CHARACTERIZATION OF PLASMIDS ISOLATED FROM *Aeromonas* SPP. OBTAINED FROM SOUTH AFRICAN AQUACULTURE SYSTEMS

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own, original work and that I have not previously in its entirety or in part submitted it at any other university for a degree

Date:.....06/03/07.....

ABSTRACT

The plasmid content of 37 *Aeromonas* spp. isolates, obtained from South African tilapia, trout and koi aquaculture systems, was evaluated and 17 *Aeromonas* spp. isolates appeared to contain one or more plasmids ranging in size from 1 to 35 kb. Large plasmids (75-200 kb) were detected using S1 nuclease pulsed-field gel electrophoresis in seven isolates. Twenty-three *Escherichia coli* transformants containing single/multiple plasmids from the 17 plasmid-containing isolates were characterized with respect to the transferred antimicrobial resistance phenotypes. Transformants displayed diverse phenotypes with the co-transfer of unrelated antibiotics. β-lactam resistance was identified as the dominant resistance phenotype displayed by 13 transformants. Co-transfer of ampicillin, amoxycillin, augmentin, ceftriaxone, cefuroxime and the first generation quinolone, nalidixic acid occurred in 47.8% of transformants. Southern hybridization experiments with *tetA*, *bla*-TEM and *strA-strB* gene probes indicated the prevalence of the respective genes in 47.1%, 35.3% and 17.6% of the 17 plasmid-containing *Aeromonas* spp., respectively. Low incidences of mobile genetic elements, i.e., Tn*1721* and *intI* were also detected. Analysis of the transformed plasmids' backbone DNA revealed the presence of possible broad-host-range plasmids based on their ability to replicate in the absence of host-encoded factors. Plasmids p31T1 and p36T2, isolated from *A. sobria* and *A. hydrophila*, respectively, were identified as potential IncQ-like plasmids based on positive hybridization signals with the *repC* gene from pRAS3.1, mobilization by the IncPα plasmid RP4, and the relatively small size (14 kb). A 26.6 kb ColE-type plasmid isolated from *A. sobria* was characterized by the transferred resistance phenotype, endonuclease restriction mapping and partial sequencing. Failure to transfer the plasmid by conjugation indicated that this plasmid was not self-transmissible, while antibiotic susceptibility testing revealed indicated the transfer of resistance to 14 antibiotics to *E. coli* DH5α. Overall, results indicated the high level of plasmid diversity and potential transfer of antibiotic resistance determinants between *Aeromonas* spp., emphasizing the role of plasmids and other mobile genetic elements in the dissemination of antibiotic resistance genes, especially in the aquaculture environment.

