the method described earlier [2]. The antibiotics and the concentrations used were as follows: amikacin (25 μg/ml), ampicillin (100 μg/ml), cefotaxim (25 μg/ml), cephalexin (25 μg/ml), chloramphenicol (100 μg/ml), gentamicin (25 μg/ml), kanamycin (50 μg/ml), netilmicin (25 μg/ml), nitrofurantoin (25 μg/ml), streptomycin (100 μg/ml), tetracycline (20 μg/ml), and tobramycin (25 μg/ml). The isolates were considered MAR if growth on at least two different antibiotic-containing plates was at least equal to that on the growth control without antibiotics.

The MAR index of each individual isolate was scored by dividing the number of antibiotics (a) to which the isolate was resistant by the total number of antibiotics (b) to which the isolate was exposed, i.e., a/b [20].

2.3. Selection of 100 MAR isolates for detecting the presence of class 1 integrons

Among a population of 3950 MAR isolates, ampicillin resistance was found in 3786 of them. One-hundred out of 3786 isolates exhibiting a MAR index of 0.41 and above were selected for detecting the presence of class 1 integrons. To identify the presence of an integron, a CS-PCR (conserved-segment polymerase chain reaction) was performed according to the method described earlier [23]. 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 of class 1 integrons, the amplicons contained inserted gene cassettes flanked on both sides by small parts of the CSs [21,24]. Primer Int_2F (5′-CTCGGGTAACATCAAGG-3′), specific for the 3′ region of the integrase gene (approximately 600 bp upstream from the 5′ CS primer site) was used in combination with the 3′ CS primer to show the proximity of inserted gene cassettes to *intI* [24,36].

The amplified products were visualized after electrophoresis through a 1% agarose gel containing ethidium bromide using TAE running buffer, and a 500-bp ladder (Bangalore Genei, India) was used as the molecular size marker.

2.4. Statistical analysis

The observations were classified simultaneously according to two attributes, the MAR index [low (0.41–0.58) and high (0.66–1.0)] and the occurrence of integrons [presence (+) or absence (−)]. The frequencies in the different categories were arranged in a two-way table (known as the 2 × 2 contingency table). The chi-square (χ^2) distribution was then used as a test for independence of attributes, i.e., to test whether the two attributes were associated or not [15].

2.5. Identification of class 1 integron-positive MAR isolates

The cultures were identified according to Bergey's Manual of Systematic Bacteriology [6]. Isolates were placed into genera or groups on the basis of cell and colonial morphology, Gram stain, motility, catalase and oxidase reactions, indole, Voges–Proskauer, methyl red, citrate reactions, gelatin liquefaction, nitrate reduction, urease test, glucose oxidation and carbohydrate fermentations.

2.6. Characterization of integrons by sequencing and restriction fragment length polymorphism (RFLP) typing

The amplicons, generated by using 5′ and 3′ CS primers, were cloned into a pGEM-T easy vector following the manufacturer's instruction and then transformed into *Escherichia coli* XL1 Blue. Transformants were selected on LB agar plates containing 50 μg/ml ampicillin, 0.5 mM isopropylthiogalactosidase and X-gal (80 μg/ml). Transformants were confirmed for the presence of the required insert by PCR reaction using 5′ CS and 3′ CS primers.

Nucleotide sequencing was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit and the reactions were analyzed on an ABI PRISM 377 DNA sequencer. The inserts were sequenced using SP6 and T7 promoter primers.

To determine whether different isolates carried identical integrons, the amplicons of similar sizes were compared by RFLP typing using the *Eco*RI enzyme. If the amplicons from two strains yielded the same RFLP pattern, two integrons were considered to be identical. If the PCR product contained a different RFLP pattern, the new product was sequenced as well.

2.7. Sequence analysis

Nucleotide sequence analysis was performed using BLAST search programs [National Center for Biotechnology Information (NCBI)]. The ProtParam (ExPASy) tool was used to determine the basic physicochemical parameters. PHD topology (Predict Protein secondary structure prediction method) was used for detecting transmembrane helices [34]. The globe prediction algorithm (Predict Protein) was used to examine whether the protein appeared as a compact globular domain or not.

The nucleotide sequences of *dfrA1*, the *dfrA1* variant, *dfrA5*, *dfrA7*, the *dfrA12* variant, *dfrA17*, *aac(6′)-Ib*, *aadA1* and *aadA6* were assigned EMBL and GenBank accession numbers (Table 2).

3. Results

3.1. The antimicrobial resistance pattern of MAR isolates

The antimicrobial resistance pattern of 100 MAR isolates was analyzed. All 100 isolates were resistant to both ampicillin and nitrofurantoin; 29 were resistant to amikacin, 73 were resistant to cefotaxim, 96 were resistant to cephalexin, 76 were resistant to chloramphenicol, 57 were resistant to gentamicin, 70 were resistant to kanamycin, 43 were resistant to netilmicin, 53 were resistant to streptomycin, 89 were resistant to tetracycline and 49 were resistant to tobramycin. Among the 100 MAR bacteria distributed into 8 groups according to the MAR index, 9 isolates were resistant to all 12 antibiotics tested (data not shown).

3.2. Detection of class 1 integrons and RFLP typing of the CS-PCR product

Class 1 integrons were sought in 100 MAR isolates. 40 (40%) of the isolates were shown to carry detectable class 1 integron structures. The remaining 60 isolates did not yield a product. The amplicon lengths, corresponding to the approximate sizes of the inserted DNA cassette, varied from 0.7 to 3.2 kb (Table 1). Among the 40 isolates, 32 yielded a single amplicon and 8 yielded two amplicons of different sizes (Table 1). PCR with a primer combination of Int_2F and 3′ CS resulted in amplicons larger by an approximate size of 600 bp than the amplicons derived from 5′ CS and 3′ CS primers (data not shown).

A 2.0-kb amplicon recorded in eight strains (TR 6, TR 10, TR 11, TR 12, TR 13, TR 14, TR 15 and TR 17), yielded

Table 1
MAR indices and sizes of inserted gene cassettes for forty Gram-negative integron-positive isolates from the River Torsa

MAR index	Isolates carrying class integron	Identification	Approximate length of amplicons generated with 5′cs and 3′cs (bp)
0.41	TR 53	*Escherichia* sp.	2700
0.50	TR 97	*Kluyvera* sp.	2000
	TR 76	*Proteus* sp.	2000
	TR 48	*Serratia* sp.	1600
0.58	TR 77	*Proteus* sp.	1200, 1800
	TR 39	*Serratia* sp.	1900, 3000
0.66	TR 6, TR 10 and TR 14	*Citrobacter* spp.	2000
	TR 81	*Providencia* sp.	1300
	TR 40	*Serratia* sp.	3200
0.75	TR 11, TR 12, TR 13, and TR 15	*Citrobacter* spp.	2000
	TR 85	*Enterobacter* sp.	800
	TR 90	*Morganella* sp.	1177
	TR 56	*Proteus* sp.	1200, 1800
	TR 18	*Providencia* sp.	1200
	TR 95	*Salmonella* sp.	718
	TR 27 and TR 92	*Pseudomonas* spp.	1200, 1800
	TR 99	*Pseudomonas* sp.	1200
0.83	TR 91	*Citrobacter* sp.	800
	TR 02	*Escherichia* sp.	750
	TR 73	*Providencia* sp.	900
	TR 79	*Salmonella* sp.	900
	TR 63	*Yersinia* sp.	900
	TR20	*Acinetobacter* sp.	1300
0.91	TR 04	*Enterobacter* sp.	800
	TR 68	*Providencia* sp.	1200, 800
	TR 78	*Serratia* sp.	900
	TR 82	*Serratia* sp.	1200
	TR 49	*Acinetobacter* sp.	1100, 900
	TR 59	*Pseudomonas* sp.	811
1.0	TR 17	*Citrobacter* sp.	2000
	TR 37	*Kluyvera* sp.	3000
	TR 58	*Providencia* sp.	1200, 800
	TR 52	*Pseudomonas* sp.	1400
	TR 62	*Pseudomonas* sp.	1400

the same RFLP pattern upon digestion with the *Eco*RI enzyme (data not shown). Similarly, a 1.4-kb amplicon of TR 52 and TR 62 digested with *Eco*RI also yielded identical RFLP patterns.

3.3. Identification of integron-positive isolates

Biochemical characterization of integron-positive isolates revealed that 32 out of 40 isolates belonged to the family Enterobacteriaceae. The other 8 Gram-negative copiotrophic isolates did not belong to this family (Table 1). The isolates were identified up to the genus level following the principles of numerical taxonomy (data not shown).

3.4. Test of significance

The number of integron-positive isolates in low and high MAR index categories was 6 and 34; similarly, the number of integron-negative isolates was 28 and 32, respectively. On the hypothesis of independence, test statistics followed χ^2 distribution with 1 degree of freedom. Since the observed statistical value, 12.18, was found to be greater than the tabulated value (value for χ^2 for 1 d.f. at 5% level is 3.84), it was significant. We therefore rejected the null hypothesis at the 5% level of significance and concluded that the attributes were not independent, i.e., data supported the hypothesis that the MAR index and integron carriage were associated.

3.5. Identification of integron-borne gene cassettes

Purified CS-PCR products of TR 02, TR 17, TR 40, TR 48, TR 52, TR 58, TR 59, TR 63, TR 85, TR 90, TR 95 and TR 97 cloned in a pGEM-T Easy Vector were subjected to DNA sequencing and gene cassettes were identified by sequence analysis (Table 1). Characterization of gene cassettes revealed a significant association between the nature of the gene cassettes and the corresponding antibiotic resistance phenotype of the isolates. All integron-carrying isolates expressed resistance to cotrimoxazole (trimethoprim + sulfonalamide). The most common carriage by integron-positive isolates involved dihydrofolate reductase cassettes, including a novel ORF showing partial homology with *dfrA1*, which represented 70% of the cassettes detected (Table 2). Aminoglycoside adenyl transferase (*aadA1* and *aadA6*) gene cassettes were detected in four isolates, while the aminoglycoside acetyltransferase (*aac(6′)-Ib*) gene cassette was detected in only two isolates (Table 2). Resistance to other antibiotics like ampicillin, cephalosporins, chloramphenicol, nitrofurantoin and tetracycline did not correspond to identified gene cassettes. The sequence derived from the 1.8-kb amplicon of *Providencia* sp. TR 58 showed 100% identity with the *Vibrio cholerae* class 1 integron *dfrA1* gene (Ac. No. AF221901). The sequence derived from *E. coli* TR 02 showed 100% identity with the *E. coli* class 1 integron *dfrA5* gene (Ac. No. AJ419169), while the *Enterobacter* sp. TR 85 sequence revealed 98% identity with the *E. coli* class 1 integron *dfrA5* gene (Ac. No. AJ419169); the sequence from *Salmonella* sp. TR 95 shared 98% identity with the *Salmonella enterica* subsp. *enterica* serovar Typhi dihydrofolate reductase type *VII* gene (Ac. No. AY245101). The resulting sequence from *Serratia* sp. TR 48 showed 98% identity with the *Salmonella* sp. S126 class 1 integron *dfrA17* gene (Ac. No. AY263739). BLASTP analysis of the sequence derived from *Pseudomonas* sp. TR 52 showed 100% identity with amino glycoside adenyl transferase AAD A6 of *Pseudomonas aeruginosa* (Ac. No. AF140629). The translated partial *aadA1* sequence of *Kluyvera* sp. TR 97 showed 97% (189/193) identity with AAD A1 of *E. coli* isolate Ec 1484R (Ac. No. AY224185) and *S. typhimurium* (Ac. No. AJ496285). The partial CDS of the *aadA1* gene as derived from *Serratia* sp. TR 40 expressed 100% identity with the same gene from the *E. coli* isolate Ec1484R class 1 integron (Ac. No. AY224185). The complete CDS of *aac(6′)-Ib* from *Pseudomonas* sp. TR 59 shared identity of 98 and 97% at the nucleotide and protein levels with amino glycoside 6′-*N*-acetyl transferase of the *Vibrio cholerae* class 1 integron (Ac. No. AY103455), while the partial CDS of the same gene from *Serratia* sp. TR 63 shared 99% identity with the same *V. cholerae* (Ac. No. AY103455) class 1 integron. The sequence derived from the 1.2-kb amplicon of TR 58 showed 99% identity with *aac(6′)-Ib* gene of the *Burkholderia cepacia* class 1 integron (Ac. No. AF371964).

