Chapter 2

REVIEW OF LITERATURE

The members of the family Vibrionaceae constitute a predominant heterotrophic bacterial group in aquatic environments (Simidu et al., 1977). *Vibrio* is one of the most important aquatic bacterial genera that are widely distributed in marine, estuarine, and fresh waters. *Vibrio* species are commonly observed in shrimp hatcheries, grow out ponds and sediments (Otta et al., 1999). The numbers of *Vibrio* species were increasing year by year now the 63 species of *Vibrio* has been recognized (Thompson et al., 2001; Thompson et al., 2005a; Thompson et al., 2005b).

2.1. Historical perspective- *Vibrio* research

*Vibrios* are among the most abundant cultivable microbes in aquatic environments (Heidelberg et al., 2002a). *V. cholerae*, the causative agent of cholera is one of the most studied of the *Vibrio* species and one which is amenable to genetic analysis and gene level research (Guidolin and Manning, 1987). Cholera is a devastating and ancient disease, occurring even today in epidemic form, in many parts of the world, claiming hundreds of thousands of lives each year (WHO Report, 2001). First described by Pacini (Pacini, 1854), the cholera *Vibrio* was extensively studied and Koch properly characterized the disease as a waterborne disease in 1884 (Jones, 1984). It is well established that the disease is seasonal and studies are beginning to elucidate the role of the coastal environment and the ecology of *Vibrio cholerae* in transmission of the disease.

The family Vibrionaceae consists of ubiquitous halophilic facultative anaerobes, which are gram-negative, motile rods. The fish/shellfish disease associated
with this genus is called Vibriosis. It affects both marine and freshwater fishes/shellfishes. *Vibrios* comprise of some extremely virulent organisms, because to their capacity to infect a wide range of aquatic organisms such as penaeid shrimp (Lightner, 1993), fish (Austin and Austin, 1999) and molluscan (Rheinheimer, 1992); while 11 other species are known to cause diseases in man (Bullock, 1987).

Members of the genus *Vibrio* are known to be marine bacteria with the exception of *Vibrio cholerae*, which is terrestrial (Sakazaki, 1981). The marine *Vibrios* are also postulated to play some roles in the degradation of organic pollutants (West et al., 1984) and nitrogen fixation (West, 1985). They exhibit halophilism or halo tolerance (Baumann et al., 1984) and harbor a wealth of diverse genomes and represents cosmopolitan and endemic species that are yet to be described (Thompson et al., 2001). The exact ecological roles of many of these groups are unknown till date.

Some of the *Vibrio* sp., viz., *V. parahaemolyticus* and *V. anguillarum*, are known to be pathogenic for fish (Blake, et al., 1980). However, other species of *Vibrios* capable of causing disease in humans have received greater attention in the last decade, which include *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio damselae*, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio hollisae*, *Vibrio metschnikovii* and *Vibrio mimicus* (Chakraborty et al., 1997). At present, it is impossible to provide realistic figures concerning the incidence of illnesses caused by *Vibrio* species worldwide; surveillance programmes, where they exist, mostly collect information on only a limited number of incidences. In the USA, *Vibrio* species have been estimated to be the cause of about 8000 illnesses annually (Mead et al., 1999). With regards to *V. cholerae*, morbidity and mortality are likely to be grossly under reported, in part owing to surveillance difficulties, but also for fear of economic and social consequences. Moreover, several cholera endemic countries are not included in the WHO report (Colwell, 1996). Globally, an estimated 120,000 deaths are caused by cholera each year (WHO, 2001).
Chapter 2

Review of Literature

The studies on both *V. cholerae* and other human pathogenic *Vibrios* have revealed the existence of these bacteria as free-living or in association with phytoplankton, zooplankton, crustaceans and molluscs in coastal and estuarine environments (Vanderzant et al., 1971; Colwell and Huq, 1994; Otta et al., 1999; Lipp et al., 2002). *Vibrio* and *Photobacterium* are also reported to be attached to the external surface of zooplankton and there exists a close partnership between these bacteria and zooplankton (Heidelberg et al., 2002b; and Lipp et al., 2002). Some of the *Vibrio* species such as *V. harveyi* and *Vibrio parahaemolyticus* are also associated with bacterial infections in shrimp (Jiravanichpaisal and Miyazaki, 1995) and are generally considered to be opportunistic pathogens causing disease when shrimp are stressed. *Vibrios* also form a part of the normal microflora of the shrimp *Penaeus vannamei* (Vandenberghe et al., 1999). Understanding the pathogenicity of certain strains of *Vibrios* is critical in aquaculture systems, where the fish and shellfish, viz., salmonids and penaeid shrimps are reared in intensive culture systems and in high densities under artificial and unstable conditions (Olafsen, 2001). A combination of these conditions automatically favors the proliferation of *Vibrios* and enhances their virulence and disease prevalence. This highly intensive aquaculture also has disastrous effect on the environment (Naylor et al., 2000; Williams et al., 2000).

Several cultivation dependent and independent studies have shown that *Vibrios* appear at particularly high densities in and/or on marine organisms, e.g., corals (Rosenberg and Ben-Haim, 2002), fish (Ringo and Birkbeck, 1999), seagrass, sponges, shrimp (Gomez-Gil et al., 1998; Vandenberghe et al., 1998; 1999; 2003) and zooplankton (Johnson and Shunk, 1936; Suantika et al., 2001). *Photobacterium leiognathi* and *P. phosphoreum* are found in symbiotic associations with fish, and *P. leiognathi*, *V. logei*, and *V. fischeri* are found in symbiotic associations with squid. These bacteria colonize the light organs of the host and play a role (via emission of light) in communication, prey attraction, and predator avoidance (Fidopiastis et al., 2000).
1998; Fukasawa and Dunlap, 1986). In the light organs of the squid Sepiolla spp., the abundance of Vibrios can be as high as $10^{11}$ cells/organ (Fidopiastis et al., 1998; Nishiguchi, 2000). Newly hatched squid excrete a mucus matrix from the pores of the light organs whereby V. fischeri cells present in seawater are found (Nyholm et al., 2000; Nyholm and Mc Fall-Ngai, 2003). Subsequently, V. fischeri migrates into the organ and colonizes the crypt epithelium. Obviously, the flagella of V. fischeri play a crucial role in the colonization of the light organs, but hyper-flagellated V. fischeri cells containing up to 16 flagella are defective in normal colonization (Millikan and Ruby, 2002). V. fischeri, V. logei, and P. leiognathi are apparently the only three organisms colonizing the light organs of squid, but this seemingly specific partnership remains to be confirmed. V. fischeri cells entrapped in the light organs of squid can sense the density of nonspecific cells by signaling molecules or pheromones (e.g., N-acyl homoserine). Copepods may, in turn, feed on these bacteria. V. cholerae moves along and attaches to surfaces with the aid of the flagellum and pili, this may act as adhesions (Moorthy and Watnick, 2004). Because V. cholerae is closely associated with plankton, it is assumed that cholera outbreaks are linked with planktonic blooms and the sea surface temperature, and so such outbreaks may be predicted by monitoring the parameters like remote sensing. The wide ecological relationships and ability to cope with global climate changes may be a reflection of the high genome plasticity of Vibrios (Lipp et al., 2002). Recently, a number of reports have highlighted the pathogenic potential of Vibrios toward humans and marine animals (e.g. corals, gorgonians, and shrimp), which may be coupled with rising of seawater temperature due to global warming (Martin et al., 2002; Rosenberg and Ben Haim, 2002; Sechi et al., 2000).