OPSOMMING

Die plasmied inhoud van 37 *Aeromonas* sp. isolate, verkry vanaf Suid-Afrikaanse tilapia, koi en forel akwakultuur sisteme was vasgestel. Sewentien *Aeromonas* sp. isolate het die teenwoordigheid van een of meer plasmiede wat gewissel het in grootte vanaf 1 tot 35 kb getoon. Die teenwoordigheid van groot plasmiede (75-200 kb) was deur S1 nuklease puls-veld gel elektroforese waargeneem in sewe isolate. Drie en twintig transformante wat *Aeromonas* sp. plasmiede bevat het, is gekarakteriseer op grond van die oordrag van antibiotika merkers. Die oordrag van antibiotika weerstandsfenotipes was baie divers, met die gesamentlike oordrag van weerstand teen onverwante anitbiotikas. B-Laktaam weerstand was geïdentifiseer as die dominante fenotipe, teenwoordig in 13 transformante. Die mede-oordrag van weerstand teen ampisillien, amoksisillien, amoksisillien te same met klavulaan suur, keftriaksoon, kefuroksiem en die eerste generasie quinoloon, naladiksiese suur was aanwesig in 47.8% van die transformante. Southern klad eksperimente met die *tetA*, *bla*-TEM en *strA-strB* gene het die voorkoms van plasmied gekodeered kopië van die onderskeie gene getoon in 47.1%, 35.3% en 17.6% van die 17 *Aeromonas* sp. wat plasmiede bevat het. Die vookoms van ander beweeglike genetiese elemente naamlik Tn*1721* en *intI* was laag. Ontleding van die DNA van die getransformeerde plasmiede het die teenwoordigheid van plasmiede wat in 'n wye verskeidenheid van gashere kan repliseer getoon. Die replikasie vermoë was waargeneem in die afwesigheid van gasheer-gekodeerde faktore. Plasmiede p31T1 en p36T2, geïsoleer vanuit *A. sobria* en *A. hydrophila*, onderskeidelik, was geïdentifiseer as potentiële IncQ-tipe plasmiede op grond van die positiewe hibridisasie syne met die *repC* geen van pRAS3.1, mobilisasie deur die IncPα plasmied, PR4, en die relatiewe klein plasmied grootte van 14 kb. Die 26.6 kb ColE-tipe plasmied geïsoleer vanuit *A. sobria* was gekarakteriseer deur die oordrag van die weerstandsfenotipe, endonuklease snydingskartering and gedeeltelike DNA volgorede bepaling. Die onvermoë van hierdie plasmied om gedurended konjugasie oorgedra te word, was 'n aanduiding dat die plasmied nie self-oordragbaar is nie. Antibiotika sensitiwiteits toetsing het die oordrag van weerstand teen 14 antibiotika middels geïdentifiseer in die nuwe gasheer, *E. coli* DH5α. Algehele resultate het 'n hoë vlak van plasmied diversiteit en die potentiële oordrag van antibiotika weerstandsgene tussen *Aeromonas* sp. getoon. Dit beklemtoon

weereens die rol van plasmiede en ander beweeglike genetiese elemente in die verspreiding van weerstandsgene in die akwakultuur omgewing.

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Introduction and Literature Review

1.1 Aquaculture systems and bacterial organisms

1.1.1. Cultured fish species

Aquaculture is the fastest growing food production activity in many countries worldwide including Saudi Arabia, Australia, Japan, Korea, Norway, Malaysia and Bangladesh (Akinbowale *et al.*, 2006; Al-Harbi & Uddin, 2005; Kim *et al.*, 2004; L'Abee-Lund & Sϕrum, 2000; Radu *et al.*, 2003; Rahman *et al.*, 2002). The demand for alternative animal protein for human consumption is ever-growing, thus placing more pressure on the production of fish and fish products. In order to face the current demand, aquaculture practices have become more intense with high densities of cultured fish being kept in close proximity to one another. Due to this close proximity; stresses on the fish are high, varying from handling stress by workers, social stress and stress as the result of the high load of micro-organisms present in the tanks.

The production of freshwater fish for human consumption has increased dramatically over the past decade due to the decline in wild fishery harvesting due to over-fishing, pollution and destruction of marine habitats (Akinbowale *et al.*, 2006). Today, aquaculture enterprises includes the farming of rainbow trout (*Oncorhynchus mykiss*), salmon (*Salmo salar*), yellowtail (*Seriola quinqueradiata*), yellowtail amberjack (*Seriola lalandi*), greater amberjack (*Seriola dumerili*), bluefin tuna (*Thunnus thynnus*), catla (*Catla catla*), Indian carp (*Labeo rohita*), grass carp (*Ctenopharyngodon idella*), African catfish (*Clarias gariepinus*), carp (*Abramis brama*), eel (*Anguilla anguilla*) and tilapia (*Oreochromis niloticus, O. mossambica*) (Al-Harbi & Uddin, 2004; Al-Harbi & Uddin, 2005; Bucio *et al.*, 2006; Furushita *et al.*, 2003; Hatha *et al.*, 2005; Kim *et al.*, 2004; Martin *et al.*, 2006; Miranda *et al.*, 2003; Rahman *et al.*, 2002; Schmidt *et al.*, 2000; Son *et al.*, 1997). Aquaculture practices are not limited to fish species, but also