BLASTN analysis of the 1177-bp sequence of *Morganella* sp. TR 90 showed the highest identity, 98% (1131/1150), with the *V. cholerae* class 1 integron sequence (Ac. No. AF455254) reported from India. Two ORFs, ORF1 and ORF2, were predicted from the TR 90 sequence to code for polypeptide sequences of 218 and 148 amino acids, respectively. The first 152 amino acid sequences of ORF1 which yielded the best scores were all DHFR type I sequences. It is interesting to note that all *dfrA1* protein sequences were 157 amino acids long and the first 152 residues of this novel ORF shared 95% identity with the existing *dfrA1* type. A stretch of 33 amino acid residues, from residue 153 to 185, did not show any resemblance to the existing sequences of the protein databases. Furthermore, another stretch of 25 amino acid residues, from residue 186 to 210, bore 60% identity to the E1 protein of *E. coli*. Again, the remaining C-terminal amino acids did not match with any of the existing protein sequences. Using the ProtParam (ExPASy) tool, the estimated half-life and the instability index (II) of the predicted protein were computed to be 10 h (*E. coli* in vivo) and 32.08, respectively, which classified the protein as stable. The dihydrofolate reductase signature sequence of this protein, revealed via the PROSITE motif search [1], was 'VIGngpdIP-Wsakg.EqIIFkaiT'. The predicted secondary structure (PHD) composition of the ORF1 protein was: *H* (denoting α-helix) = 19.25%; *E* (denoting extended β-strand/sheet) = 29.58%; and *L* (denoting others, loop) = 51.17%, which was comparable to the *dfrA1* protein composed of *H* = 19.75%, *E* = 31.21%, and *L* = 49.04%. Both the ORF1 protein and the *dfrA1* protein appeared as compact globular domains in the Globe prediction algorithm and no bonded cysteine was found in them, as revealed by the CYSPRED algorithm (Predict Protein). The predicted secondary structure and the presence of a dihydrofolate reductase signature indicated that the TR 90 protein might function like other DHFRs, similarly to the *dfrA1* protein, and might also confer resistance to trimethoprim.

Table 2
Characterization of gene cassettes in MAR isolates from the River Torsa

Isolate	Antimicrobial resistance profile[a]	Nature of the gene cassette	Cassette-encoded antibiotic resistance	Accession No.
TR 97	AMP, CEF, CEP, CHL, COT, NIT, TET	*aadA1*	STR	AJ698461
TR 48	AMP, CEF, CEP, COT, NIT, TET, TOB	*dfrA17*	TMP	AJ868226
TR 6	AMP, CEF, CEP, CHL, COT, KAN, NIT, STR, TET	*dfrA12*	TMP	–[b]
TR 10	AMP, CEF, CEP, COT, GEN, NIT, STR, TET, TOB	*dfrA12*	TMP	–[b]
TR 14	AMP, CEF, CEP, CHL, COT, GEN, NIT, STR, TET	*dfrA12*	TMP	–[b]
TR 40	AMP, CEP, COT, GEN, KAN, NIT, STR, TET, TOB	*aadA1*	STR	AJ938160
TR 11, TR 12, TR 13	AMP, CEF, CEP, COT, GEN, NET, NIT, STR, TET, TOB	*dfrA12*	TMP	–[b]
TR 15	AMI, AMP, CEF, CEP, COT, GEN, NET, NIT, TET, TOB	*dfrA12*	TMP	–[b]
TR 85	AMI, AMP, CEP, COT, GEN, KAN, NET, NIT, TET, TOB	*dfrA5*	TMP	AJ867252
TR 90	AMP, CEF, CEP, CHL, COT, GEN, KAN, NET, NIT, TET	*dfrA1* variant	TMP	AJ698460
TR 95	AMP, CEF, CEP, COT, KAN, NET, NIT, STR, TET, TOB	*dfrA7*	TMP	AY604170
TR 02	AMP, CEF, CEP, CHL, COT, GEN, KAN, STR, TET, NIT, TOB	*dfrA5*	TMP	AJ620333
TR 63	AMI, AMP, CEP, CHL, COT, GEN, KAN, NET, NIT, TET, TOB	*aac(6′)-Ib*	AMI	AJ937775
TR 59	AMI, AMP, CEF, CEP, CHL, COT, GEN, KAN, NET, NIT, TET, TOB	*aac(6′)-Ib*	AMI	AJ697861
TR 17	AMI, AMP, CEF, CEP, CHL, COT, GEN, KAN, NET, NIT, STR, TET, TOB	*dfrA12*	TMP	AY604169
TR 58	AMI, AMP, CEF, CEP, CHL, COT, GEN, KAN, NET, NIT, STR, TET, TOB	*dfrA1*,	TMP	AJ937774
		aac(6′)-Ib	AMI	AJ938159
TR 52	AMI, AMP, CEF, CEP, CHL, COT, GEN, KAN, NET, NIT, STR, TET, TOB	*aadA6*	STR	AJ620334
TR 62	AMI, AMP, CEF, CEP, CHL, COT, GEN, KAN, NET, NIT, STR, TET, TOB	*aadA6*	STR	–[c]

[a] AMI: amikacin; AMP: ampicillin; CEF: cefotaxim; CEP: cephalexin; CHL: chloramphenicol; COT: cotrimoxazole; GEN: gentamicin; KAN:kanamycin; NET: netilmicin; NIT: nitrofurantoin; STR: streptomycin; TET: tetracycline; TMP: trimethoprim; TOB: tobramycin.
[b] RFLP pattern similar to TR 17 amplicon.
[c] RFLP pattern similar to TR 52 amplicon.

4. Discussion

In the present study, we examined 100 Gram-negative MAR bacteria from the Torsa River and found that 40% contained a detectable class 1 integron structure. The proportion of strains in this collection of antibiotic-resistant bacteria carrying integrons is comparable to that of other studies. For example, 43–75% of antibiotic-resistant clinical bacteria contained class 1 integrons [8,18,19,23,24], while non-selected Gram-negative bacteria from an estuarine environment revealed the presence of the *intI1* gene among 3.6% of the isolates [33]. A study conducted on the Rio Grande River (which separates the United States from Mexico) showed that 10% of the *E. coli* isolates were MAR and 13% of MAR contained class 1 integron sequences [32]. These results, together with those obtained in the present study, indicate that class 1 integrons are widespread in clinical as well as in environmental samples. The MAR index of the 100 isolates under study ranged from 0.41–1.0. The rationale behind the criterion of selecting MAR isolates with such MAR indices for detecting class 1 integrons lay in the fact that the calculated MAR index exhibited by 82–100% of class 1 integron-bearing isolates was in the range of 0.4–0.9 [14,26,37]. In the present study, we did not find any significant difference in the incidence of integrons among members of the *Enterobacteriaceae* (32 integron-positive out of 81 MAR isolates) and non-enterobacterial members (8 integron-positive out of 19 MAR isolates), which is similar to the observation made by earlier authors [33]. The presence of class 1 integrons in six *Pseudomonas* spp. and two *Acinetobacter* spp. is significant in the context of gene transfer and dissemination of resistance gene cassettes in the environment. An earlier study on two European rivers expressed clinical concern over the development of drug resistance in mesophilic *Aeromonas* spp. [13]. Among enteric members, the genus *Citrobacter* represented the highest proportion of integron-positive isolates (8 of 40). Although, in this study, copiotrophs were cultured from river water samples and were deemed to be environmental samples, it is possible that some of the strains, particularly those identified as col-

[20] P.H. Krumperman, Multiple antibiotic resistance indexing of *Escherichia coli* to identify high risk sources of fecal contamination of foods, Appl. Environ. Microbiol. 46 (1983) 165–170.

[21] M.A. Leverstein-van Hall, A.T.A. Box, H.E.M. Blok, A. Paauw, A.C. Fluit, J. Verhoef, Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant enterobacteriaceae in a clinical setting, J. Infect. Dis. 186 (2002) 49–56.

[22] M.A. Leverstein-van Hall, H.E. Blok, A.R. Donders, A. Paauw, A.C. Fluit, J. Verhoef, Multidrug resistance among enterobacteriaceae is strongly associated with the presence of integrons and is independent of species and isolate origin, J. Infect. Dis. 187 (2003) 251–259.

[23] C. Levesque, L. Piche, C. Larose, P.H. Roy, PCR Mapping of integrons reveals several novel combinations of resistance genes, Antimicrob. Agents. Chemother. 39 (1995) 185–191.

[24] P. Martinez Freizo, A.C. Fluit, F.J. Schmitz, V.S.C. Grek, J. Verhoef, M.E. Jones, Class 1 integrons in gram negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds, J. Antimicrob. Chemother. 42 (1998) 689–696.

[25] P. Martinez Freizo, A.C. Fluit, F.J. Schmitz, J. Verhoef, M.E. Jones, Many class I integrons comprise distinct stable structures occurring in different species of enterobacteriaceae isolated from widespread geographic regions in Europe, Antimicrob. Agents. Chemother. 43 (1999) 686–689.

[26] D. Mazel, B. Dychinco, V.A. Webb, J. Davies, Antibiotic resistance in the ECOR collection: Integrons and identification of a novel *aad* gene, Antimicrob. Agents. Chemother. 44 (2000) 1568–1574.

[27] S. Nandi, J.J. Maurer, C. Hofacre, A.O. Summers, Gram-positive bacteria are a major reservoir of class 1 antibiotic resistance integrons in poultry litter, Proc. Nat. Acad. Sci. USA 101 (2004) 7118–7122.

[28] J.C. Park, J.C. Lee, J.Y. Oh, Y.W. Jeong, J.W. Cho, H.S. Joo, W.K. Lee, W.B. Lee, Antibiotic selective pressure for the maintenance of antibiotic resistant genes in coliform bacteria isolated from the aquatic environment, Water Sci. Technol. 47 (2003) 249–253.

[29] E.D.J. Peters, M.A. Leverstein-van Hall, A.T.A. Box, J. Verhoef, A.C. Fluit, Novel gene cassettes and integrons, Antimicrob. Agents. Chemother. 45 (2001) 2961–2964.

[30] A. Peterson, L. Guardabassi, A. Dalsgaard, J.E. Olsen, Class 1 integrons containing a *dhfrI* trimethoprim resistance gene in cassette in aquatic *Acinetobacter* spp., FEMS Microbiol. Lett. 182 (2000) 73–76.