Seafood-associated illnesses are mainly associated with the consumption of molluscan (viz., mussels, oysters, squids, cuttlefish, clams) owing to the filter feeding habit of these organisms that concentrates particulate matter and bacteria present in
surrounding waters. Two groups of pathogenic bacteria present in coastal seawater may be entrapped by bivalves: firstly the exotic bacterial pathogens like *Salmonella* and *Shigella* that is shed into the water from infected animals and humans and the other is autochthonous members of the family Vibrionaceae (Potasman *et al*., 2002). *Vibrio* resistance to depuration procedures of edible bivalves is a further reason for the worldwide incidence of *Vibrio* related seafood borne diseases. In order to decrease the number of unwanted microorganisms to acceptable levels for human consumption, bivalve depuration in controlled waters is used worldwide. (Perkins *et al*., 1980; Richards, 1988; Prieur *et al*., 1990; Olafsen *et al*., 1993; Marino *et al*., 1999). Interestingly, some *Vibrio* species have been reported to be resistant to depuration and are able to persist and multiply in bivalve tissues (Jones *et al*., 1991; Shumway, 1992; Murphree and Tamplin., 1991), supporting the hypothesis that these bacteria represent a bivalve-specific community. Outbreaks of cholera have been associated with consumption of seafood including oysters, crabs and shrimp (Kaper, 1995). Given their abundance in water, 100-fold higher concentrations are found in filter-feeding shellfish such as edible bivalves than in the surrounding water (Wright *et al*., 1996). During the warm summer months, virtually 100% of oysters can carry *V. vulnificus* and *V. parahaemolyticus* (Wright *et al*., 1996; Cook *et al*., 2002). Factors that favor active filter feeding by shellfish increase the probability that shellfish in a given area will take up the pathogen (Murphree and Tamplin, 1991).

*Vibrio tapetis* is the causative agent of brown ring disease (BRD), an epizootic disease that causes high mortalities in the introduced Manila clam, *Ruditapes philippinarum* cultured in Western Europe. *Vibrio tapetis* adheres to and disrupts the production of the periostracal lamina, causing anomalous deposition of periostracum around the inner shell (Prieur *et al*., 1990; Novoa *et al*., 1998). *Vibrio alginolyticus* infections have been related to production of heat labile and heat-stable toxins, with lethal and both lethal and ciliostatic activity respectively (Di Salvo *et al*.,
1978; Brown and Roland, 1984) *V. parahaemolyticus* causes gastroenteritis in which the hemolysins, thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH), have been considered to play a crucial role (Nishibuchi and Kaper, 1995). Terrestrial and aquatic animals (including plankton, birds, fish, reptiles) may harbor virulent strains of *V. parahaemolyticus* and play a role as intermediate hosts and vehicles for spread (Sarkar et al., 1985). *V. parahaemolyticus* can be introduced into non-contaminated areas by relaying shellfish prior to commercial harvesting. Sewage discharge may indirectly influence the densities of *V. parahaemolyticus* present in shellfish growing areas (Watkins and Cabelli, 1985). *V. parahaemolyticus* favors the presence of particulates, zooplankton and other chitin sources (Kaneko and Colwell, 1973). *V. parahaemolyticus*, a chitinoclastic organism adsorbs onto the exoskeleton of copepods. This association has been reported to be the most important in dictating the annual cycle of *V. parahaemolyticus* in temperate and estuarine areas (Kaneko and Colwell, 1978). The isolation of *V. parahaemolyticus* from freshwater samples is by no means novel. There have been occasional reports on the recovery of this organism from freshwater areas (Sayler, 1976). Introduction of the pathogen into such closed water bodies through ambulatory cases or carriers can be presumed since these waters are constantly used for domestic and ablutionary purposes. Since *V. parahaemolyticus* is among the more salinity dependent Vibrios, one might expect the halophile to survive for a very short period in such alien environments. Adsorption onto plankton might, perhaps, prolong its survival conferring some kind of protection (Sarkar et al., 1985). In Japan and eastern Asian countries, *V. parahaemolyticus* has been recognized as a major cause of food borne gastroenteritis. It has spread throughout Asia and to the United States elevating the status of *V. parahaemolyticus* to pandemic (Miyamoto et al., 2000).

The first account of Vibriosis in impounded lobsters was that of (Sanyal et al., 1983), who reported the isolation of both *Vibrio parahaemolyticus* and *Vibrio
algginolyticus from moribund aquarium-held lobsters. *Vibrio fluvialis* like organisms were isolated from diseased lobsters. Although the emergence of this pathogen poses economic threat that merits additional studies, the causative *V. fluvialis* like strains are probably not infectious for humans (Sanyal *et al.*, 1983).

*V. vulnificus* is an important etiologic agent of wound infections and septicemia in humans (Finkelstein, 2002). *V. vulnificus* has been associated with primary septicemia in individuals following consumption of raw bivalves which is a serious, often fatal, disease. To date, *V. vulnificus* disease has almost exclusively been associated with oysters (Oliver and Kaper, 1997). *Vibrio mimicus* has also been established as a pathogenic member of the genus *Vibrio* (Davis *et al.*, 1981). Isolation of the pathogen from clinical samples has been made in different countries including the United States, Japan, Bangladesh, New Zealand, and Canada (Davis *et al.*, 1981). Association of toxigenic *V. mimicus* with freshwater prawns has been described in Bangladesh (Chowdhury, 1986).

Other *Vibrios*, e.g., *V. hollisae, V. damselae, V. alginolyticus, V. cincinnatiensis, V. fluvialis, V. furnissii, V. harveyi, V. metchnikovii*, and *V. mimicus*, have been sporadically found in human infections (Yamane *et al.*, 2004; Farmer and Brenner, 1992; Farmer, 1992; Carnahan *et al.*, 1994; Davis *et al.*, 1981; Brenner *et al.*, 1983; Abbott and Janda, 1994). Apparently, they are less important as human pathogens (Farmer, 1992; Farmer and Brenner, 1992).

A *Vibrio* surveillance system maintained by the Centre for Disease Control and Prevention reported 296 cases of infection caused by *Vibrios* in the United States in 2000 (CDC, 2001). Most strains were isolated from stool, wound, and blood samples and were identified as *V. parahaemolyticus, V. vulnificus, and V. cholerae*, respectively. Most patients who died were infected by *V. vulnificus*. 
Some of the *Vibrio* species, viz., *Vibrio alginolyticus*, have been characterized as probionts (Gomez-Gil *et al.*, 2002) as well as pathogens (Lee *et al.*, 1996). Certain *Vibrio* strains have been reported to be potential probiotics for this shrimp (Gomez-Gil *et al.*, 1998, 2002). Use of probiotics, i.e. live microorganisms that, when administered in adequate amounts, confer a health benefit on the host, has been reported to reduce the need for medication (e.g. antibiotics and pesticides) and water exchange, which are used massively in intensive shrimp-rearing (Verschuere *et al.*, 2000). In India, the occurrence of various *Vibrio* species in water, sediment and shrimp samples from multiple shrimp farm environments from the east and west coast of India was studied (Shubha *et al.*, 2005).

### 2.2. Aquaculture and *Vibrios*

Aquaculture emerged as one of the most promising food production in the later part of 20th century, with annual growth rate exceeding 11% per annum (Pillay, 1997). However, the industry is unfortunately encountering serious diseases during the culture period. Of the different diseases, Vibriosis is one of the most frequent disease affect ing fishes, molluscs and crustaceans, which needs further attention.

It is a common practice in aquaculture systems to incorporate antibiotics to treat the bacteriological infections. The dispersion of antibiotics after treatment in shrimp ponds or hatcheries can lead to the development of resistance among the pathogens, and a changed microorganism composition in the aquatic environment (Molina Aja *et al.*, 2002).

### 2.3. Use of antibiotics

Bacteria have adapted defense mechanisms against the antibiotics and continue to develop new resistances, even as new antibiotics are being developed. Although penicillin was the first natural antibiotic to be discovered, the idea of using
microorganisms therapeutically was not new. A series of different antibiotics were quickly discovered after penicillin came into use. In 1943, one of Waksman's students discovered streptomycin (Schatz et al., 1944), leading to a flood of researchers combing the world for new drugs. It was in this same period the gramicidin, the first antibiotic active against gram-positive bacteria was discovered (Hotchkiss and Dubos, 1941). Chlortetracycline, Chloramphenicol and others were discovered shortly thereafter (Garrod, and O'Grady, 1971). Many drugs discovered were too toxic for human use, or that had already been discovered. Nevertheless, this work lead to the discovery of many new drugs and within a decade, drugs comprising the major classes of antibiotics were found (Greenwood, 2000). Some antibiotic-producing bacteria were isolated from a wound infection and others from sewage, a chicken's throat, and a wet patch of wall in Paris (Garrod and Grady, 1971). In 1962, one of the later discoveries was a synthetic drug nalidixic acid, the first of the quinolones to be described, and although not therapeutically important by itself, modification of nalidixic acid led to the production of the highly effective fluoroquinolones. Members of this class, such as ciprofloxacin, norfloxacin, enrofloxacin, and ofloxacin, have become very important in the treatment of diseases in both humans and animals (Mitsuhashi, 1993.). Since 1960's, there have been few discoveries of new antibiotic drugs. The drugs developed since have mostly been chemical modifications of existing drugs. These modifications have been very useful in treating infectious diseases, leading to the enhanced killing of pathogens, increased spectrum of action, reduced toxicity, and reduced side effects.