include the production of tiger shrimp (*Penaeus monodon*), fairy shrimp (*Streptocephalus dichotomus*), skeleton shrimp (*Caprella* sp.) freshwater prawns (*Macrobrachium rosenbergii* de Man), rock lobster (*Panulirus Cygnus*) and various mollusk species including mussels (*Mytilus edulus*), Pacific oysters (*Crassostrea gigas*), clams (*Hiatella actica, Menetrix lusoria*) and scallops (*Agropecten irradians*) (Hsia & Liu, 2003; Johnston *et al.*, 2006; Lalitha & Surendran, 2006; Le *et al.*, 2005; Swift *et al.*, 2006; Velu & Mususwamy, 2006). The production of the above-mentioned species in aquaculture systems is currently expanding to meet the demand set by the ever-increasing human population.

1.1.2. Bacterial diversity in aquaculture systems

Aquatic micro-organisms influence not only the water quality in fish hatchery tanks, but have an important effect on the physiology of the cultured fish, diseases and post-harvest quality (Al-Harbi & Uddin, 2005). Microbial populations in aquaculture systems are very diverse due to the high nutrient content (fecal matter, fish feed, digestive tracts of fish) and consists of both commensal and pathogenic bacteria. Various bacterial species have been isolated from different fish parts including the intestines, gills, fins and skin, and from tank sediment as well as tank water (Al-Harbi & Uddin, 2005; Munro & Hastings, 1993; Robersts, 1993). Gram-negative and Gram-positive organisms have been isolated from the aquaculture setting indicative of the wide diversity of micro-organisms present (Akinbowale *et al.*, 2006; Al-Harbi & Uddin, 2005).

Al-Harbi and Uddin (2005) characterized the bacterial flora of cultured tilapia fish and emphasized the diverse bacterial populations present on the gills and in the intestinal tract of sampled fish. Bacteria isolated included *Aeromonas hydrophila, Chryseomonas* spp., *Pasteurella pnemotropica, Photobacterium* spp., *Pseudomonas* spp., *Serratia liquefaciens, Shewanella putrefaciens, Staphylococcus* spp., *Streptococcus* spp. and seven different *Vibrio* spp. isolates. The dominant *Vibrio* spp., *V. parahaemolyticus, V. alginolyticus* and *V. vulnificus* composed 17.7%, 13% and 10.5% of the microflora of the intestinal tract, respectively. The diverse number of bacteria and the high incidence of

Vibrio spp. could be the result of increased metabolic activities associated with the increased feeding rates and high water temperatures (Al-Harbi & Uddin, 2005). The dominant presence of *Vibrio* spp. in cultured fish was further supported by findings by Akinbowale *et al.* (2006). Sixty percent of bacteria isolated from freshwater and marine water fishes belonged to the *Vibrionaceae* family.

Aeromonas spp. have been isolated from a wide variety of fish species including trout, koi and salmon (Akinbowale *et al.*, 2006; L'Abee-Lund & Sϕrum, 2000). Motile aeromonads are the dominant bacterial species frequently isolated when assessing the microflora of cultured fish (Al-Harbi & Uddin, 2004). Hatha *et al.* (2005) isolated *A. hydrophila, A. caviae* and *A. sobria* as the dominant bacterial spp. from diseased fish.

Although Gram-positive bacteria are isolated at a lower frequency than the Gram-negative *Vibrio* and *Aeromonas* spp., they form part of the complex bacterial populations within aquaculture systems (Akinbowale *et al.*, 2006; Al-Harbi & Uddin, 2004; Al-Harbi & Uddin, 2005). Gram-positive lactic acid bacteria are frequently isolated from the intestines of mammals, birds and freshwater-farmed fish (Bucio *et al.*, 2006). The dominant spp. *Lactobacillus alimentarius* and *L. sakei,* were isolated from economically important fish species, which could have important implications in product quality, storage and trade capablilties.