[31] G.D. Reechia, R.M. Hall, Gene cassettes: A new class of mobile element, Microbiology 141 (1995) 3015–3027.

[32] M.T. Roe, E. Vega, S.D. Pillai, Antimicrobial resistance markers of class 1 and class 2 integron bearing *Escherichia coli* from irrigation water and sediments, Emerg. Infect. Dis. 9 (2003) 822–826.

[33] S.J. Rosser, H.K. Young, Identification and characterization of class 1 integrons in bacteria from an aquatic environment, J. Antimicrob. Chemother. 44 (1999) 11–18.

[34] B. Rost, C. Sander, Third generation prediction of secondary structure, Methods Mol. Biol. 143 (2000) 71–95.

[35] A.S. Schmidt, M.S. Bruun, I. Dalsgaard, J.L. Larsen, Incidence, distribution and spread of tetracycline resistance determinants and integron associated antibiotic resistance genes among motile aeromonads from a fish farming environment, Appl. Environ. Microbiol. 67 (2001) 5675–5682.

[36] F.J. Schmitz, D. Hafner, R. Geisel, P. Follmann, C. Kirschke, J. Verhoef, K. Kohrer, A. Fluit, Increased prevalence of class 1 integrons in *Escherichia coli*, *Klebsiella* species and *Enterobacter* species isolated over a 7 year period in a German University hospital, J. Clin. Microbiol. 39 (2001) 3724–3726.

[37] M. Thungapathra, Amita, K.K. Sinha, S.R. Chaudhuri, P. Garg, T. Ramamurthy, G.B. Nair, A. Ghosh, Occurrence of antibiotic resistance gene cassettes *aac(6)-Ib*, *dfrA5*, *dfrA12*, and *ereA2* in class 1 integrons in Non-O1, Non-O139 *Vibrio cholerae* strains in India, Antimicrob. Agents. Chemother. 46 (2002) 2948–2955.

[38] P.A. White, C.J. McIver, W.D. Rawlinson, Integrons and gene cassettes in the enterobacteriaceae, Antimicrob. Agents. Chemother. 45 (2001) 2658–2661.

Stress Biology
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TRIMETHOPRIM STRESS RESISTANCE BY CLASS I INTEGRON BORNE ALTERED DIHYDROFOLATE REDUCTASE GENE CASSETTE OF AN *E. COLI* ISOLATE OF TORSA RIVER

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INTRODUCTION

Bacteria collect and exchange genetic information with extraordinary facility and lack of species specificity, permitting antibiotic resistance already present in the environment to be "picked up" and passed on from one microbe to the other (Davies, 1994). During times of stress, commensal and pathogenic bacteria demonstrate the ability to become hyper mutable (Shapiro, 1999) and they duplicate and share survival information (Roberts, 1999), such as resistance genes that can be encoded on plasmids, transposons and integrons. Given the inevitability that resistant strains of bacteria are emerging in response to widespread use of antibiotics, progress has been made for the development of new structural class of synthetic molecules with broad spectrum and acceptable potency. Trimethoprim is such a broad-spectrum synthetic antimicrobial agent, which came into human therapeutic use in 1968 in combination with sulfonamides (Bushby, 1968). In the 1970s TMP alone came into use first for the prophylaxis of urinary tract infections and later for the treatment of acute urinary tract infections as well (Kasanen, 1982). Trimethoprim interferes with folate synthesis in susceptible bacteria. It competes with the normal substrate of dihydrofolate reductase as it bears structural analogy to dihydrofolate. Dihydrofolate reductase has been isolated from almost every type of living cell, with the exception of archaebacteria and a few parasitic protozoa. The main function of this enzyme is to convert dihydrofolate to tetrahydrofolate, which is a methyl group shuttle, required for the denovo synthesis of purines, thymidylic acid and certain amino acids. The enzyme from animals and some microorganisms also slowly reduces folate to 5, 6, 7, 8-tetrahydrofolate. As trimethoprim acts by blocking the production of tetrahydrofolate from dihydrofolate by binding to and reversibly inhibiting the enzyme dihydrofolate reductase, this antibiotic selectively interferes with bacterial synthesis of nucleic acids (thymidylate) and proteins (Houvinen *et al,* 1995).

Bacterial resistance to trimethoprim is due to a variety of mechanisms. Mobile genetic elements including plasmids, transposons and integrons that encode trimethoprim resistance lead to the generation of an additional mutant DHFR that is less sensitive to inhibition by trimethoprim. Chromosomal resistance to trimethoprim maybe due to the

chromosomal location of transposon Tn 7, or due to some kind of mutational loss which hinders the bacterial ability to methylate deoxyuridylic acid to thymidylic acid causing a low level resistance to TMP or it maybe due to mutational changes in the gene for DHFR. All these changes could be combined with regulatory mutations, leading to the cellular overproduction of the enzyme and very high levels of TMP resistance. Plasmid mediated resistance is caused by non-allelic and drug insusceptible variants of chromosomal DHFR.

Now-a-days there is growing concern regarding the emergence of TMP resistant microorganisms. The mechanisms of resistance and of its spread among pathogenic bacteria show a remarkable evolutionary adaptation. Different *dfr* genes seem to have arisen in different parts of the world and then spread to geographically distant regions. Horizontal genetic exchange plays a vital role in this dissemination. Epidemiological studies reveal the location of various TMP resistance genes on a type of transferable unit called a cassette, which maybe exchanged between different integrons. Two of the first observed integron cassettes were those carrying the TMP resistance gene *dhfr* IIb and *dhfr* V (Sundstrom *et al,* 1988). To date more than 16 DHFRs have been characterized in gram-negative facultative bacilli.

In this paper we report the detection of the presence of class I integron in a multiple antibiotic resistant (MAR) environmental isolate from River Torsa of North Bengal, India. The MAR isolate was identified as *Escherichia coli* strain TR2 possessing self-transmissible plasmid bearing antibiotic resistance genes. The gene cassette, loaded in the integron structure identified in this isolate, was sequenced and analyzed. The indigenous dihydrofolate reductase gene cassette conferred resistance to withstand trimethoprim stress on TR2 isolate.

MATERIALS AND METHODS

Isolation of MAR bacteria: Culturable bacteria were recovered by spread plating 0.1 ml volumes of diluted river water samples onto LB agar plates. Thousands of bacterial colonies were picked at random & their MAR profile was determined against 12 different antibiotics by a replica plate method (Armstrong *et al* ,1981). This method uses colonies on a master plate upon which nearly 100 isolates had been inoculated, followed by incubation at 37 c for 24 hours. These master plates were then replicated onto LB agar plates supplemented with antibiotics. Each master plate was also replicated onto LB agar plates without antibiotics as a growth control. The antibiotics & the concentrations used were as follows: Aminoglycosides: amikacin (25μg/ml), gentamicin (25μg/ml), kanamycin (50μg/ml), netilmicin (25μg/ml), streptomycin (100μg/ml), & tobramycin (25μg/ml); Cephalosporins: cephalexin (25μg/ml), cefotaxim (25μg/ml); Penicillin: ampicillin(100μg/ml); Others: chloramphenicol (100μg/ml), tetracycline (20μg/ml), & nitrofurantoin (25μg/ml).

Screening of MAR isolates on MacConkey agar plates : About 40 to 50 colonies were picked at random from MAR population & streaked onto MacConkey agar (HiMedia, India) plates. After 24 hours of incubation at 37°C, ten typical *E .coli* colonies were randomly selected by lifting them from the plate with sterile wooden toothpicks &

transferred them onto a LB agar plate to construct a master plate. This master plate was replicated on Simmons Citrate agar (HiMedia, India) plates & evaluated after 96 hours of incubation at 37°C. Citrate negative isolates were randomly selected for further diagnostic evaluation to confirm the consistent selection of *E.coli* isolates.

Physiological and biochemical characterization: Detailed biochemical & physiological characterization was done according to Bergey's manual of systematic bacteriology to confirm the status of *E .coli* isolates.

Conjugation study : This study was performed using *E .coli* DH5α Rif^r as a recipient, following the method described by Son *et al*, 1997.

Detection of the presence of Class I integrons : PCR reactions were carried in 25il volume following the method previously described by Roy *et al*, 1995 with minor modifications. The primers used were as follows –

5′CS – 5′-GGC ATC CAA GCA GCA AG – 3′
3′CS – 5′-AAG CAG ACT TGA CCT GA – 3′
Int_2F – 5′-TCT CGG GTA ACA TCA AGG – 3′

Cloning: pGEM-T easy vector was used for cloning of PCR products. Cloning was done according to manufacturer's instruction and was then transformed into *E. coli* XL1 blue [*rec* A *rec* A1 *lac end* A1 *gyr* A46 *thi hsd* R17 sup E44 *rel* A1 (F′ *pro* A Blac $I^q Z^d$ M15 Tn 10 {Tet^r})]. Transformants were selected on LB agar containing 100μg/ml Amp, 2% X-gal and 20% I PTG.

Sequencing: Nucleotide sequencing was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit and the reactions were analyzed on an ABI PRISM 377 DNA sequencer.

Sequence analysis: Initial nucleotide sequence analysis was performed by using Blast N and Blast X tool (National Center for Biotechnology Information). Multiple sequence alignments were performed using CLUSTAL W program (EMBL Computational service).

Nucleotide sequence accession number : Nucleotide sequence of *dfr* V gene cassette (TR 2) has been deposited in the GenBank under the accession number AJ620333.

RESULTS AND DISCUSSION

The isolate TR 2 included in this study was one amongst the 100 clonally purified gram-negative bacilli, which had been randomly selected from the pool of thousands of multiple antibiotic resistant bacteria. The taxonomic characterization which was done by using the key of Bergey's manual , led to the identification of the isolate TR 2 as an *E. coli* as it bear 99% similarity with a type *E. coli* strain (*E. coli* K12). When tested against a panel of 12 different antibiotics (aminoglycosides: amikacin, gentamicin, kanamycin, netilmicin, streptomycin and tobramycin; cephalosporins: cephalexin and cefotaxim; penicillin: ampicillin; others: chloramphenical, tetracycline and nitrofurantoin) exhibited resistance against 10 antibiotics of this panel (Table.1). Conjugation study was performed using a plasmidless laboratory *E. coli* strain: *E. coli* DH5α Rif.^{r} as a recipient.

Table 1: Characteristic features of *E. coli* TR 2

MAR profile	Amp^r Cef^r Cep^r Chl^r Gen^r Kan^r $Nitro^r$ Str^r Tet^r Tob^r
Resistance transferred	Amp^r Cef^r Cep^r Gen^r Tet^r Tob^r
Conjugal transfer frequency	1.44×10^{-3}
Type of integron carriage	Class I
Nature of gene cassette	dfr V
Accession No.	AJ 620333

Amp: Ampicilin; Cef: Cefotaxim; Cep: Cephalexin; Chl: Chloramphenicol; Gen:Gentamicin; Kan: Kanamycin; Nit: Nitrofurantoin; Str: Streptomycin; Tet: Tetracycline; Tob: Tobramycin.