In order to comprehend the problem of antibiotic resistance, as it exists today, it is useful to understand the history and development of both antibiotics and antibiotic resistance. Antimicrobial drugs have generally been classified into two categories- one includes the synthetic drugs, such as the sulfonamides and the quinolones; and the second includes the antibiotics synthesized by microorganisms. In recent years, increasing numbers of semi-synthetic drugs have been developed which are chemical
derivatives of antibiotics, thereby blurring the distinction between synthetic and natural antibiotics.

Unfortunately, since the 1970's, only one new class of antibiotics has been introduced (Lipsitch, 2002.) and a recent trend in antibiotic therapy has been to employ combinations of drugs with different mechanisms of action, in order to increase their effectiveness and to overcome the problem of drug resistance.

2.4. Antibiotics in veterinary and aquaculture: Drug use and antibiotic resistance

Antibiotics are added to various feeding mixtures used in poultry and animal farming as a preventive measure to keep the animals in good health. There are seven chemicals approved for sale when labeled for food fish use in Canada, including four antibiotic drugs (oxytetracycline, florfenicol, sulfadimethoxine plus ormetoprim, sulfadiazine plus trimethoprim), one anaesthetic (tricaine methanesulphonate) and two fungicides/disinfectants (formaldehyde and hydrogen peroxide) (Health Canada, 2001a). Oxolinic acid has been widely used in salmonid culture outside of Canada, including the United States, and off-label prescription potential exists where veterinarians can legally prescribe it. In addition, oxolinic acid provides a wide degree of information regarding fate and effect data, which could be relevant to other antibiotics. As with all intensive animal husbandry, aquaculture practices create an opportunity for the proliferation and spread of pathogens that can lead to significant mortality of stock and subsequent loss of revenue (Dixon, 1994). Antibiotics can be administered directly by injection or by releasing feed containing antibiotics directly into the aquatic ecosystem. Unconsumed medicated feed is available to wild animals. In addition, antibiotic containing feed can accumulate in the sediments or unabsorbed antibiotics can be released in fish faeces or urinary waste (Bjorklund et al., 1990 and 1991). Subsequently influencing the natural bacterial flora, an important component of ecological food webs. It is estimated that 1.4 to 40.5% of fish feed passed uneaten
through an Atlantic salmon sea cage (Thorpe et al., 1990). However, this may be a conservative estimate since diseased fish rarely feed (Bjorklund et al., 1990) and the majority of the active form antibiotic passes unabsorbed through the gastrointestinal tract of fish (Cravedi et al., 1987; Bjorklund et al., 1991).

The evolution of drug resistant strains of pathogenic bacteria is perhaps the most important implication of antibiotic use in aquaculture. Resistance to antibiotics is present in bacterial populations naturally (McPhearson et al., 1991; Johnson and Adams 1992; Spanggaard et al., 1993) and antibiotic use gives resistant strains the opportunity to proliferate and spread. Studies that examined antibiotic resistance following drug therapy at fish farms (Bjorklund et al., 1990, 1991; McPhearson et al., 1991; Nygaard et al., 1992; Samuelsen et al., 1992a; Spanggaard et al., 1993; Ervik et al., 1994; Kerry et al., 1996a) and in microcosms (O’Reilley and Smith, 2001) shows an increased frequency of resistance to several drugs across a variety of bacterial species. However, Kapetanaki et al., (1995) and Vaughan et al., (1996) suggest that increased levels of bacterial drug resistance can arise independently of the presence of a drug (through sterile fish feed, sediments added to microcosm studies, uneaten fish food).

The emergence and spread of antimicrobial resistance poses a major challenge to the quality and cost of healthcare systems worldwide. Effective interventions are urgently needed to contain emerging resistance without these the problem will inevitably worsen, with dramatic human and financial consequences. The WHO Global Strategy for the Containment of Antimicrobial Resistance provides a practical framework and helps to prioritize those interventions that are likely to be most effective. The future containment of antimicrobial resistance requires a coordinated multidimensional approach in which effective change in antimicrobial usage; infection control and epidemiologically sound resistance surveillance are key concerns, which is to be addressed in future.
2.5. History of antibiotic resistance

There is evidence that although resistant microorganisms existed in nature before the use of antibiotics, such microorganisms were mostly absent from human flora (Hughes and Datta, 1983). However, in the intervening years, antibiotic resistant microorganisms have become frighteningly common. Almost as soon as antibiotics were discovered, researchers began to find microorganisms resistant to the new drugs. Even by 1909, when Ehrlich first began to study dyes and arsenicals, he found drug resistant trypanosomes. Resistant strains of *Staphylococcus aureus* in hospitals grew initially from less than 1% incidence, when penicillin first came into use, now resistance increases to 14% in 1946; to 38% in 1947, to more than 90% today (Greenwood, 2000). Worldwide, ampicillin and penicillin resistance can be found together in more than 80% of *S. aureus* strains. Within 30 years of their discovery, sulfonamides ceased to be an effective treatment for meningococcal disease (O’Brien, 1987). In the years since, reports of resistance have grown increasingly common and pathogens that are resistant to almost all antibiotics have been found. It has become painfully obvious that antibiotic resistance has reached a crisis stage and some clinicians have even forecasted that we are facing a return to the devastating diseases of the pre-antibiotic era (Hughes and Datta, 1983; Lipsitch et al., 2002).

Antibiotics have been widely used in aquaculture worldwide to treat infections caused by a variety of bacterial pathogens of fish: *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella tarda*, *Pasteurella piscicida*, *Vibrio anguillarum*, and *Yersinia ruckeri*. Use of antimicrobial agents in aquaculture directly doses the environment, which results in selective pressures in the exposed ecosystem (Aarestrup, 1999 and 2000). The emergence of antimicrobial resistance following the use of antimicrobial agents in aquaculture has been identified in fish pathogens. In several countries *A. salmonicida* is frequently resistant to multiple drugs including sulphonamidcs, tetracycline, amoxycillin, trimethoprim, sulfadimethoxine and
quinolones (Aarestrup and McNicholas, 2002). Similar correlations between antimicrobial agents used in aquaculture and antimicrobial resistance are also reported among other fish pathogens (Akiba et al., 1960)

During certain periods of the year, pathogenic *Vibrios* would endure adverse environmental conditions within aquaculture systems and when favorable environmental conditions are re-established, they are able to cause disease in wild animals (Ben-Haim et al., 1997). The spread of antibiotic resistance from aquaculture settings to natural environments has recently been shown (Hameed and Balasubramanian, 2000; Liu, 1999). About 70% of the *Vibrios* isolated from aquaculture settings in Mexico were multiple-drug resistant. Several *Vibrio* isolates have also acquired resistance to the most commonly employed antibiotics (e.g., enrofloxacin, florfenicol, trimethoprim, and oxytetracycline) in shrimp rearing, suggesting that the recently initiated application of these antimicrobials has led to the generation of resistant strains of *Vibrios* (Molina Aja et al., 2002).

Use of antimicrobial agents in aquaculture also selects for antimicrobial resistance among bacteria that are not fish pathogens. Several studies have assessed the impact of use of antimicrobial agents in aquaculture on the bacteria in the sediment and within fish in the local environment. Bacteria resistant to antimicrobial agents used on specific fish farms have been isolated from sediment beneath the fish "net pens" on those fish farms (Allen et al., 1977); in contrast, no resistance was present among bacteria from the intestinal contents of the fish from untreated areas (Allen et al., 1977).

Many antibiotic resistance determinants in fish pathogens are frequently carried on transferable R plasmids. Horizontal spread of plasmids from fish pathogens may therefore transfer resistance genes to other bacteria including those that are pathogenic to humans. Horizontal transfer of resistance genes on plasmids has been
Chapter 2

Review of Literature

demonstrated between bacteria in the water of fish ponds (Anderson and Datta, 1965) and in marine sediments (Anderson and Sandaa, 1994). Plasmids carrying resistance determinants have also been transferred in vitro from fish pathogens to human pathogens including *Vibrio cholerae* (Angulo et al., 2000). *Vibrio parahemolyticus* (Apgar et al., 2005) and potential human pathogens including *Escherichia coli* (Armstrong et al., 1990; Arvanitidou et al., 2001).