Bacterial prevalence and diversity show variations when comparing cell density of the water and sediment with the cell numbers present in and on the fish itself (Al-Harbi & Uddin, 2005). Seasonal variation in the concentrations and diversifications in the intestines exist in cultured tilapia (Al-Harbi & Uddin, 2004). Although *A. hydrophila, Sh. putrefaciens, Corynebacterium urealyticum, E. coli* and *V. cholerae* were present throughout the year, *Pseudomonas* spp., *Micrococcus* spp., *Flavobacterium* spp. and *Streptococcus* spp. were only isolated during the autumn and winter months. The dominance of *Aeromonas* and *Vibrio* spp. in aquaculture is of importance since these organisms often are implicated in the outbreak of various infectious diseases.

1.1.3. Fish pathogens

Different bacterial species have been implicated in the outbreak of diseases in the aquaculture setting impacting production, trade and effecting economic development in many countries (Verschuere *et al.*, 2000). Bacterial populations within fish hatchery tanks are very complex consisting of both commensal and pathogenic bacteria. Bacterial pathogens are capable of surviving outside of the fish, but some are obligatory fish pathogens. Organisms resides within the host tissue for long periods of time without a detrimental effect on the health of the fish and clinical infections occur only as a result of extreme changes in the host physiology which is correlated with different stress factors (Hjeltnes & Roberts, 1993; Munro & Hastings, 1993). Primary pathogens such as *V. anguillarum* and *A. salmonicida* infect fish causing vibriosis and furunculosis, respectively (Hjeltnes & Roberts, 1993; Munro & Hastings, 1993). Secondary pathogens such as *A. hydrophila*, *Edwardsiella* spp. and various other bacteria are responsible for infections of compromised fish, either due to the onset of a primary infection or high stress levels (Munro & Hastings, 1993; Robersts, 1993).

Members of the genus *Vibrionaceae* cause a wide variety of diseases in different fish species. Although *V. anguillarum* is the dominant fish pathogen, other species such as *V. vulnificus* and *V. ordalii*, are implicated in the outbreak of diseases in aquaculture systems (Hjeltnes & Roberts, 1993). Traumatized fish, both in the aquaculture setting and in the wild, are sensitive to possible *Vibrio*-related infections. These infections involve haemorrhagic ulcers on the mouth or skin surface, necrotic lesions in the muscle tissue and/or on the orbit or along the edge of the fins (Hjeltnes & Roberts, 1993). The role of these bacteria as fish pathogens is emphasized by the high rate at which they are isolated from diseased fish.

The role of members belonging to the genus *Aeromonas* as fish pathogens is well known. *A. salmonicida* is the causative agent of furunculosis, a septicaemic infection, principally in salmonids (Munro & Hastings, 1993). Furunculosis is a complex disease and is displayed in different forms depending on the health, age, environmental conditions, temperature and fish species (Boyd *et al.*, 2003). This bacterium may even

cause diseases in a wider variety of freshwater and marine species than previously thought. In addition to salmonids, disease outbreaks in carp and Atlantic cod have also been reported (Munro & Hastings, 1993). Onset of this disease is stress-related which poses a real threat to fish farming, since handling, high fish densities and the close proximity in which fish are bred, all increase the potential of a disease outbreak. The high mortality rate of furunculosis leads to great losses in fish hatcheries and farms annually. It is believed that the secondary pathogen *A. hydrophila* is the causative agent of ulcerative disease syndrome (UDS) in a wide variety of fish species (Hatha *et al.*, 2005). Another *Aeromonas* spp., *A. veronii* biovar sobria, is thought to be the causative agent of epizootic ulcerative syndrome among a wide variety of different fish including African catfish (Rahman *et al.*, 2002). *A. hydrophila* causes septicaemia in cultured fish disabled through other infections (Robersts, 1993). The high frequencies at which different *Aeromonas* spp. are isolated from diseased fish emphasize the importance of these organisms as primary and secondary fish-pathogens (Al-Harbi & Uddin, 2004; Munro & Hastings, 1993; Robersts, 1993).

Both *A. hydrophila* and several *Vibrio* spp. isolates have been implicated in zoonotic diseases, i.e., diseases which can be spread from animals to humans and vice versa (Daskalov, 2005). This characteristic of *A. hydrophila* and *Vibrio* spp. isolates poses a real threat to human health, since the consumption of diseased fish or fish contaminated with these organisms, but which appeared healthy upon consumption, could lead to human illness.