PCR amplification with 5′CS and 3′CS primers produced on amplicon of 750 bp (Fig. 1A). Several workers have used these two primers in combination for the identification of the presence of integrons, as well as for size determination of the inserted gene cassettes (Chang *et al.*, 2000; Fluit *et al.*, 1998). Integrons are potential mobile DNA elements capable of integrating or mobilizing individual gene cassettes encoding antibiotic resistance through site specific recombination (Hall & Collis, 1995). Four classes of integrons with different *int* genes have been identified so far, of which class-I integrons are more prevalent in nature. There have been a number of reports describing the presence of class-I integrons within gram negative clinical isolates (Chang *et al.* 2000; Fluit *et al.* 1998; Rawlinson *et al.* 2001; Fluit *et al.* 2001), while reporting on the occurrence of integrons in bacteria other than those of clinical are very rare. In one of the studies, the occurrence of integrons in bacteria from an estuarine environment was confirmed (Rosser *et al.* 1999). The amplicon generated with Int_2F and 3′CS primers was 600 bp larger than the amplicon generated with 5′CS and 3′CS primers (Fig. 1B).

This result confirms the general structure of the class I integron (Fluit *et al.* 1999). We have performed the same PCR reaction with the transconjugants expressing Amp^r Tet^r phenotype, but failed to get any amplification of the variable region of class-I integrons. This has led us to derive the conclusion that the class-I integron found in *E. coli* TR 2 is not present on the self-transmissible plasmid but probably located in the genome. The general structure of the class-I integron consists of a variable region bordered by a 5′ and 3′ conserved regions. The 5′ region is made up of the *int* I gene (integrase), *att*I site and a promoter region expressing the inserted gene(s) (Peter, 1999). Int_2F primer is specific for the *int*I region of class-I integron and the location of this region is about 600bp upstream of the 5′CS boundary. The 3′ region contains the defective quaternary ammonium resistance gene

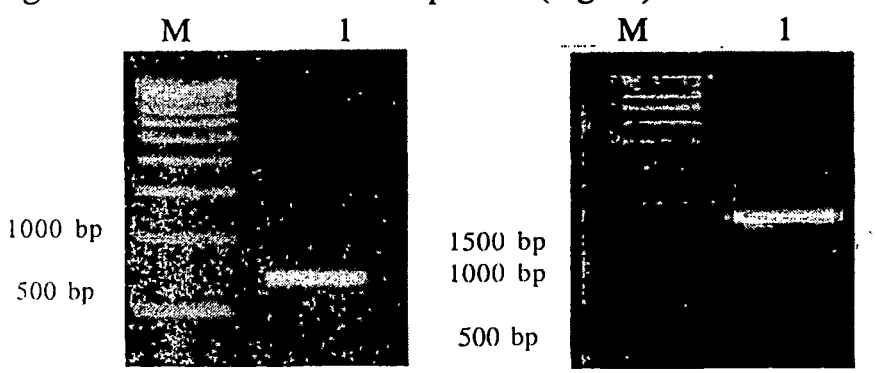

Fig. 1: PCR amplification of TR2 using two sets of primers. A: Amplicon generated using 5′CS & 3′ CS primers: Lanes: M, 500 bp ladder; 1, TR 2 and B : Amplicon generated using Int_2F & 3′ CS primers . Lanes: M, 500 bp ladder; 1, TR 2

*qac*EA1, a sulfonamide resistance gene (*sul* I) and an open reading frame containing a gene of unknowing function. The variable region which amplifies in PCR reaction using 5'CS and 3'CS primers constitutes the promoterless gene cassettes acquired through integration. These cassettes are mobile, promoterless, nonreplicating elements, comprise of an open reading frame together with a 3' associated integrase specific recombination site, *att* C [59-base element] (Hall and Collis, 1995; Hall, 1997). As the expression of the gene cassettes are dependent on a common promoter located upstream of the 5'Cs region, the cassette closest to the promoter will be maximally expressed. In naturally occurring integrons there appears to be no restrictions on the number or order of inserted gene cassettes (Hall and Collis, 1995). This genetic flexibility allows numerous cassette rearrangements under antibiotic selective presence. It has been observed that chances of getting empty integrons (*i.e.*, without gene cassettes) are much more in the absence of sustained antibiotic stress (Rosser *et al.* 1999). It is, therefore, presumable that antibiotic stress plays a significant role in promoting the incorporation and maintenance of gene cassettes in the variable region of integron. The most common types of cassette carried by class-I integrons are those for amoniglycosides or trimethorpim resistance.

Purified PCR product was subjected to DNA sequencing to identify the nature of the inserted gene cassette and the cassette present within the variable region of *E. coli* TR 2 was *dfr* V (coding trimethoprim resistance). The cassette borne TMP resistance genes seem to be more widespread than those borne on plasmids and chromosome (Huovinen, 1995). The emergence of TMP resistance genes among pathogenic bacteria is very likely due to the recruitment of metabolic genes from unidentified organisms by horizontal genetic exchange (Houvinen *et al,* 1995)). Phylogenetic analysis has revealed two families for transferable DHFRs. The *dfr* V gene is included in family 1 which also includes type I, VI, VIII and Ib. All the members of family 1 share about 64 to 88% identity, mediate resistance to very high levels of TMP and the polypeptide length is about 150 to 200 amino acids (Huovinen *et al.,* 1995). The software tool 'transeq' was used to determine all possible reading frames. Out of 6 reading frames , reading frame +2 represents the longest uninterrupted polypeptide sequence of 147 amino acids (Fig.2).The start of the ORF begin with an GTG start codon coding valine at positions 101 to 103 (valine also acts as start codon in some cases).

```
1    cgctgttacgccgtgggtcgatgtttgatgttatggagcagcaacgatgttacgcagcag
61    ggcagtcgccctaaaacaaagttaacccggaaccaaaattgtgaaagtatcattaatggc
                                                  V  K  L  S  L  M  A
121  tgcaaaagcgaaaaacggagtgattggttgcggtccacacataccctggtccgcgaaagg
       A  K  A  K  N  G  V  I  G  C  G  P  H  I  P  W  S  A  K  G
181  agagcagctactctttaaagccttgacgtacaaccagtggcttttggtgggccgcaagac
       E  Q  L  L  F  K  A  L  T  Y  N  Q  W  L  L  V  G  R  K  T
241  gttcgaatctatgggagcactccctaataggaaatacgcggtcgttactcgctcagcctg
       F  E  S  M  G  A  L  P  N  R  K  Y  A  V  V  T  R  S  A  W
301  gacggccgataatgacaacgtaatagtattcccgtcgatcgaagaggccatgtacgggct
       T  A  D  N  D  N  V  I  V  F  P  S  I  E  E  A  M  Y  G  L
361  ggctgaactcaccgatcacgttatagtgtctggtggcggggagatttacagagaaacatt
       A  E  L  T  D  H  V  I  V  S  G  G  G  E  I  Y  R  E  T  L
421  gcccatggcctctacgctccatatatcgacgattgatattgagccggaaggagatgtttt
          P  M  A  S  T  L  H  I  S  T  I  D  I  E  P  E  G  D  V  F
481  ctttccgaatattcccaataccttcgaagttgtttttgagcaacactttagctcaaacat
       F  P  N  I  P  N  T  F  E  V  V  F  E  Q  H  F  S  S  N
```

Fig. 2 : Nucleotide sequence and translated peptide of TR 2 amplicon.
5-81: 5' conserved segment; 31-81: *att* I site (integrase binding site); 101-538 Predicted translation product of frame +2;140-208: Signature sequence of DHFR (VIGcgphIPWsakgEqllFkalT)

A typical *dfr V* gene cassette consists of 157 amino acids, as is found in plasmid pLM020. Sequence of the gene cassette of TR 2 is read up to 147th amino acid & the ORF is expected to extend further by 10 amino acids in the region yet to be sequenced. Even if the chain length is reduced by 10 amino acids, it will not hamper the activity or the stability of the functional protein, since these amino acids are not in contact with many other residues that participate in numerous interactions and thereby do not affect the reaction rate by several orders of magnitude (Maranas et al, 2003). The amino acid at the 27th position of this polypeptide is glutamate like other members of family1 DHFRs and the typical signature sequence of DHFR is also present at the N terminal end.
The isolates like the strain TR2 bearing class I integrons isolated from Torsa River of Northern West Bengal, India, speaks of the possibility of dissemination and propagation of antibiotic genes in nature and further contribute to the growing concern regarding the emergence of trimethoprim resistant microorganisms.

REFERENCES

Chang, C.Y., Chang, L.L., Chang, Y.H., Lee, T.M. and Chang, S. F. (2000). Characterization of drug resistance gene cassettes associated with class-I integrons in clinical isolates of *E. coli*. From Taiwan, ROC. *J. Med. Microbiol.* 49: 1097-1102.

Davies, J. (1994). Inactivation of antibiotics and dissemination of resistance genes. *Science.* **264**: 375-382.

Hall, R.M. (1997). Mobile gene cassettes and integrons: moving antibiotic resistance genes in gram negative bacteria. *Ciba. Found. Symp.* **207**: 192-202.

Hall, R.M. and Collis, C. M. (1995). Expression of antibiotic resistance genes in the integrated cassettes of integrons. *Antimicrob. Agents. Chemother.* **39 :** 155-162.

Huovinen, P., Sundström, L., Swedberg, G. and Skold, O. (1995). Trimethoprim and sulfonamide resistance. *Antimicrob. Agents. Chemother.* **39 (2)**: 279-289.

Kasanea, A. and Sundqvist, H. (1982). Trimethoprim alone in the treatment of urinary tract infections: eight years of experience in Finland. *Rev. Infect. Dis.* **4**: 358-365.

Levesque, C., Piche, L., Larose, C. and Roy, P.H. (1995). PCR mapping of integrons reveals several novel combinations of resistence genes. *Antimicrob. Agents. Chemother.* 39 **(1)**: 185-191.

Martinez-Freizo, P., Fluit, A.C., Schmitz, F. J., Grek, V.S.C., Verhoef, J. and Jones M.E. (1998). Class I integrons in gram negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. *J. Antimicrob. Chemother.* **42**: 689-696.

Pelleitier, J. N., Campbell-Valosis, F.X. and Michnick, S.W. (1998). Oligomerization domain directed reassembly of active dihydrofolate reductance from rationally designed fragments. *Biochemistry.* **95:** 12141-12146.

Peter, A.W., McIver, C. J. and Rawlinson, W. D. (2001). Integrons and gene cassettes in the interobacterial. *Antimicron. Agents. Chemother.* **45**: 2658-2661.

Peter, M.B. (1999). Integrons and gene cassettes: a genetic construction kit for bacteria. *J. Antimicrob. Chemother.* **43**: 1-4.

Roberts, M. C., Chang, W.O., Roe, D., Xia, M., Marques, C., Borthgaray, G., Whittington, N.L. and Homes, K.K. (1999). Erythromycin resistant *Neisseria gonorrhoeae* and oral comensal *neisseria* sp. carry known rRNA methylase genes. *Antimicrob. Agents. Chemother.* **43**: 1367-1372.

Rosser, S. J. and Hilary, K.Y. (1999). Identification and Characterization of class I integrons in bacteria from an aquatic environment. *J. Antimicron. Chemother.* **44**: 11-18.

Schmitz, F. J., Hafner, D., Geisel, R., Follmann, P., Kirschke, C., Verhoef, J., Kohrer, K. and Fluit, A. D.C. (2001). Increased prevalence of class I integrons in *Escherichia coli*, *Klebsiella* species and *Enterobacter* species isolates over a 7 year period in a German University Hospital. *J. Clin. Microb.* **39** : 3724-3726.