Furthermore, plasmids carrying multiple antimicrobial resistance determinants have been transferred in simulated natural microenvironments between bacterial pathogens of fish, humans, and other animals, demonstrating that resistance determinants on plasmids can spread from fish pathogens to human pathogens (Arzese et al., 2000). These studies indicate that the horizontal transfer of plasmids between related and diverse bacteria may facilitate dissemination of antimicrobial resistance determinants. Bacteria present in aquaculture systems may be transmitted to humans who come in contact with this ecosystem. For instance, *Vibrio* spp can cause wound infections in persons with open wounds or abrasions exposed to seawater or marine life (Ash, 2002). In 1991, an epidemic of *Vibrio cholerae* O1 infections affected Latin America; the epidemic strain in Latin America was susceptible to the 12 antimicrobial agents tested except in coastal Ecuador where the epidemic strain became multi drug-resistant (Ashkenazi et al., 2003).

The cholera epidemic in Ecuador began among persons working in shrimp farms. Multidrug-resistance was present in non-cholera *Vibrio* infections that were pathogenic to the shrimp. The resistance may have been transferred to *V. cholerae* O1 from other *Vibrios* (Ashkenazi, et al., 2003). Bacteria from the aquaculture ecosystem may also been transmitted directly to humans through handling of fish. Recently, the fish pathogen *Streptococcus iniae* has caused invasive infections in persons who handled store-bought aquacultured tilapia and *S. iniae* was isolated from the aquaculture ecosystem and on fish in grocery stores (Bager et al., 1999). Similarly a
new biotype of *Vibrio vulnificus* caused hundreds of serious infections among persons handling live tilapia produced by aquaculture in Israel (Baquero and Blazquez, 1997). Bacteria in fish also might have transmitted to humans when the cultured fish were eaten or when other foods, which got cross contaminated by bacteria from fish, were eaten. For instance, *V. parahaemolyticus* is a common food borne disease in Japan where infections have been linked to the consumption of aquacultured finfish (Baquero et al., 1991). Furthermore, *Salmonella* spp., a common cause of food borne disease has been isolated from aquacultured fish and shrimp ponds (Bass, et al., 1999). There were other reports, which indicate that bacteria present in aquaculture ecosystems can be transmitted to humans. Newly available molecular characterizations of antimicrobial resistance determinants provide further evidence of the transmission of antimicrobial resistance between aquaculture ecosystems and humans.

Some of the antimicrobial resistance determinants in *Salmonella* serotype *typhimurium* definitive type 104 may have originated in aquaculture. *S. typhimurium* DT 104 is typically resistant to ampicillin, chloramphenicol, florofenicol, streptomycin, sulfonamides and tetracycline. The strain was first isolated from a patient in 1985 and emerged during the 1990s as a leading cause of human *Salmonella* infections. Tetracycline resistance in *S. typhimurium* DT104 is due to a class G resistance gene (Baxter et al., 1998). Class G was first identified in 1981 in tetracycline-resistant isolates of *Vibrio anguillarum*, a pathogen of fish (Baya et al., 1986). Furthermore, the recently described novel florofenicol resistance gene, *floR*, in *S. typhimurium* DT 104, which also confers resistance to chloramphenicol, is almost identical, by molecular sequence, to the florofenicol resistance gene first described in *Photobacterium damselae*, bacteria found in fish. This resistance gene is rare and has not previously been reported from *Salmonella* isolates (Bayne et al., 1983). Finally, all of the antimicrobial resistance determinants in *S. typhimurium* DT104 are grouped
on the chromosome within two distinct integrons and an intervening plasmid-derived sequence. The Class G and floR determinants are located within the intervening plasmid-derived sequence. By molecular sequence, the plasmid-derived sequence is closely related (94% identity) to a plasmid identified in Pasteurella piscicida, a pathogen of fish (Bergh et al., 1989). These and other reports indicate that antimicrobial resistance determinants selected for in aquaculture ecosystems can be transmitted to bacteria that cause illness in humans, perhaps at a greater frequency than previously suggested (Blanch et al., 2003).

With the expansion of fish culture in recent years, problems associated with bacterial fish pathogens have increasingly occurred. Concomitantly, a variety of important properties of microorganisms have been proven to be plasmid mediated. The use of antibiotics in the treatment of infectious diseases of fishes has resulted in the expansion of R plasmids in commercial aquaculture (Aoki et al., 1977 and 1981; Chen, 1978; Watanabe, 1971), owing to the selective pressure exercised by chemotherapeutic agents when used over an extended period of time (Aoki, et al., 1974; 1975 and 1981). The presence of plasmids in bacterial fish pathogens may pose a potential public health hazard, since plasmids from animals may be transferred to humans either directly, by infection with pathogens such as Aeromonas hydrophila or Edwardsiella tarda (Jordan and Hadley, 1969; Joseph et al., 1979), or indirectly, if they are transferred to human pathogens such as Vibriocholerae or Escherichia coli by way of pathogenic fish bacteria (Aoki et al., 1974 and 1977).

2.6. Plasmids

The term plasmid was originally used by Lederberg to describe extra chromosomal hereditary determinants and it is currently used to describe autonomously replicating extra chromosomal DNA of bacteria. They are found both in gram-negative and gram-positive bacteria as well as in some yeast and other fungi.
They range in size from 1 to more than 200 kb and are present in a wide variety of prokaryotic and eukaryotic organisms, but are mainly in bacteria. Plasmids are found in a variety of microorganisms and it is difficult to generalize about plasmids. Although most of them are covalently closed circular double stranded DNA molecules, some linear plasmids have also been recently isolated from bacteria. In general plasmids are not essential for the survival of bacteria, but they may nevertheless encode a wide variety of genetic determinants, which permit host bacteria to survive better in an adverse environment or to compete better with other organisms occupying the same ecological niche (Luis et al., 1999).

The existence of plasmid was initially revealed as the "F factor" in *Escherichia coli* even before the double-helix structure of DNA was elucidated by Watson and Crick (Hayes, 1953; Lederberg, 1998). The occurrence of plasmids has been well documented among the majority of gram-negative and gram-positive isolates from the Eubacteria, and recently in a hyperthermophilic Archaeon (Erauso et al., 1996).

The first linear plasmid was found in *Streptomyces rochei* in 1979 (Hayakawa et al., 1979) and now they have been detected in several bacterial genera such as *Agrobacterium*, *Borrelia*, *Nocardia*, *Rhodococcus*, *Thiobacillus*, and *Escherichia* (Hinnebusch and Tilly, 1993). Most of the plasmid-encoded genes that have been characterized impart some growth advantages to the host. They are also responsible for nitrogen fixation in certain bacteria, resistance to heavy metals and radiation, production of certain endonucleases, metabolism of compounds such as toluene and camphor, plant tumor development, and also for the production of bacterial virulence determinants. The genetic determinants encoded by plasmid enable the bacterial host to survive better in adverse environments and to compete better to occupy ecological niches. Examples of some typical plasmid-encoded traits include protection from UV light damage (Rochelle et al., 1989), resistance to heavy metals
(Hansen et al., 1984; Schutt, 1989), proliferation in the presence of antibiotics (Aviles et al., 1993) and catabolism of xenobiotic compounds (Hada and Sizemore, 1981; Sayler et al., 1990).

2.7. Role of plasmids

Plasmids display an amazing diversity of characteristics, such as size, modes of replication and transmission, host range, and the presence of variety of genes. They have adopted a variety of strategies to ensure their own faithful replication, maintenance, and transfer. They impart a wide assortment of phenotypes to the cells that harbor them. From a human perspective, some phenotypes may be problematic, for example, the expression of antibiotic resistance and expression of antibiotic resistance genes and pathogenicity genes that hinder human and animal health. Other properties may be beneficial, such as the ability to fix elemental nitrogen or features that can be exploited and used to improve soil fertility. It is the rich diversity of their form, function, and utility that can be explored in plasmid biology.

An obvious way of classifying plasmids was by function. There are five main types:

- **Fertility plasmids**, which contain only *tra* genes. Their only function is to initiate conjugation.

- **Resistance plasmids**, which contain genes that can build a resistance against antibiotics or poisons.