1.2. The genus *Aeromonas*

1.2.1. Classification and identification of *Aeromonas* spp.

The complexity of the *Aeromonas* genus has led to conflicting opinions regarding identification in the laboratory setting. This problem has arisen because taxonomic studies often report only selected biochemical tests on newly identified environmental isolates which are then compared to existing data from previous studies. Often, the

conditions under which the biochemical tests are performed are not uniform and therefore, have an impact on the results. Abbott *et al.* (2003) suggested the use of an identification scheme to minimize the list of possible *Aeromonas* spp. isolates and thus a starting point to simplify the identification procedures, since a high level of phenotypic diversity among the genus has been observed (Fig. 1.1).

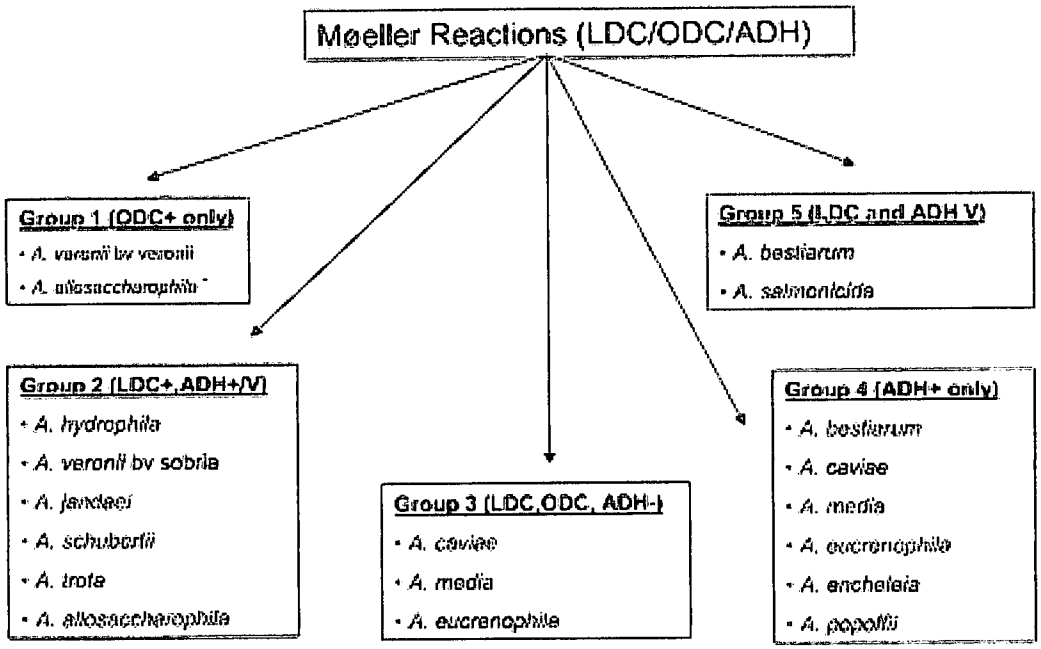

Figure 1.1. The Møeller identification scheme as a screening tool to group possible *Aeromonas* spp. Abbreviations: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; ADH, arginine dihydrolase; V, variable (Abbott *et al.*, (2003).

Over the past two decades, there have been a number of taxonomic changes and revisions of the genus *Aeromonas* and increased interest in the genus due to the emergence of these organisms as human pathogens. *Aeromonas* spp. were originally placed in the family *Vibrionaceae* (Popoff, 1984), which included *Vibrio*, *Photobacterium* and *Plesiomonas*, however, phylogenetic investigation suggested that *Aeromonas* formed a distinct group and it was subsequently moved to a new family, *Aeromonadaceae* (Janda, 1991). Further biochemical characteristics are used to group this complex genus into the four phenospecies namely, *A. hydrophila, A. salmonicida, A. sobria* and *A. caviae* with additional genetic clusters found in each group (Janda, 1991).