Shapiro, J.A. (1999). Views about evolution are evolving. *American Society of Microbiology News.* **65**: 201-207.

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Unregulated use of antibiotics in Siliguri city *vis-a-vis* occurrence of MAR bacteria in community waste water and river Mahananda, and their potential for resistance gene transfer

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Abstract : *The unregulated use of antibiotics, including therapeutic and prophylactic prescribing, in the fastest growing city of West Bengal, Siliguri, was studied indirectly from a random survey conducted on retail medicine sellers at their counters. Ciprofloxacin, ampicillin, norfloxacin and amoxycillin were the highest retailed antibiotics and 58% of the city pharmacies sold antibiotics even without prescriptions. To understand the influence of the extent of antibiotic use by the community on the collective bacterial flora in the aquatic environment, we have determined the fraction(s) of Standard Plate Count (SPC) bacteria resistant to different antibiotics and multiple antibiotic resistance (MAR) profile of resistant SPC isolates from two municipal open drains and Mahananda river water samples of Siliguri. Within the MAR groups of Drain I and Drain II samples, 37.44% and 77.43% respectively were resistant to all seven antibiotics (ampicillin, chloramphenicol, ciprofloxacin, kanamycin, netilmicin, streptomycin and tetracycline) used in the study. Twenty Gram-negative SPC MAR isolates were examined for the presence of plasmids. Antibiotic resistance was shown to be associated with a carriage of a 47 kb (D1QN – 9), 48 kb (D2QN – 14) and 49.4 and 3.6 kb (MR – 1) plasmids, which were transmissible to the Escherichia coli DH5α recipient. The rapid spread of antibiotic resistance genes in bacterial population as a consequence of indiscriminate use of antibiotics, which can be partly attributed to plasmid-mediated horizontal transfer was discussed.*

Key words : *Antibiotics, Multiple antibiotic resistance, Self transmissible R plasmids, Gene-transfer.*

Introduction

Siliguri, the fastest growing city of West Bengal, with a population of 5,00,000 in 1999, is situated in the foothills of Himalayas on the banks of the river Mahananda. The advantageous location of Siliguri, bounded by Darjeeling Hills on the north, Bangladesh on the south, Jalpaiguri on the east and Nepal on the west, has made it an ideal centre for trade, commerce and transit tourist office and therefore attracted people from its vast hinterland stretching as far as Bihar, eastern fringes of Uttar Pradesh and Assam (Chakraborty, 1999). With a decadal growth rate of 119.5% during 1981-91, the Siliguri municipality has been promoted to a Corporation in the year 1994 (Chanda, 1999). The flow of effluents into the river Mahananda, illegal settlements on its banks and the unscientific waste management system including disposal of medical waste, expose the inhabitants of Siliguri to the risk of several diseases (Biswas, 1999; Lama, 1999). There is a tendency among common people to self-medicate themselves with various antibiotics even for the diseases where antibiotic use is often redundant. This is true in many parts of the world where antibiotics are available over the counter and even in countries in which use is more controlled; their use is often unnecessary (Cohen, 2000; Institute of Medicine, 1998; Levy, 1998; Hart and Kariuki, 1998; Walsh, 2000).

Although many factors can influence whether bacteria in a person or in a community will become insensitive to an antibiotic, the two principal forces are the occurrence of resistance genes and the extent to which antibiotics are used. If the collective bacterial flora in a community possesses resistance genes and the community uses the drug persistently, bacteria acquiring the ability to combat eradication by the compound will emerge and multiply (Levy, 1998). Antimicrobial drug use and transmission of resistant pathogens in humans are well-recognized contributors to the increase in antimicrobial resistance (McGeer, 1998). The majority of antimicrobial resistance phenotypes are obtained by the acquisition of external genes that may provide resistance to an entire class of antimicrobials. In recent years, a number of these resistance genes have been found to be associated with large, transferable plasmids, bearing transposons and integrons (White and McDermott, 2001).

The percentage of antibiotic – resistant bacteria in the intestinal flora of children exposed to large amounts of antibiotics has been shown to be much higher than that in children exposed to smaller amount of these drugs (Lester *et al.*, 1990). Moreover, the greater the number of resistant bacteria in the intestinal flora, the greater the likelihood that genes encoding resistance will be transferred to potentially pathogenic bacteria and disseminated into the environment (Van den Bogaard, 1997).

In this study, we have presented an assessment of unaccounted medication availed by the inhabitants of Siliguri reflecting the primary health picture and widespread consumption of antibiotics (with and without prescription) from a random survey conducted on retail medicine sellers at the counter, based on personal enquiry method with a short questionnaire. We have also reported the occurrence and abundance of multiple antibiotic resistant (MAR) bacteria within the standard plate count (SPC) populations from two municipal drains and Mahananda river waters of Siliguri. A few selected Gram-negative MAR SPC isolates were investigated to determine the association of antibiotic resistance gene(s) with self-transmissible plasmids.

Materials and Methods

***Method of enquiry to study the unregulated use of* antibiotics :** Personal enquiry method rather than mailed questionnaire method was chosen on the basis of the objective of the study and the nature of the respondents. A question sheet/schedule was prepared. We personally asked the questions to the respondents and noted down the replies on the schedule or the respondents filled in the questionnaire with their own handwriting. Basically, sample survey method was followed. Seventy-four retail medicine counters out of a total of 600 registered pharmacies, were selected at random representing nearly all the principal roads and streets of urban Siliguri.

Tabulation and analysis of data : The information collected through the enquiry were analyzed and presented in the form of statements and statistical diagrams so that some comparisons can be made and some conclusions can be arrived at. In course of analysis, care was taken so that no information relevant to the decision process was lost in the primary data reduction.

Waste water from two concrete Municipal open drains and river water used for sampling : Because the urban settlement is spread on both banks of river Mahananda (Fig.1), two big 'Pucca' municipal drains of the drainage network, one (Drain I) cross-secting Ward No. 17 and the other (Drain II) cross-secting Ward No.3 (Fig.1) were selected. River waters were collected from the Mahananda stretch at two sampling spots in between the Mahananda bridge on the Hill Cart Road and the railway bridge ahead of it over the river (Fig.1).

Collection of samples : Water samples were collected in peak summer, rainy and winter months according to standard methodology (APHA, 1989). Mahananda river water samples were collected in May 2000, Aug 2000 and Jan 2001. Wastewater samples from municipal drains were collected in Jan 2001, May 2001 and Aug 2001 respectively.

Enumeration of total SPC bacteria and the fraction(s) resistant to different antibiotics : Serial dilutions of each water sample were made in sterile distilled water, which served as the diluent of known volume. Standard plate count bacteria and their fraction(s) resistant to different antibiotics were enumerated on Luria Bertani (LB) agar and LB agar plates containing a separate antibiotic, after incubation at 37°C for 48 h. The antibiotics used were ampicillin (100μg/ml), chloramphenicol (50 μg/ml), ciprofloxacin (25 μg/ml), kanamycin (50 μg/ml), netilmicin (25 μg/ml), streptomycin (100 μg/ml) and tetracycline (20 μg/ml), as per concentration generally used to characterize resistant strains.

Enumeration of multiply antibiotic–resistant (MAR) bacteria out of resistant population : MAR was enumerated by the method described earlier (Armstrong *et al.*, 1981). Bacterial colonies were randomly picked up from antibiotic plates with sterile toothpicks and were transferred to a master plate of LB agar. Master plate colonies incubated for 24 h at 37°C were replicated on LB agar plates, each containing a separate antibiotic at the concentration mentioned above. The final plate replicated was one of plain LB agar. The results from this plate confirmed the successful replication of colonies on the preceding replicated plates. Isolates were considered multipe antibiotic resistant (MAR) only if their growth on more than one different antibiotic plate were as well developed as their growth on the control plate. Any sign of inhibition or sensitivity was considered to be indicative of non-resistance.

Plasmid isolation : Several gram-negative MAR isolates were screened for the plasmid DNA by the procedure of Birnboim and Doly, 1979. Extracted plasmids were

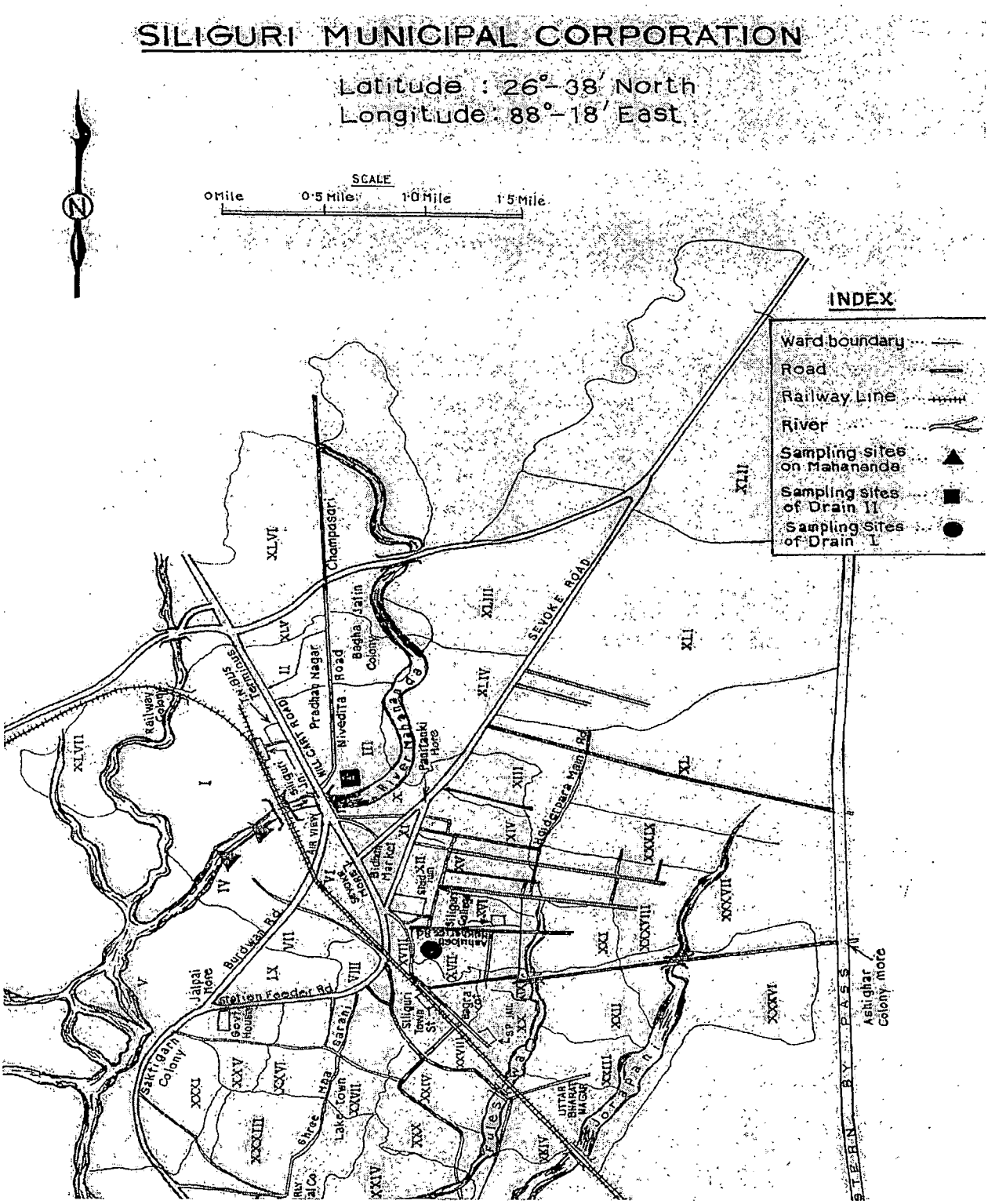

Fig. 1 : Map of Siliguri Municipal Corporation showing distribution of municipal wards on both sides of the river Mahananda. ●Sampling spot of Drain-I; ▣, Sampling spot of Drain-II; ▲, Sampling spot on the river Mahananda