- **Col plasmids**, which contain genes that code for the production of colicins, proteins etc that can kill other bacteria.

- **Degradative plasmids** which enable digestion of unusual substance toluene or salicylic acid.

- **Virulence plasmids** that turn the bacterium into a pathogen.
Plasmids may also be categorized into one or two major types: conjugative or non-conjugative, depending on whether or not they carry a set of transfer genes, called the *tra* genes that promote bacterial conjugation. Generally, conjugative plasmids are of high molecular weight and low copy number i.e. they are present in one to three copies per chromosome whereas non-conjugative plasmids are of low molecular weight and are high copy number i.e. present as multiple copies per chromosome (Willetts and Wilkins, 1984).

Plasmids can also be categorized on the basis of their being maintained as multiple copies (relaxed plasmids) or as limited copies (stringent plasmids) per cell. Frequently, plasmids contain some genes advantageous to the bacterial host. Plasmids which do not have any phenotypic traits ascribed to them are called cryptic plasmids. The number of copies also varies among plasmids, and bacterial cells can harbor two different types of plasmids, with hundreds of copies of one plasmid type and only one copy of other type. Unlike chromosomes, plasmids generally encode genes whose functions benefit the bacterium under certain specific circumstances (Tolmasky et al., 1992).

### 2.8. Role of Plasmids in resistance

It took several decades to appreciate the existence of plasmid-mediated antibiotic resistance. The key to identifying plasmids as resistance factors was their property of providing simultaneous protection to multiple antibiotics of inherently different nature. But in hindsight, resistance based on plasmids as well as chromosomally based resistance, indubitably was an important mechanism early after the antibiotics were introduced and used commercially. Plasmids bearing antibiotic resistance markers can be found in organisms isolated before the antibiotic era (Falkow, 1975). Resistance based on transmissible plasmids affords the significant advantage of flexibility to the microorganism. Resistance to several antibiotics can be
brought together in single plasmids. Antibiotic resistance genes can be amplified when needed and deamplified when not needed. Plasmids can be stored in a minimum portion of the microbial population and regained as needed. Plasmids can serve as vectors to transfer genes. Plasmids serve an evolutionary role in the rearrangement of genetic parts both between and within organisms.

However, gene transfer does little to account for the resistance characters themselves (Bennett and Richmond, 1978). But plasmid-borne resistance has the disadvantage to the bacterium that it cannot easily function to alter the target of the antibiotic within the cell, since the structural genes for the cell's essential proteins in almost all cases are carried on the chromosome. In gram-negative organisms, much less enzyme suffices to detoxify penicillin or cephalosporin because the enzyme is retained within the periplasm itself. *Escherichia coli* strain K-12 contains a gene that codes for a penicillinase, but this gene does not afford much protection, suggesting that the enzyme really has another unknown function, and therefore highly possible that it does function to protect against penicillin (Boman *et al*., 1967; Burman *et al*., 1973). One possibility is that at low levels of penicillin and high cell densities, it protects against penicillin in the amounts that may occur naturally. The second possibility is that this gene protects the strain against high levels of penicillin only after it has become tandemly duplicated. Still, gene amplification may be needed to protect the cell. Besides the gram-negative envelope structure, there are other aspects of bacterial biology that serve to increase the effectiveness of plasmid borne resistance mechanisms. For example, the derivatives of amino glycoside antibiotics produced by resistance mechanisms are such that they interfere with the transport of the unmodified drug (Demerec, 1948).
2.9. Role of mechanisms facilitating genetic rearrangements within an organism

In earlier paper (Koch, 1972), it was reported that the mobilization of genetic material would greatly slow the fundamental rate of evolution by supplying alternate but preexisting mechanisms to overcome a particular growth limitation rather than forcing a new solution to be sought. Some of these mechanisms lead to the mutation and therefore also faster de novo evolution provided that the genetic burden is not high. Early evolution could have taken place via point mutations and by chance through primitive replications, but mechanisms allowing duplication of more than several base pairs and rearrangement of segments of DNA may have involved biological evolved mechanisms, such as insertion sequences, at a very primitive stage. Numerous studies have demonstrated that genetic exchange by conjugation as well as transduction and transformation occurs between bacteria in the environment (O'Morchoe et al., 1988; Ogunseitan et al., 1990; Paul et al., 1991; Kinkle et al., 1993). All the three transfer mechanisms have been shown to occur in marine systems (Maruyama et al., 1993; Barkay et al., 1995).

2.10 Molecular properties of R plasmids

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. This type of plasmids is widespread in bacteria and can be transferred between different microorganisms, a genetic property that represents a very serious medical problem in human and animal medicine. R plasmids can be isolated from host bacteria as circular DNA in both closed and nicked forms. There has been some debate as to the proportions of closed and nicked circles that arise during the isolation procedures. Nevertheless there is little doubt that both forms exist in the cell. The closed circular structure is probably adopted by R plasmids when not in replication. The R plasmid
sometimes may dissociate into its conjugative and resistance determinants (Franklin and Snow, 1989).

The numbers of R plasmids harbored by individual bacteria is influenced by the properties of the plasmid and host as well as by the culture conditions. As a general rule that larger plasmids are present only in a limited number of copies per chromosome in *E.coli*, whereas in *Proteus mirabilis* the number varies during the growth cycle (Franklin and Snow, 1989). In stationary phase, replication of the R plasmid continues for some times after chromosomal replication has ceased. Conditions that give rise to an increased number of R plasmid copies are sometimes associated with enhanced resistance. However the level of resistance does not always reflect the number of resistance gene copies in the cell (Franklin and Snow, 1989).

In general, plasmids are not essential for the survival of bacteria, but nevertheless encode a wide variety of genetic determinants, which may confer on their bacterial hosts, better prospects of survival in an adverse environment or to compete better with other microorganisms occupying the same ecological niche. Resistance plasmids (R-Plasmids) harbor a variety of genes encoding resistance to a wide spectrum of antimicrobial compounds, which include antibiotics, heavy metals, resistance to mutagenic agents like ethidium bromide, and even disinfectant agents such as formaldehyde (Foster, 1983). The medical importance of plasmids that encode for antibiotic resistance, as well as specific virulence traits has been well documented and demonstrated the important role that the bacterial genetic elements play in nature (Luis *et al.*, 1999). Although they encode specific molecules required for initiation of their replication, plasmids rely on host-encoded factors for their replication. Plasmid replication initiates in a predetermined cis-site called ori and can proceed either by a rolling circle or a theta replication mechanism. Chromosomal replication origin, (ori C) was isolated from plasmid of *V. harveyi*. The ori C was found to be functional in *E. coli* (Zyskind *et al.*, 1983).
Molecular and genetic work on plasmids resulted in extraordinary contributions to the modern fields of molecular genetics and molecular biology (Cohen, 1993). 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 important findings about basic aspects of initiation of DNA replication and its regulation, DNA partitioning, and plasmid copy number and incompatibility (Helsinki, 1996). In pathogenic microorganisms, plasmids that contribute directly to microbial pathogenicity in plants and animals, such as for instance iron transport in several pathogens or the presence of adhesins, invasins or antiphagocytic proteins, is well documented (Crossa et al., 1989). In a more applied vein, plasmids played a central role in the initial development of recombinant DNA technology, gene cloning, and the constant evolution of molecular biology (Cohen, 1993). Furthermore, genetic and molecular analysis of plasmids proved to be essential in understanding the structure of transposons and integrons and the role these genetic elements play in the transmission of resistance to antimicrobial agents (Hall, 1995).

The presence of these mobile genetic elements in transmissible plasmids, some of them capable of replicating in bacterial strains belonging to different species, makes matters quite serious since they contribute to the transmissibility of resistance genes from strains to strains as well as between different replicons within any given strain (Luis et al., 1999).

2.11. Other roles of plasmids

Virulence factors of certain bacterial pathogens are encoded by the plasmids. The correlation between enhanced virulence and presence of a 50 M Da plasmid in V.anguillarum was reported (Crossa et al., 1977). Plasmid plays an important role in adaptation of Pseudomonas spp to chronic petroleum pollution. 50% of the isolated oil
degrading bacteria from oil spills on industrial bay and off shore oil field and grown on liquid enriched media of crude oil and poly nuclear aromatic hydrocarbons had multiple plasmids (Deverix et al., 1982).