Members belonging to the family *Aeromonadaceae* are Gram-negative, heterotrophic, facultative anaerobes. They are rod-shaped, motile bacteria that produce oxidase and catalase. Optimum growth temperature of these organisms is between 22 – 28°C, and they prevail in a wide variety of environmental niches. *A. salmonicida* is the only non-motile member of this family and is an obligate fish pathogen with limited survival outside host tissue. Identification of *A. salmonicida* is simplified by the production of a diffuse brownish pigment when grown on nutrient media containing 0.85% salt and the psychrophilic nature of this organism (Munro & Hastings, 1993). Nine biochemical tests are uniform among the *Aeromonadaceae* family and are thus used for identification to genus level. These include the presence of cytochrome oxidase and nitrate reductase, fermentation of D-glucose and trehalose, failure to utilize mucate, and inability to produce acid from D-arabitol, dulcitol, erythritol and xylose (Abbott *et al.*, 2003). The *A. hydrophila* complex is separated from other species based on the ability to produce the enzymes elastase, pectinase, and/or stapholysin, while the *A. sobria* complex is identified based on the failure to hydrolyze esculin, failure to utilize L-arabinose and salicin, and inhibition by KCN (Abbott *et al.*, 2003).

Currently, 14 distinct genospecies have been identified in the present phenospecies through extensive DNA-DNA hybridizations (De Gascun *et al.*, 2006). Some discrepancies still remain between observed DNA homology groups and those obtained by phenotypic characterization, which has paved the way for the development of reliable molecular techniques to successful identify presumptive *Aeromonas* spp. (Borrell *et al.*, 1997).

1.2.2. Molecular identification of *Aeromonas* spp.

The use of molecular techniques to identify possible *Aeromonas* spp. has been shown to be a more reliable method and is more frequently used than the general biochemical method of identification. Restriction fragment length polymorphism (RFLP) of PCR-amplified 16S rRNA has been used as an identification tool to identify clinical *Aeromonas* spp. isolates from diverse origins (Borrell *et al.*, 1997; Dorsch *et al.*, 1994).

This method was able to distinguish the different strains to the level of phylogenetic species, although some discrepancies were observed upon biochemical testing. Other DNA fingerprinting methods such as random amplified polymorphic DNA PCR (RAPD), repetitive extragenic palindromic sequence PCR (REP-PCR) and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), which have a high discriminatory power and are approximately 100% reproducible, are frequently used to identify aeromonads to species level (Szczuka & Kaznowski, 2004). These techniques present fast, reliable and cost-effective methods to differentiate between different *Aeromonas* spp. (Bin Kingombe *et al.*, 2004; Borrell *et al.*, 1997; Dorsch *et al.*, 1994).

The amplification of the *aroA* gene, encoding the 3-phosphoshikimate-1-carboxyvinyltransferase is a key enzyme in both the aromatic amino acid and folate biosynthesis pathways, is another tool used in the identification of members of the genus *Aeromonas* (Soriano *et al.*, 1997). The *aroA* gene sequence is very well conserved within Gram-negative bacteria and has been shown to be a powerful tool for characterization of *Aeromonas* spp. isolates to genus level. Combined with restriction fragment length polymorphism (RFLP) of the amplicon, it was possible to identify representatives of all 14 DNA-hybridization groups (Soriano *et al.*, 1997).

1.3. Virulence and pathogenesis of *Aeromonas* spp.

1.3.1. Food and water-borne *Aeromonas* spp.

Seafood and fish products, aimed for human consumption, are known to contain a wide variety of different micro-organisms including *Aeromonas* spp. (Conzalez-Rodrigues *et al.*, 2002; Davies *et al.*, 2001; Lalitha & Surendran, 2006; Ottaviani *et al.*, 2006). In France, Greece and Great Britain, 40% of sampled fresh fish contained the opportunistic human pathogen, *A. hydrophila,* while *A. sobria, A. caviae, A. eucrenophila* and *A. hydrophila* were isolated from pre-packed salmon, and *A. schubertii* from pre-packed trout (Conzalez-Rodrigues *et al.*, 2002; Davies *et al.*, 2001). Other seafood products such as mussels collected from natural beds in the Adriatic Sea contained

virulent *Aeromonas* spp. (Ottaviani *et al.*, 2006). The prevalence of these virulent *Aeromonas* spp. is important since human illnesses could arise after consumption of food from the aquatic environment where these organisms are prevalent.