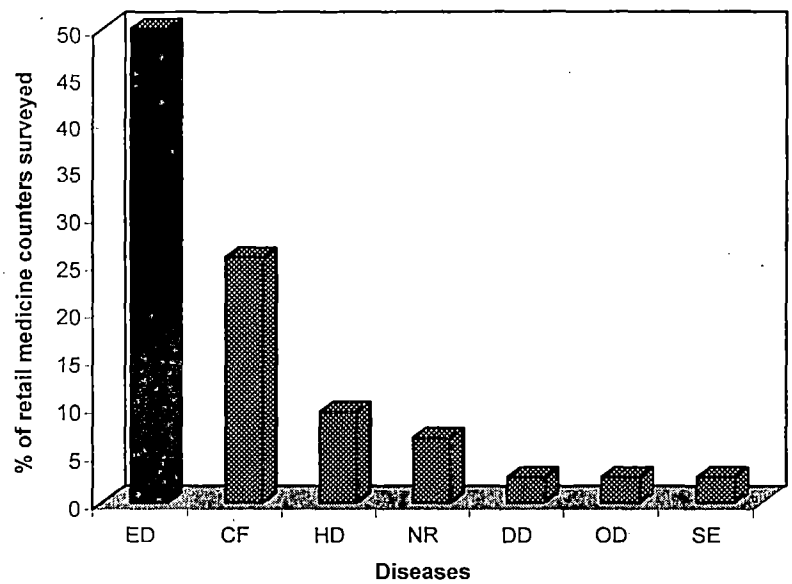

Fig. 2 : Frequencies of surveyed retail medicine counters stated to experience average maximum sale of medicine for a particular group of disease(s) irrespective of seasonal variation.
ED, Enteric diseases and intestinal disorders; CF, Common illnesses (fever); HD, Heart/coronary diseases; NR, Neurological diseases; DD, Diabetic disorders; OD, Orthopedic disorders; SE, Skin and ENT diseases.

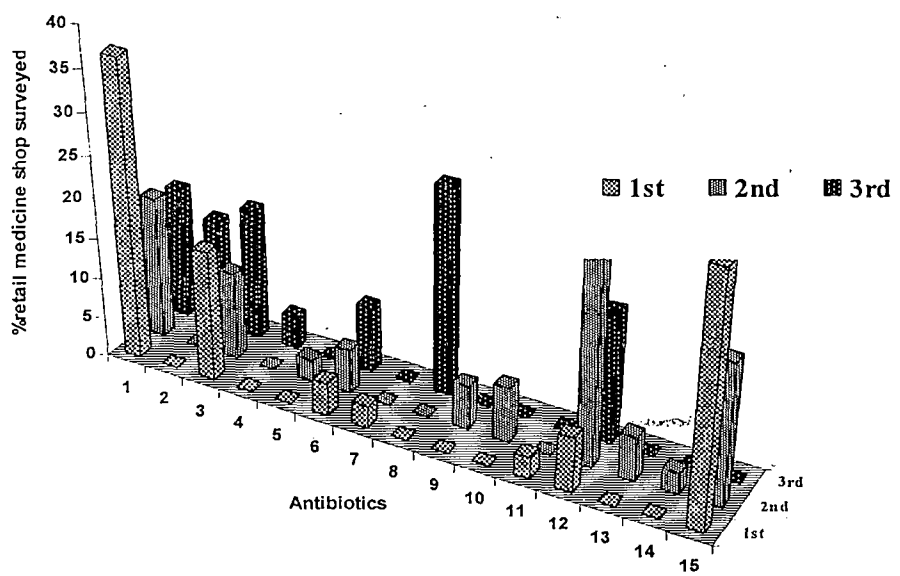

Fig. 3 : Frequencies of surveyed retail medicine shops expressing categories of first, second and third highest selling antibiotics over the counters.
1, Ciprofloxacin; 2, Tetracycline; 3, Amoxycillin; 4, Althrocin; 5, Septran (Trimethoprim + Sulphamethoxazole); 6, Cephalexin; 7, Alcipro (Ciprofloxacin + Tinidazole); 8, Cloxacillin; 9, Co-trimoxazole; 10, Doxycycline; 11, Erythromycin; 12, Norfloxacin; 13, Roxithromycin; 14, Cefadroxil; 15, Ampicillin.

electrophoresed at 20 mA on a 0.7% agarose gel in TAE as described by Sambrook *et al.*, 1989. The approximate molecular size of each plasmids were determined by comparison with the size standards and sizes of the self transmissible R plasmid(s) isolated from the transconjugants were confirmed by summing up the respective restricted fragment lengths.

Conjugation studies : Mating experiments were performed according to the method described by Son *et al.*, 1997. Selected MAR but rifampicin sensitive, plasmid containing Gram negative SPC isolates and a rifampicin resistant, plasmidless strain of *E. coli.* DH5α were used. Potential donor strains and recipient cells were grown to mid log phase (10^7 cfu ml^{-1}) in LB broth at 37°C in 100 ml flasks. A 1ml sample containing the donor strain was added to 1 ml of the recipient sample, and the mixture was incubated for 5h at 37°C without shaking. Plate counts were performed for estimates of donor and recipient population. Transconjugants were selected on LB agar containing 50μg ml^{-1} of rifampicin and inhibiting concentration of the antibiotic to which donor strain had been resistant. Antibiotic resistant, plasmid bearing *E. coli.* DH5α isolates were scored as transconjugants. The frequency was computed as the number of presumptive transconjugants in relation to the initial number of donor cells.

Results and Discussion

Siliguri, the most populous city in the northern part of West Bengal, has one pharmaceutical retail counter per 824 persons at present compared to one per 2000 persons in 1951 (this study). Because of the inadequate civic medical facilities, most middle class and poor people render themselves to be treated empirically and usually with antibiotics. As we found a general tendency among the pharmacy-owners to suppress the exact turn over value in quantity and rupee figures, we have excluded questioning them in quantitative terms rather insisted them to respond to our questions in relative terms. Medicine retail counters of Siliguri could be broadly categorized into seven groups on the basis of average maximum sale of medicines for particular group of disease irrespective of seasonal variations (Fig. 2). It has been found that the pharmacies selling mostly the medicines related to enteric diseases constituted the half of the total retail counters surveyed (Fig. 2). Over 60% of the retailers stated high trend of selling medicines for alleviating enteric diseases in summer and rainy seasons of Siliguri (data not shown). The predominance of water borne diseases in Siliguri reported in print media (Lama, 1999) can also be substantiated from our data. It is an important fact that diarrhoeal disease still remains as a major cause of morbidity and mortality in developing countries (Hart and Cunliffe, 1997; Hossain *et al.*, 1998). We have noticed a wide range of antibiotics including the third generation antibiotics being sold over the counters (Fig. 3). Categories of highest, second highest and third highest selling antibiotics from the city pharmacies were shown in Fig.3. Ciprofloxacin, Ampicillin, Norfloxacin and Amoxycillin were the highest retailed antibiotics in the city. The survey has also revealed an important fact that 58% of the retailers sold antibiotics to the consumers even without medical prescription. The highest sold self-medicated antibiotics were Ampicillin and Cloxacillin. A similar survey in Bangladesh conducted on 'bare foot doctors' has shown that such medical practitioners prescribed antibiotics to 60% of their patients (on an average of 380 patients/doctor/month) on the basis of symptoms alone (Murray and Lopez, 1997). It was also shown in the study that out of a total of 291500 doses of antibiotics (prescribed to 14950 patients in a month), only 109500 doses had been dispensed by pharmacies, and a further 100,000 doses had been dispensed without a prescription (Murray and Lopez, 1997).

Siliguri city has to do without any underground system and a huge quantity of community wastewater and other kinds of waste pass through the estimated 250 km long open drains and ultimately discharges into main river Mahananda (Lama, 1999). River water samples were taken from two spots of Mahananda River between the Mahananda bridge and railway track ahead of the bridge over the river. The selection of two drains was based on their location in two congested wards representing community usages on both banks of the river. Sampling spot, Drain I, was located in the heart of the administrative core of the city cross-secting Ward No. 17 and Drain II was located in the increasingly populous Ward No. 3 which is nearer to the other bank of Mahananda (Fig. 1).

Standard plate counts (SPCs) of Mahananda River and Siliguri municipal drain waters recorded in three seasonal months (Summer, May; Rainy, August; and Winter, January) of 2000-2001 displayed very wide variations and therefore larger were the dispersion (measured in terms of standard deviation) values (Table 1). We have considered geometric mean (GM) because it

Table – 1 : Antibiotic resistance of SPC bacteria isolated from Municipal drains and Mahanda river waters of Siliguri city.

	Average SPC density (cfu ml^{-1})	Average % resistant population (taking SPC value in plates without antibiotics as 100 %)						
		Ap	Cm	Cf	Km	Nt	Sm	Tc
Mahananda river water[a]	5.46×10^6 (12.5×10^6)	2.32 (15.02)	0.10(0.16)	n.d.	0.11(0.09)	n.d.	1.76(5.27)	0.31(1.07)
Drain I[b]	3.82×10^5 (1.17×10^6)	4.96 (7.38)	5.29(14.61)	3.32(12.46)	6.09(8.99)	3.05(3.88)	6.51(5.88)	4.6(4.6)
Drain II[c]	5.38×10^6 (12.5×10^6)	16.92 (12.97)	14.11(12.40)	8.29(12.54)	14.24(7.21)	10.04(15.91)	9.66(5.57)	5.87(12.28)

[a], Geometric mean of 6 samples (2 samples from two points of river in each sampling month);

[b] and [c], Geometric mean of 3 samples (1 sample from one point in each sampling month)

The standard deviation (s) are given in the first bracket.

Ap, Ampicillin; Cm, Chloramphenicol; Cf, Ciprofloxacin; Km, Kanamycin; Nt, Netilmicin; Sm, Streptomycin; and Tc, Tetrecycline. n.d. Not done.

is less affected by the presence of extremely large or small values. The SPC geometric mean values from the river and drain water samples ranged from 3.82×10^5 tc 5.46×10^6 cfu ml^{-1}. The SPC GM values for Drain II and Mahananda River are similar (Table 1). Since the coefficient of variation for SPC density of river water (CV=200) was higher than drain water samples (CV=115 for Drain I and CV=111 for Drain II), SPC density of Mahananda river water was more variable than drain waters. Though seasonal variation in SPC density was observed in another river, Torsa of North Bengal (Bhadra *et al.*, 2003), cutting across two growing townships, Jaigaon and Hashimara at Indo Bhutan border, the SPC GM value (5.3×10^4) was found at least two orders lower than that of Mahananda at Siliguri.