Marine bacteria are able to survive in polluted environment due to the presence of self-transmissible plasmids as they transfer plasmid DNA coding for ecologically advantageous functions such as detoxification of heavy metals, oxidation of manganese, etc. Heterotrophic manganese oxidizing bacteria lost the capacity along with the loss of plasmids when maintained in laboratory (Gregory and Stanley, 1982).

Plasmids can be used for the transformation of bacteria as vectors (Hackett and Sarma, 1989). Studies on plasmids in wastewater bacteria showed that most of them had multiple plasmids 2 to 4 and have direct correlation between the number of plasmids and crude oil degradation by bacteria. (Flood gate, 1991). Plasmids in V.parahaemolyticus controls biodegradation, polymyxine resistance and low-level halophilism like characteristics (Chakraborty et al., 1994). A plasmid measuring 70-100 kb was isolated from Pseudomonas capcia G4 (Shields et al., 1995). The plasmid may also affect the temperature tolerance in acido thermophilic Archaebacterium thermoplasma (Yasuda et al., 1995). But there is little effect of plasmids on production of siderophore by the Aeromonas hydrophila strains obtained from diarrheal samples of human patients and fresh water ponds (Naidu, 1997).

In the isolates of Aeromonas hydrophila collected from shellfish and water, it was shown that 60% of strains simultaneously possessed the plasmids and haemolytic activity (Borrego et al., 1991). It was observed that A. salmonicida harbored 17 plasmids in size ranging from 12 -90 kD which encodes various proteins (Bell and Trust, 1989). Virulence plasmid of V.anguillarum is known to mediate a restriction system that prevents the conjugal transmission of plasmid DNA from E.coli donor to V.anguillarum (Singer et al., 1992). Plasmids of V. fisheri carry genes that are
important for the survival of these strains outside the squid symbiont (Boettcher and Ruby, 1994).

### 2.12. Plasmid profile

Various workers have studied the plasmid profiles of a number of bacterial species earlier. Many of studies have covered the different strains within species isolated from different locations. Plasmid profile of five fish pathogens- *A. salmonicida, A. hydrophila, Vibrio anguillarum, Pasteurella piscicida, Yersinia ruckeri, Edwardsiella tarda* and *Renibacterium salmoninarum*, showed that 75% of the strains were found to harbor one or more plasmids, with the majority of strains having multiple plasmids (Toranzo et al., 1983). The study also showed that some of the strains of *A. salmonicida* harbored six plasmids, *P. piscicida* were with three plasmids and *A. hydrophila* strains harbored a single plasmid having a molecular weight of 20 to 30 kb. In this study the highest molecular weight plasmids of 145 and 130 kb were detected in *V. anguillarum* (Toranzo et al., 1983). Plasmids from halophilic bacteria were shown to have varying molecular sizes ranging 6.4 kb to 8.75 kb (Hong, 1985). In *Edwardsiella ictaluri*, the causative agent of enteric septicemia of Channel catfish, two plasmids of 5.7 kb and 4.9 kb were reported (Lobb and Rhoades, 1987). Strains of *A. salmonicida* the agent of furunculosis also have fairly uniform plasmid pattern. The pattern consists of four small plasmids of 4.2, 3.6, 3.5 and 3.3 M Da and a larger plasmid. The larger plasmid was most often 50-56 M Da, but sometimes it was even larger. An additional plasmid was seen in a few species (Bast et al., 1988). A similar plasmid profile was also revealed in the *A. salmonicida* (Toranzo et al., 1991). However, Bell and Trust (1989) observed 2-9 plasmids in strains of *A. salmonicida*. Thermophilic bacteria have been reported to harbour plasmids; 6 M Da and 47 M Da were isolated from *Thermus thermophilus* (Fee and Mathew, 1988). A plasmid pTAI of 15.2 kb was reported in isolates of an acidothermophilic bacterium archaeabacterium, *Thermoplasma acidophilum* (Yasuda et al., 1995). Zhao and Aoki (1992) reported
that *Pasteurella piscida* isolated from *S. quinqueradiata* had one large plasmid of 110 kb and two small plasmids of a 5 and 5.1 kb which were shared by all strains. However, Margarinos *et al.*, (1992) found common plasmid band of 20 and 7 M Da in all *Pasteurella piscicida* studied. But the European strains were found to have an additional 50 M Da plasmid. Jain and Tiwari (1993) screened the plasmid pattern of 32 isolates of *Shigella dysenteriae* from different parts of India and reported that all the strains had at least five plasmids of following sizes viz 120 M Da, 57 M Da, 10.5 M Da, 6.5 M Da and 2.5 M Da. Maximum numbers of plasmids seen were eight while minimum numbers of plasmids present were five. Studies carried out by the Pederson *et al.*, (1996) revealed that *Aeromonas salmonicida* strains have 2 to 3 plasmids and all of them share a common small sized plasmid. The strains of *A. salmonicida* from Atlantic coast was found to posses 4 to 6 plasmids within the size range of 4.3 to 8.1 kb and while the strains from Pacific coast possessed 6 plasmids in the range of 14.2 to 0.9 kb. In strains of Aeromonas, 36% had plasmids and the most plasmid containing strains with multiple were less than 12 kb in size (Pettibone *et al.*, 1996). But plasmids were observed only in two hundred ninety seven isolates out of the more than thousand aerobic hydrophilic bacteria isolated from coastal California marine sediments.

While majority of the isolates typically contained one large plasmid of 40 to 100 kb size, some contained multiple small plasmids, three to five numbers with 5 to 10 kb size (Sobecky *et al.*, 1997). *Vibrio salmonicida* isolated from Cod and Atlantic salmon have 61, 21, 3.4 and 2.8 M Da plasmids and a 61 M Da plasmid were found exclusively in *V. salmonicida* strains of Northern Norway (Amaru *et al.*, 1998). Twenty-five *A. hydrophila* strains isolated from fresh water fish and water samples were screened for the presence of plasmids (Noterdaeme *et al.*, 1991). Ten strains were found without plasmids while eleven harbored one plasmid of 20 kb and four strains contained two or three plasmids. The 20 kb plasmid was common to all plasmid
positive strains. Borrego et al., (1991) studied the plasmid profile of sixty strains *A. hydrophila* isolated from shell fish and water and found that forty strains harbored one or more plasmids. The plasmid profile most frequently detected (15%) was the association of three small plasmids of 4.2, 3.2 and 2.8 M Da. Thirty four *A. hydrophila* strains isolated from various fish species and several geographical locations were examined (Ansari et al., 1992) for their plasmid carriage and reported that the plasmid occurrence rate was only 14.7 % with size range from 2.6 to 6 M Da. Multiple carriage of plasmid is more likely with strains having three or four plasmids.

### 2.13. Importance of plasmid profiling

Plasmid profiling can be used in the characterization and identification of bacteria. Plasmid profiles can be taken as a fingerprint in identifying the bacteria. Reud and Bayle (1989) used the plasmid profiling, since it is simple, easy to perform and useful to identify *Edwardsiella ictaluri* that causes enteric septicemia in channel catfish. According to Lobb and Rhoades (1987) restriction profiles of the separated plasmids reveal whether the plasmids are closely related or nor. Typing based on the plasmid profiling appears to be the most effective method for grouping strains with the same serotype obtained from clinical source and from environmental sources (Borrego et al., 1991).

In recent years, a molecular-based approach, referred to as replicon typing, has been used to assign plasmids to incompatibility groups using specific DNA probes containing replication control genes from well characterized plasmids (Couturier et al., 1988). The primary sources of the majority of these well-characterized plasmids have been bacteria from clinical and animal origins. This more direct and less time consuming method for classifying plasmids is possible due to the nature of the basic replicon of plasmids. The basic or minimal replicon of a plasmid consists of the genes and sites necessary to ensure and control autonomous replication. The genes essential
for plasmid replication and maintenance are typically clustered on a contiguous segment of DNA usually not more than 2–3 kb in size (Helsinki et al., 1996). It is this compact nature of plasmid replication origins that has facilitated the isolation and characterization of replicons from plasmids obtained from bacteria of clinical and animal origins. The bank of replicon probes developed by Couturier et al., (1988) contain unique DNA sequences derived from 19 different basic replicons cloned in high copy number plasmid vectors. These collections of replicon (inc/rep) probes have been shown to be suitable for the molecular typing of plasmids from bacteria of medical importance. Interestingly, recent studies that have attempted to use these clinically based replicon probes to type plasmids from bacterial isolates obtained from terrestrial soils (Kobayashi and Bailey, 1994), as well as sediments (Sobecky et al., 1997), bulk water, air water interfaces and biofilms of marine environments have been unsuccessful (Dahlberg et al., 1997). None of the hundreds of plasmid containing isolates from these different environments shared homology to the inc/rep group-specific DNA probes currently available for plasmid typing. Such findings indicate that plasmids isolated from bacterial populations occurring in terrestrial soils and marine aquatic and sediment systems encode novel replication and incompatibility loci that lack homology to clinically-derived plasmid incompatibility groups. Moreover, the extent of plasmid diversity occurring in natural microbial communities, such as marine sediments, cannot be determined using the present molecular classification system based on plasmids of clinical and animal origins. Therefore, inc/rep probes specific for replicons isolated from the marine environment are necessary to characterize naturally occurring plasmid distribution and diversity (Sobecky et al., 1997).