The prevalence of *Aeromonas* strains in not limited to seafood, fish and fish products, but was also found in other food sources including vegetables and pork products (Li *et al.*, 2006; Palu *et al.*, 2006). *Aeromonas* spp. was not the dominant species isolated from packaged pork products, but the presence of these organisms in different food products is alarming. The dominant species isolated from raw vegetables, *A. hydrophila* and *A. caviae,* are both considered opportunistic human pathogens and have previously been isolated from other food products (Conzalez-Rodrigues *et al.*, 2002; Davies *et al.*, 2001; Palu *et al.*, 2006).

Aeromonas spp. are ubiquitous to aquatic environments. Water samples collected from the Ebrié lagoon showed the presence of *Aeromonas* spp. with *A. sobria, A. hydrophila* and *A. caviae* isolates comprising 49%, 21% and 30% of the total population, respectively (Marcel *et al.*, 2002). High numbers of *Aeromonas* spp. isolates were observed in unchlorinated well-water aimed for human use, i.e. drinking and food preparation (Massa *et al.*, 2001). The dominant species, *A. hydrophila* and *A. sobria*, displayed the presence of putative virulence properties, emphasizing the possible role of these organisms as opportunistic human pathogens and the outbreak of potential illnesses due to consumption (Massa *et al.*, 2001).

The presence of *Aeromonas* sp. in food products and water supplies is undeniable. The point at which food becomes contaminated, whether it forms part of the natural occurring microflora or whether it is the result of breaches during food processing is important. However, the presence of different *Aeromonas* spp. in different food products may be seen a possible threat to human health.

1.3.2. Clinical *Aeromonas* spp.

The role of motile aeromonads as human pathogens is still under considerable debate. Discrepancies in the classification of the genus *Aeromonas*, led to various human infections caused by other bacteria being attributed to various *Aeromonas* spp. (Janda, 1991). However, *Aeromonas* spp. are capable of causing disease and infection in humans, with the majority of clinical pathogens being identified as *A. hydrophila, A. sobria* and *A. caviae* spp. (Borrell *et al.*, 1997). Wu *et al.* (2006) isolated the three major *Aeromonas* pathogens from a clinical setting and found *A. hydrophila* to be the dominant causative agent of human infections, followed by *A. sobria* and *A. caviae* resulting in 24% and 23% of cases, respectively.

Gastroenteritis-causing pathogens are the second leading cause of morbidity and mortality worldwide (Streit *et al.*, 2006). These pathogens are transmitted through food, water, environmental contact and direct contact between people. Fecal samples, revealed that *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* were the dominant species suggesting their role in causing diarrhea in humans (Altwegg *et al.*, 1990; Kuijper *et al.*, 1989). When surveying the prevalence of *Aeromonas*-associated gastroenteritis in Europe, *Aeromonas* spp. were not the predominant isolates and showed a prevalence of only 6% (Streit *et al.*, 2006). The same holds true in Latin America where *Aeromonas*-associated gastroenteritis was not dominant (Streit *et al.*, 2006). Although *Aeromonas* spp. were not the predominant causative agent of gastroenteritis, the incidence in different countries is still significant.