Antibiotic resistance is a direct consequence of antibiotic use (Finch, 1998). Both continue to escalate despite many calls for moderation of antibiotic use, in the hospital and in the community (Gould *et al.*, 1994; Gould, 1999; Kunin, 1997; Shales *et al.*, 1997; Swartz, 1997). With clonal spread, cross infection and environmental contamination are also important and surely also due to the contribution of water as a means of spreading bacteria bearing resistance-genes can defeat attempts to contain resistance by antibiotic restriction (Bell *et al.*, 1980; McGowan, 1994). On an average ampicillin resistant bacteria represented the highest fraction of SPC bacteria resistant to any of the antibiotics (Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, Streptomycin; and Tc, tetracycline were used for analyzing Mahananda river water and on receiving the feedback from the survey of medicine retail counters, we introduced two more antibiotics, Cf, ciprofloxacin and Nt, netilmicin for analysis of drain water samples) in Mahananda as well as Drain II samples while streptomycin resistant bacteria represented the highest occurrence in Drain I samples (Table 1). Antibiotics with long-term use in the same environment can produce perceptible change in the proportion of resistant and susceptible bacteria not only in the treated individual but also in the environment and society at large (Levy, 1998). Though ciprofloxacin and netilmicin were newer antimicrobial drugs, the frequencies of their resistance among SPC isolates (Table 1) were alarming in view of the extensive use of these compounds (Fig. 3). The occurrence of high frequencies of antibiotic resistant bacteria within the SPC populations from several distribution water system (Armstrong *et al.*, 1981; Armstrong *et al.*, 1982) has led to a closer examination and assessment of the significance of these organisms as there is concern that several SPC genera may pose a hazard to public health (Lamka *et al.*, 1980; Le Chevallier and Seidler, 1980). It was also shown that SPC bacteria isolated from the tank holding the finished drinking water at the treatment facility were more frequently antibiotic resistant than were respective river water populations (Armstrong *et al.*, 1982). The present study is relevant to the context that a large drinking water treatment plant from the supply of Mahananda river water has recently been installed for the city and therefore presumably runs a risk of occurrence of antibiotic-resistant SPC in the drinking water supply system of Siliguri.

The screening of 268 resistant Mahananda river water SPC solates (picked up randomly from plates containing single antibiotic) by a replica plate method that

Table – 2 : Characterization of transconjugants.

Donor	Plasmid size(kb)	Source	MAR profile	Resistance transferred	Frequency of transfer*	Plasmid size(kb)+
MR –I	49.4,3.6	Mahananda river	Ap^r Cm^r Km^r Sm^r	Ap^r Cm^r Km^r	$6.2x10^{-4}$	49.4,3.6
DIQN-9	49.5,48.4,47.0, 6.8,5.7,3.8,3.23	Drain I	Ap^r Cm^r Km^r Sm Tc^r	Ap^r Cm^r	$4.8x10^{-5}$	47.0
D2QN-14	50.0,48.0,47.3, 22.9,20,7.7,3.6	Drain II	Ap^r Cm^r Km^r Sm^r Tc^r	Ap^r	$7.6x10^{-4}$	48.0

Ap, Ampicillin; Cm, Chloramphenicol; Km, Kanamycin; Sm, Streptomycin; Tc, Tetrecycline.
* Frequencies are expressed as the number of transconjugants per input donor cells.
+ plasmid detected in the transconjugants obtained.

uses colonies on a master plate as inocula, the resistance of each isolate to five antibiotics being determined by growth on antibiotic plates, yielded 82.1% multiple-antibiotic resistance (MAR, i.e., resistant to two or more of the screening antibiotics) phenotype. Among singly resistant isolates, the most predominant was ampicillin (representing one of the highest retailed antibiotics in Siliguri) resistant bacteria (12.31%). Within the population of 220 MAR isolates out of 268 isolates, 33.63% were doubly resistant, 32.27% were triply resistant, 26.81% were quadruply resistant and 7.27% were quintuply resistant to all of the five antibiotics used in the study. Ap^r Tc^r (20% of MAR), Ap^r Sm^r Tc^r (17.7% of MAR) and Ap^r Cm^r Sm^r Tc^r (17.3% of MAR) were the most frequent combinations scored within doubly, triply and quadruply resistant MAR groups. In one of the study related to MAR SPC, it was shown that 18.6% of the SPC bacteria isolated from the intake for untreated water and 67.8% of the drinking water SPC bacteria isolated from the distribution system expressed multiple antibiotic resistance (Calomiris *et al.*, 1984).

Of a total of 2030 antibiotic resistant SPC bacterial isolates from six water samples from two municipal open drains of Siliguri city, 100% and 99.8% were MAR in Drain I and Drain II respectively. Within the group of MAR isolates of Drain I, 38.86% were hexadly resistant, 37.44% were heptadly resistant, 15.73% were quintuply resistant, 4.41% were quadruply resistant, 2.87% were triply resistant, and 0.55% were doubly resistant to the antibiotics which were used. In Drain II samples, 77.43% of the resistant SPC bacteria were resistant to all seven antibiotics (heptadly resistant) tested followed by 15.19% hexadly, 5.87% quintuply, 0.94% quadruply, 0.25% triply, no doubly resistant and 0.17% singly resistant represented uniquely by ampicilin resistant bacteria. The percentage distribution of MAR groups in two drains (Drain I and II) bears a positive rank correlation coefficient of 0.928. As pointed by early authors (Hsu *et al.*, 1992), differences in percentage of bacterial resistance to various antibiotics may reflect the history of antibiotic application and hence there is a possibility of using bacterial drug resistance as an indicator of antibiotic application. It is interesting to note that out of a total 43 different combinations of MAR in drain samples, the number of occurrences of Ap^r, Cm^r, Tc^r, Sm^r, Km^r, Cf^r, and Nt^r in the combinations were 36, 35, 27, 24, 24, 20 and 20 respectively which may also indicate the chronology of the usage. Among the most populous MAR group of Drain I (the hexadly resistant group), the frequency of occurrence of Ap^r Cm^r Km^r Nt^r Sm^r Tc^r bacteria (22.5%) was exceedingly higher than the other combinations of the group. Likewise, Ap^r Cm^r Km^r Sm^r Tc^r, Ap^r Cm^r Sm^r Tc^r, Ap^r Cm^r Tc^r and Ap^r Cm were the most frequent patterns observed within quintuply, quadruply, triply and doubly resistant group respectively of Drain I MAR SPC. In Drain II samples, Ap^r Cm^r Cf^r Km^r Sm^r Tc^r and Ap^r Cm^r Km^r Nt^r Tc^r were the most frequent patterns observed within hexadly and quintuply resistant MAR groups. In an earlier study related to the incidence and extent of MAR among gram negative SPC bacteria, screened against the commonly used antibiotics: ampicillin, chloramphenicol, kanamycin, neomycin (Nm), streptomycin and tetracycline, isolated from various sources including water and sewage samples in Karachi, revealed that the most common resistance pattern was Ap^r Cm^r Sm^r Tc^r followed by Cm^r Sm^r Tc^r and Ap^r Cm^r Km^r Nm^r Sm^r Tc^r (Khatoon *et al.*, 1981). However, isolation of bacterial pathogens of human and animal origin that are increasingly resistant to most frontline antibiotics, including third generation cephalosporins, aminoglycoside (e.g. Netilmicin) and even fluoroquinolones (e.g. Ciprofloxacin) has further contributed to the increased

public and scientific interest regarding the administration of therapeutic and subtherapeutic antimicrobials to living systems (White and McDermott, 2001).

The loss of antibiotic efficacy through the emergence and transfer of bacterial antibiotic resistance is an increasing reality (Kruse and Sorum, 1994; Salyers and Amiable-Cuevas, 1997). In fact, resistant bacteria were detected soon after the introduction of commercial antimicrobials, but it was not until the 1960s when transferable multiple drug resistance was described (Salyers and Amiable-Cuevas, 1997). The rapid dissemination of antibiotic resistance genes in bacterial populations can be partly attributed to plasmid-mediated horizontal transfer. Plasmids capable of being transferred and stably maintained in a wide range of bacteria are of special interest with respect to interspecies gene exchange (Gotz *et al.*, 1996). Most of the current knowledge comes from plasmids isolated from clinical material or from studies on plasmid-mediated dissemination of antibiotic resistance genes (Tschape, 1994), in which plasmids were usually isolated after selective cultivation of bacteria with subsequent screening for the presence of plasmids. Twenty gram negative MAR isolates representing all MAR groups of Drain I, Drain II and Mahananda river water SPC bacteria were examined for the occurrence of plasmid DNA. All twenty MAR isolates harboured plasmid DNA of varying sizes from 2.0 kb to 100 kb, and seven of them had multiple plasmids (data not shown). However, in this report, three isolates MR1 (Mahananda river isolate), D1QN-9 (Drain I isolate), and D2QN-14 (Drain II isolate) harbouring potentially self transmissible R plasmids (molecular size greater than 23 Kb) were chosen to perform matings with plasmidless *E. coli* DH5α Rif^r as potential recipient. Table 2 and Fig. 4 showed the resistance patterns and plasmid content of the three donor isolates as well as the transferred plasmids in transconjugants associated with the expression of antibiotic resistance(s) in them. However, only plasmids of high molecular size of 49.4 kb (MR1), 47 kb (D1QN-9) and 48 kb (D2QN-14) were transferred to the recipient *E. coli* DH5α Rif^r. Mobilization of a small (3.6 kb) non-conjugative plasmid in donor strain MR-1 was also detected (Table 2 and Fig. 4). The self transmissibility of the transferred plasmid were confirmed by further conjugal transfer from *E. coli* DH5α transconjugants to another plasmidless *E. coli* recipient strain, *E. coli* HB101 Str^r (data not shown). Presence of multiple plasmids has been documented in *Escherichia coli* isolates of Malaysia and transferable/transmissible antibiotic resistance was demonstrated to be associated with carriage of one plasmid out of multiple plasmids present in individual isolates (Son *et al.*, 1997).

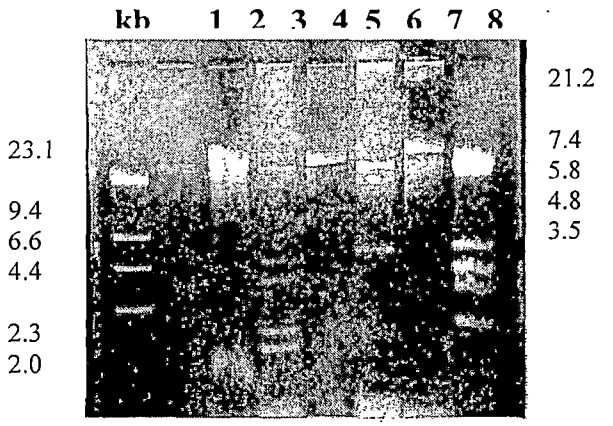

Fig. 4a : Agarose (0.7%) gel electrophoresis of plasmid DNA from MAR-SPC isolates and their respective transconjugants, Lanes : 1 & 8, λ DNA digested with *Hind* III and *Eco* R1 respectively, used as size standards; 2, Donor MR-1; 3, Transconjugant of donor MR-1; 4, Donor D1QN-9; 5, Transconjugant of donor D1QN-9; 6, Donor D2QN-14; 7, Transconjugant of donor D2QN-14.