To better understand plasmid distribution, diversity and abundance in marine microbial communities, the isolation and characterization of replication sequences from naturally occurring plasmid populations is necessary. Ideally, such information could be used to develop a collection of environmentally based
incompatibility group specific replicon probes suitable for typing plasmids from non-clinical environments. An increasing body of literature, based largely on the analysis of plasmids from culturable bacteria from diverse environments, supports the existence of new plasmid groups which appear to have evolved along separate lines from plasmid groups occurring in clinical bacterial populations (Kobayashi and Bailey, 1994; Top et al., 1994; Dahlberg et al., 1997; Sobecky et al., 1997; Van Elsas et al., 1998). Therefore, studies designed to isolate and characterize plasmid replication and incompatibility sequences from environmental isolates will aid in the determination of plasmid diversity, as well as to provide more detailed insights into gene movement in microbial communities. The basic plasmid characterization study may further lead to the development of such replication probes that will help to identify the environmental isolates itself rather than from available clinical probes.

Wilk (1989) reported that plasmid profile along with serological and biological properties is helpful in isolating *V. anguillarum* strains from diseased fish. He classified the *V. salmonicida* using the same technique. Zhao and Aoki (1992) reported that plasmid profile could be used as a fingerprint of bacteria. They found that a plasmid of 5.1 kb size is specific to *Pasteurella piscida*, which causes influenza. Austin and Adams (1995) are of the opinion that plasmid profiling can be used as a rapid diagnostic technique for detection of furunculosis caused by Aeromonas salmonicida. However Dahlberg et al., (1997) observed the plasmid types isolated from different habitats and from different sampling occasions showed little similarity indicating high variation.

2.14. Plasmids in *Vibrios*.

In *Vibrio*, plasmids have been found and their involvement in resistance to many antibiotics has been proven (Toranzo *et al.*, 1983). Antibiotic resistance
plasmids have been reported in strains of *Vibrio* isolated from cultured marine fish (Aoki *et al.*, 1973). Cryptic plasmids have been isolated from enteropathogenic strains of *Vibrio parahaemolyticus* (Guerry and Colwell, 1977). Sizemore has observed a bacteriocinogenic plasmid in *Vibrio harveyi* (Mc Call and Sizemore, 1979). The plasmid presence in the Danish isolates of *Vibrio anguillarum* with phenotypic properties of haemagglutination, and biochemical activities was also reported (Larsen, 1991). A 50 M Da plasmid pJM1 was present in avirulent strains encodes a very efficient iron sequestering mechanism that helps to compete with iron binding proteins in serum (Crossa *et al.*, 1980). It is to be observed that plasmid-carrying bacteria could grow under conditions of iron limitations. Non-virulent bacteria without plasmids were unable to produce siderophore activity, which is plasmid mediated nature (Tolmasky, 1985). A plasmid from *V cholerae* strain isolated in Malaysia encoding the CT toxin was detected. (Mohammad and Haque, 2002).

The complete sequence analysis of pPS41 from *Vibrio splendidus* showed that this plasmid could be mobilized by RK2 transfer system (Leigh *et al.*, 2000). The role of plasmids in the degradation of hydrocarbons was reported. *Vibrio parahaemolyticus* possessed a degradative plasmid, which helped in the break down many aromatic compounds. The halophilic characteristic of microbe has also been linked to a plasmid (Chakraborty, 1994). In the non-marine environments where there is low concentration of sodium chloride, the plasmids were acquired by the *Vibrios* and retained them by due to selection pressure. As soon as the *Vibrio parahaemolyticus* return to marine environment, the plasmid may be lost because of the lack of selection pressure of halophilism. But in some clinical isolates of *Vibrios*, a plasmid determined the growth of those isolates in less concentration of sodium chloride (Chakraborty, 1994). *Vibrio anguillarum* isolated from diseased trout carried a 67 kb plasmid similar to plasmid pJM, the virulence plasmid and also a 98 kb
Larsen and John 1991 studied certain strains and observed that 42% isolates from healthy fish and from environment were without plasmids. Environmental authorities and fish farmers are naturally concerned about the increasing spread of pathogenic bacteria in the aquatic environment and hence the plasmid profiling should be used as an epidemiological trait in such cases.

The antibiotic susceptibility differences, plasmid content and RAPD analysis of *Vibrio vulnificus* isolated from *Anandra granosa* (cockles) in Malaysia however yielded no plasmid profile predictive of particular antibiotic susceptibility. A non-conjugative plasmid of 2 M Da was observed during the characterization of *Vibrio cholerae* O139 Bengal isolated from sewage drainage water in Malaysia. Although strains contain plasmids, they were not genetically identical. The genetic diversity within the group demonstrated that the isolates were isolated from a single location, has almost genetic diversity among them and finally they found out that isolates appeared to be heterogeneous. They also studied the ability to transfer resistance is a potentially serious health hazard not only because of consequent problems regarding therapy but also because of risk of resistance spreading to other enter bacterial organisms including normal flora. Further the transmissibility of antimicrobial resistance may assist in establishment of persistence of organism in the host. (Son *et al.*, 1997).

A plasmid encoding histidine decarboxylase gene *angH* that is essential for biosynthesis of siderophore Anguibactin was also reported from the fish pathogen *Vibrio anguillarum* (Courtney *et al.*, 1998). The fatal hemorrhagic septicemic disease in salmonids and other fish is caused by *Vibrio anguillarum*. The pathogenic strains survive within the host due to the possession of a 65 kbp virulence plasmid, which provides the bacteria with an iron sequestering system that is crucial. This confers upon them the synthesis of the siderophore Anguibactin, an iron scavenging compound and subsequent transport of ferric Anguibactin complex in the cell.
Anguibactin synthesis requires expression of gene from chromosome and the virulent plasmid pJM1.

The plasmid profiles of fish pathogenic isolates such as *Aeromonas* and *Vibrio ordalii* from Canada revealed that the resistance to oxytetracycline and streptomycin do not appear to be plasmid mediated. In this the antibiotic resistance was altered following plasmid curing and resistance was not transferable to *E.coli*. (Giles et al., 1995). *Vibrio ordalii* is one of major causes of *Vibriosis* in wild and cultured marine salmonids in Japan and the USA (Actis et al., 1985). The molecular characterization of different isolates of *Vibrio ordalii* showed that they contain a plasmid, designated as pMJ101. The pathogenic *Vibrios* isolated from moribund silver sea bream in fish farms in Hong Kong were screened for plasmids and they describe the antibiotic resistance associated plasmids. The different *Vibrio* strains studied had similar antibiotic resistant profiles (Liu et al., 1999). It is reported that the antibiotic susceptibility profiles for the different *Vibrio* species in clinical and environmental setting and observed 29% bacteria gave large plasmid with molecular weights ranging from 9-123 kb. Bacterial resistance is usually associated with the presence of plasmids and the ability of plasmids for trans conjugation. But in general, plasmids, which can be trans conjugated usually, possess a high molecular weight (French et al., 1989).