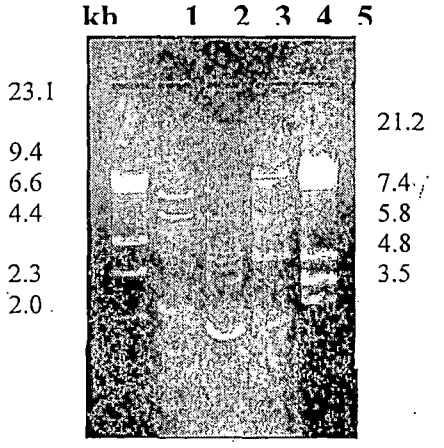

Fig. 4b : Agarose (0.7%) gel electrophoresis of *Eco* R1 digested transferred plasmid DNAs. Lanes : 1 & 5, λ DNA digested with *Hind* III and *Eco* R1 respectively, used as size standards; 2, 3, and 4, *Eco* R1 digested transferred plasmid DNA from transconjugants of the donor D1QN-9, MR-1, and D2QN-14 respectively.

The *Eco*R1 restriction patterns of the transferred plasmids were different in three transconjugants (Fig. 4B), indicating the presence of diverse conjugative R plasmids amongst the MAR isolates. The small 3.6 kb plasmid, mobilized along with the 49.4 kb plasmid from MR1 to the *E. coli* DH5α Rif^r, showed no reduction in size after *Eco*R1 digestion (compare lane 2 and 3 of Fig 4A and lane 3 of Fig 4B) suggesting either absence of any or presence of an unique *EcoR*1 site. Thus it was apparent from this study that MAR SPC isolates of municipal drains and river harboured transmissible R-plasmid DNA. The widespread existence of plasmids in natural isolates and their apparent stability, even where antibiotics are not present (Gotz *et al.*, 1996; Top *et al.*, 1994; Vander *et al.*, 1996), argue against the widely held belief that plasmids and other gene transfer elements are readily lost in the absence of selection. Moreover, the abundance of antibiotic-resistant strains in environmental settings where the bacteria presumably do not come into contact with antibiotics (Andersen and Sandaa, 1994; McKeon *et al.*, 1995), suggests that resistance genes can also be stably maintained in the absence of antibiotic selection. Conjugal transfer itself can contribute to stable maintenance of antibiotic resistance genes in a bacterial population by continually reseeding members of population that have lost a resistance gene-transfer element (Salyers and Amiable-Cuevas, 1997). Any successful attempt to curb the spread of resistance will have to take into account the fact those resistance genes and transmissible elements that carry them could be easily received but difficult to get rid of.

We would like to render a view that wide spread use of antimicrobial drugs is no way a substitute for good sanitation and personal hygiene. Antimicrobial agents are losing their effectiveness because of the spread and persistence of drug resistant organisms. It is, therefore, imperative to increase awareness of the dangerous consequences of antibiotic misuse at all level of usage: consumer, prescribers, dispensers, manufacturers, and regulatory agencies.

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References

American Public Health Association. : Standard methods for examination of water and waste water. 17th Ed. *APHA* Washington D.C. (1989).

Andersen, S.R. and R.A. Sandaa : Distribution of tetracycline resistance determinants among gram negative bacteria isolated from polluted and unpolluted marine sediments. *Appl. Environ. Microbiol.*, **60**, 908-912 (1994).

Armstrong, J.L., D.S. Shigeno, J.J. Calomiris and R.J. Seidler : Antibiotic resistant bacteria in drinking water. *Appl. Environ. Microbiol.*, **42 (2)**, 277-283 (1981).

Armstrong, J.L., J.J. Calomiris and R.J. Seidler : Selection of antibiotic resistant standard plate count bacteria during water treatment. *Appl. Environ. Microbiol.*, **44 (2)**, 308-316 (1982).

Bell, J.B., W.R. Macrae and G.E. Elliott : Incidence of R factors in Coliform, Fecal Coliform and *Salmonella* populations of the Red River in Canada. *Appl. Environ. Microbiol.*, **40**, 486-491 (1980).

Bhadra, B., S. Mukherjee, R. Chakraborty and A.K. Nanda : Physico-chemical and bacteriological investigation on the river Torsa of North Bengal. *J. Environ. Biol.*, **24 (2)**, 125-133 (2003).

Birnboim, H.C. and J. Doly : A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic. Acid. Res.*, **7**, 1513-1523 (1979).

Biswas, P. : Insidious intent! *The Statesman*, Suppl. NB Plus. 1-5. Dt. 7.5.99 (1999).

Calomiris, J.J., J.L. Armstrong and R.J. Seidler : Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. *Appl. Environ. Microbiol.*, **47**, 1238-1242 (1984).

Chakraborty, R. : Not Seeing the wood for trees. *The Statesman.* Suppl. NB Plus, 2, dt. 20.8.99 (1999).

Chanda, S. : Turning the trumps. *The Statesman*, Suppl. NB Plus, 1, dt. 14.05.99 (1999).

Cohen, M.L. : Changing patterns of infectious disease. *Nature*, **406**, 762-767 (2000).

Finch, R.G. : Antibiotic resistance. *J.Antimicrob.Chemother*, **42**, 125-128 (1998).

Gotz, A., R. Pukall, E. Smit, E. Tietze, R. Prager, H. Tschape, J.D. van Elsas, K. Smalla : Detection and characterization of broad-host range plasmids in environmental bacteria by PCR. *Appl Environ. Microbiol.*, **62**, 2621-2628 (1996).

Gould, I.M. : A review of the role of antibiotic policies in the control of antibiotic resistance. *J. Antimicrob. Chemother.*, **43**, 459-465 (1999).

Gould, I.M., J. Hampson, E.W. Taylor and M.J. Wood : Hospital antibiotic control measures in the UK. *J. Antimicrob. Chemother.*, **34**, 21-42 (1994).

Hart, C.A. and N.A. Cunliffe : Viral gastro-enteritis. *Curr. Opin. Infect. Dis.*, **10**, 408-413 (1997).

Hart, C.A. and S. Kariuki : Antimicrobial resistance in developing countries. *BMJ.*, **317**, 647-650 (1998).

sain, M.A., M. Rahman, Q.S. Ahmed, M.A. Malek, R.B. Sack and M.J. Albert : Increasing frequency of mecillinam-resistant *Shigella* solates in urban Dhaka and rural Matlab, Bangladesh: A 6-year observation. *J. Antimicrob. Chemother*, **42**, 99-102 (1998).
C.H., S.C. Hwang and J.K. Liu : Succession of bacterial drug resistance as an indicator of antibiotic application in aquaculture. *J. Fish. Soc. Taiwan*, **19**, 55-64 (1992).
titute of Medicine, Antimicrobial Resistance. : Issues and Options. National Academy Press, Washington D.C. (1998).
hatoon, H., S.A. Ali and S.M. Najeeb : Molecular biology, pathogenecity and ecology of bacterial plasmids (*Eds:* S. D. Levy *et al.*). Plenum Press, New York, pp. 621 (1981).
Kruse, H. and H. Sorum : Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural environments. *Appl. Environ. Microbiol.*, **60**, 4015-4021 (1994).
nin, C.M: : Antibiotic armageddon. *Clin. Infec. Dis.*, **25**, 240-241 (1997).
ma, N. : Down the Drain. *The Statesman*, Suppl. NB Plus, 1, dt. 14.05.99 (1999).
mka, K.G., M.W. Le Chevallier and R.J. Seidler : Bacterial contamination of drinking water supplies in a modern rural neighborhood. *Appl. Environ. Microbiol.*, **39 (4)**, 734-738 (1980).
e Chevallier, M.W. and R.J. Seidler : *Staphylococcus aureus* in rural drinking water. *Appl. Environ. Microbiol.*, **30**, 739-742 (1980).
ester, S.C., M. del Pilar Pla, F. Wang, I. Perez Schael, H. Jiang and T.F. O'Brien : The carriage of *Escherichia coli* resistant to antimicrobial agents by healthy children in Boston, in Caracas, Venezuela and In Qin Pu, China. *New. Eng. J. Med.*, **323**, 285-289 (1990).
vy, S.B. : The challenge of antibiotic resistance. *Scient. Amer.*, **275**, 46-53 (1998).
cGeer, A.J. : Agricultural antibiotics and resistance in human pathogens : villain or scapegoat? *CMAJ.*, **159**, 1119-1120 (1998).
owan, J.E. : Do intensive hospital antibiotic control programs prevent the spread of antibiotic resistance? *Inf. Cont. Hos. Epidem.*, **7**, 478-483 (1994).
Keon, D.M., J.P. Calabrese and G.K. Bissonette : Antibiotic resistant gram negative bacteria in rural groundwater supplies. *Water Res.*, **29**, 1902-1908 (1995).
Murray, C.J. L. and A.D. Lopez : Mortality by cause for eight regions of the world: Global burden of disease study. *Lancet.*, **349**, 1269-1276 (1997).
Salyers, A.A. and C.F. Amiable-Cuevas : Why are antibiotic resistance genes so resistant to elimination? *Antimicrob. Agent. Chemother*, **41**, 2321-2325 (1997).
Sambrook, J., E.F. Fritsch and T. Maniatis : Molecular Cloning: A laboratory Manual. Cold Spring Harbor, New York; Cold Spring Harbor Laboratory Press (1989).
Shales, D.M., D.N. Gerding, J.F. John, W.A. Craig, D.L. Bornstein and R.A. Duncan : Society for healthcare epidemiology of America and Infectious Diseases Society of America Joint Committee on the prevention of antimicrobial resistance: guidelines for the prevention of antimicrobial resistance in hospitals. *Inf. Contr. Hos. Epidem.*, **18**, 275-291 (1997).
Son, R., G. Rusul, and M.I.A. Karim : Conjugal transfer of plasmids and antibiotic resistance among *Escherichia coli* isolated from animals in a rural area in Sarawak (Malaysia). *J. Appl. Microbiol.*, **82**, 240-244 (1997).
Swartz, M.N. : Use of antimicrobial agents and drug resistance. *New Eng. J. Med.*, **337**, 491-492 (1997).
Top, E.I., I. Desmet, W. Verstracte, R. Diijkmans and M. Mergeay : Exogenous isolation of mobilizing plasmids from polluted soils and sludges. *Appl. Environ. Microbiol.*, **60**, 831-839 (1994).
Tschape, H. : The spread of plasmids as a function of bacterial adaptability. *FEMS Microbiol. Ecol.*, **15**, 23-32 (1994).
Van den Bogaard, A.E. : Antimicrobial resistance-relation to human and animal exposure to antibiotics. *J. Antimicrob. Chemother*, **40**, 453-454 (1997).
Vander Bij, A., L.A. Weger, W.J. Tucker and B.J. Lugtenberg : Plasmid stability in *Pseudomonas fluorescens* in the rhizosphere. *Appl. Environ. Microbiol.*, **62**, 1076-1080 (1996).
Walsh, C. : Molecular mechanisms that confer antibacterial drug resistance. *Nature*. **406**, 775-781 (2000).
White, D.G. and P.F. McDermott : Emergence and transfer of antimicrobial resistance. *J. Dairy. Sci.*, **84** (E. Suppl.), E 151-E 155 (2001).

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