*Vibrio ordalii* is a major cause of *Vibriosis* in wild and cultured marine salmonids in Japan and carries pMJ101, a 30 kb cryptic plasmid that replicates in the absence of DNA polymerase I without producing single-stranded intermediates. *V. ordalii* is phenotypically and genetically distinct from *V.anguillarum* 775 (Schiewe et al., 1981), which also causes disseminated infections in salmonids (Schiewe et al., 1981). The molecular characterization of different isolates of *V. ordalii* revealed the presence of a high-copy-number plasmid in all strains examined to date (Schiewe et al., 1981). This plasmid, which was designated pMJ101, is a 30-kb extra
chromosomal element that has no DNA sequence homology with the pJM1 virulence plasmid present in *V. anguillarum* 775 (Crossa *et al.*, 1980). This plasmid is essential for the virulence of *V. anguillarum* and encodes a high-affinity siderophore-mediated iron acquisition system that allows this fish pathogen to acquire this essential micronutrient from the infected host (Crossa, 1989).

2.15. PCR based molecular characterization of microbes

PCR is a technique for the *in vitro* amplification of DNA, which lies between the regions of known sequence. This technology has proven to be a revolutionary method, which gives scientist the great advantage of generating a large number of target DNA sequences from trace amounts of DNA material. Since its introduction was first reported by Saiki (Saiki *et al.*, 1985). PCR has already become a wide spread technique in research laboratories.

2.16. Horizontal gene transfer

The horizontal transfer of genetic material within microbial communities has been instrumental in the emergence of novel functions and species (De la Cruz and Davies, 2000; Ochman *et al.*, 2000). The ‘antibiotic resistance phenomenon’ is perhaps the most striking recent example of the impact of horizontal transfer on microbial adaptation. This phenomenon refers to the rapid and widespread emergence of similar antibiotic resistance profiles among phylogenetically diverse gram negative clinical isolates over the last half-century (Davies, 1997).

Antibiotic resistance is the best-known example of rapid adaptation of bacteria to a new ecosystem. The ability of bacteria to expand their ecological niche, also in the presence of certain antibiotics, can be explained by the acquisition of resistance genes by horizontal gene transfer and/or by the accumulation of point mutations leading to the modification of existing genes. Several studies on bacterial pathogens of human and animal origin concluded that multiple antibiotic resistance is
a consequence of horizontal gene transfer (Sundstrom, 1998; Trieu and Courvalin, 1986). The principal mechanisms facilitating horizontal gene transfer among bacteria are transformation, transduction and conjugation.

Conjugation is the most frequently recognized mechanism for horizontal gene transfer. This mechanism exists in a wide variety of bacterial species and genera. In this process, mobilizable DNA molecules (plasmids, episomes, conjugative transposons) can be transferred from a donor to a recipient cell, via a contact-dependent transmission. The self-transmissible conjugative F-plasmid of *Escherichia coli* is the best-known example of an autonomously replicating molecule, which encodes all necessary factors required for conjugation. Some non-self-transmissible plasmids can also be mobilized *in trans* by an associated self-transmissible plasmid, which is not normally transferred at the same time. Conjugation can also mediate chromosomal exchange following the integration of a self-transmissible plasmid into the bacterial chromosome.

The conjugation involves physical contact between donor and recipient cells and can mediate the transfer of genetic material between domains (for example, between bacteria and plants, and between bacteria and yeast) (Buchanan et al., 1987; Heinemann and Sparge, 1989). Typically, DNA is transferred from a donor to a recipient strain by either a self-transmissible or mobilizable plasmid.

The intercellular spread of the genetic determinants of resistance to antimicrobial agents is facilitated by mobile genetic elements, such as conjugative plasmids and conjugative transposons. Plasmids or transposons borne integrons are a key player in this process, being able to acquire, rearrange, and express genes, in this case, those conferring antibiotic resistance (Stokes and Hall, 1989). Whether integrons are located on a plasmid or chromosome, their structure and function are similar (Michael et al., 2005).
Naturally occurring gene expression elements, called *integrons*, have been described as a very efficient genetic mechanism by which bacteria can acquire resistance genes (Hall and Collis, 1995; Martinez and De la Cruz, 1990; Stokes and Hall, 1989). Integrons promote the capture of one or more gene cassettes within the same attachment site, thereby forming composite clusters of antibiotic resistance genes. Over the past few years, the analysis of many antibiotic resistance genes identified in clinical and veterinary isolates of gram-negative organisms (particularly *Enterobacteriaceae*) established the importance of integrons in the dissemination of resistance among bacterial pathogens from different geographical origins (Falbo et al., 1999).

Horizontal transfer of antibiotic resistance genes provides a potentially saving ecological impact on any bacterial population exposed to an antibiotic treatment. However, the transferred resistance gene must be expressed in a manner that benefits the recipient microorganism. Many recent investigations on the molecular basis for antibiotic resistance have highlighted the link between resistance determinants embedded in units of DNA called *integrons* and broad-host range plasmids. This novel class of specialized DNA elements was initially described mainly from comparisons of the DNA sequence surrounding different antibiotic resistance genes found in naturally occurring gram negative bacteria. Early attempts to describe integrons suggested that they consisted of two conserved regions flanking a variable region containing one resistance gene or more (Stokes and Hall, 1989).

Horizontal transfer of resistance genes is a successful mechanism for the transmission and dissemination of multiple drug resistance among bacterial pathogens. The impact of horizontally transmitted genetic determinants in the evolution of resistance is particularly evident when resistance genes are physically associated in clusters and transferred to the recipient cell. Recent advances in the
molecular characterization of antibiotic resistance mechanisms have highlighted the existence of genetic structures, called integrons, involved in the acquisition of resistance genes. These DNA elements have frequently been reported in multi-drug resistant strains isolated from animals and humans, and are located either on the bacterial chromosome or on broad-host-range plasmids. The role of integrons in the development of multiple resistances relies on their unique capacity to cluster and express drug resistance genes. Moreover, the spread of resistance genes among different replicons and their exchange between plasmid and bacterial chromosome are facilitated by the integration of integrons into transposable elements. The associations of a highly efficient gene capture and expression system, together with the capacity for vertical and horizontal transmission of resistance genes represents a powerful weapon used by bacteria to combat the assault of antibiotics (Falbo et al., 1999).

The capture and spread of antibiotic resistance determinants by integrons underlies the rapid evolution of multiple antibiotic resistances among diverse Gram negative clinical isolates. The association of multiple resistance integrons (MRIs) with mobile DNA elements facilitates their transit across phylogenetic boundaries and augments the potential impact of integrons on bacterial evolution (Dean et al., 2002).

2.17. Integrons and Sulfamethaxazole Trimethoprim element (SXT) as mobile elements for antibiotic resistance

After the introduction of antibiotics in the treatment of infectious diseases, antibiotic resistance has spread dramatically among microbes. The occurrence of drug resistant strains of *Vibrio cholerae* has been reported in India with increasing frequency (Bag et al., 1998). Spread of antibiotic resistance in microbes has been attributed to the mobilization of drug-resistance markers by a variety of agents (e.g.,
plasmids, transposons, and integrons).

Integrons are DNA elements capable of mobilizing individual gene cassettes into bacterial chromosomes by site-specific recombination. Integrons consist of a central variable region that often harbors antibiotic-resistance gene cassettes, flanked by 5' and 3' conserved sequences (CS). Integrons have been categorized into four different classes on the basis of the distinctive integrase (int) genes they carry on their 5'-CS (Recchia and Hall, 1995). Among the different integron families, class I integrons are found to be most prevalent in drug-resistant bacteria. Class I integrons have been detected in *V. cholerae* O1 strains isolated in Vietnam, Thailand, and Italy (Dalsgaard *et al.*, 2000; Falbo *et al.*, 1999). Amita *et al.*, 2003, however, have previously reported the presence of integrons in *V. cholerae* O1 strains isolated in India. The indiscriminate usage of antibiotics in human medicine and animal husbandry promotes the spread of multiple antibiotic resistance.

SXT is representative of a family of conjugative transposon like mobile genetic elements that encode multiple antibiotic resistance genes. The term conjugative transposon (CTn) encompasses a diverse and growing group of mobile genetic elements. Chromosomal integration by CTns is mediated by recombinases of either the integrase (Salyers *et al.*, 1995; Scott and Churchwood, 1995) or resolvase family (Wang and Mullani, 2000). In the laboratory, SXT is transmissible by conjugation to a variety of gram-negative organisms, and it can mediate the transfer of certain mobilizable plasmids, as well as chromosomal DNA, in an IIfr-like fashion. This integrase is required for SXT transfer but not for SXT-dependent transfer of mobilizable plasmids or chromosomal DNA (Hochut *et al.*, 2000).