A. hydrophila was isolated from a chronic pancreatitis abscess (De Gascun *et al.*, 2006), while *A. popoffii* was identified as a human pathogen and implicated in an urinary tract infection (Hua *et al.*, 2004). Wound infections due to the colonization of *A. hydrophila* seem to be very uncommon, although *A. hydrophila* strains were isolated from patients suffering from various degrees of burn wounds (Skoll *et al.*, 1998). One of these patients also presented with a positive blood culture, suggesting the opportunistic nature of *A. hydrophila* in causing severe infections with more serious consequences. Another serious wound infection resulting from *Aeromonas* spp., occurred after

medicinal leech therapy (Janda, 1991). Aeromonads live symbiotically in the gut of the leech, and may cause human infections after therapy. This finding further supports the opportunistic nature of *Aeromonas* spp. Other infections caused by *A. hydrophila* include ocular infections, infections of the respiratory tract, skin and soft-tissue infections, bacteremia and hepatobiliary tract infections (Ketover *et al.*, 1973; Wu *et al.*, 2006). Although septicemia from an *Aeromonas* infection rarely occurs, this is the most invasive form of infection caused by this bacteria (Borger van der Burg *et al.*, 2006).

The severity of an *Aeromonas*-associated infection should not be underestimated especially among non-immunocompromised individuals. A recent case of severe necrotizing fasciitis due to the exposure to stagnant water contaminated with *A. hydrophila* has been reported (Borger van der Burg *et al.*, 2006). The relatively quick onset (24 h after exposure) and the severity of the *Aeromonas*-associated infection led to the transfemoral amputation of the infected leg. Furthermore, the initial colonization by *A. hydrophila* resulted in secondary *Pseudomonas aeruginosa* infections and ultimately the death of the patient due to organ failure (Borger van der Burg *et al.*, 2006). The rapid onset of necrotizing fasciitis in the above-mentioned case could be compared to similar infections caused by other human pathogens such as *Clostridium perfringens* and β-hemolytic streptococci. Although gastrointestinal infections caused by *Aeromonas* spp., are more common than sporadic cases of serious infections, colonization by *Aeromonas* spp. may lead to life-threatening conditions.

1.3.3. Virulence factors

Bacterial virulence is a complex mechanism involving a number of interplaying factors such as the production of extracellular enzymes, cell-associated factors and structural features/properties including the presence of pili, S-layers, lipo-polysaccharides (LPS), outer membrane proteins (OMP) and flagella (Janda, 1991; Sinha *et al.*, 2004; Stuber *et al.*, 2003a). Most aeromonads produce a large number of extracellular enzymes that are responsible for the degradation of complex proteins, polysaccharides, mucopolysaccharides and lipid-containing compounds (Janda, 1991). Adhesion to and

colonization of mucosa, followed by fluid accumulation, or epithelial changes are likely events leading to the onset of disease (Daskalov, 2005). The function of these enzymes in the physiology of the bacterium still remains largely unclear, but it is thought that these factors/enzymes might play a role in the virulence of the organism.

Hemolysin production in *Aeromonas* spp. enables strains to hemolyze erythrocyte-containing media and is related to the enterotoxigenicity of an organism which is ultimately an indication of virulence (Hatha *et al.*, 2005; Janda, 1991). Other than hemolysin, *Aeromonas* spp. isolates are capable of producing other potential virulence factors including enterotoxins, proteases, siderophores and cytotoxins (Janda, 1991; Tsai *et al.*, 1997). Aerolysin, a cytotoxic enterotoxin which displays enterotoxic, cytotoxic and hemolytic activities, has been described as the most powerful virulence factor associated with *Aeromonas* species (Martins *et al.*, 2002). Most *Aeromonas* spp. are capable of hemolysin production as observed for 100%, 50% and 78% of *A. hydrophila, A. sobria* and *A. caviae* species isolated from farm-raised freshwater fish, respectively (Hatha *et al.*, 2005). *A. hydrophila* strains isolated from drinking water and food products displayed a higher frequency of hemolytic activity than aquatic isolates, while cytotoxin activity was higher in the food strains (92%) than in the water-borne strains (73%) (Handfield *et al.*, 1996).

The ability to produce virulence factors does not seem to be limited to a specific *Aeromonas* spp. nor to specific environmental and geographical isolates. Enterotoxic activity was more frequently detected among clinical than food-borne *Aeromonas* spp., while *A. veronii* and *A. sobria* were the most enterotoxic isolates among the clinical and food isolates (Martins *et al.*, 2002). All 194 isolates of clinical and food origin produced hemolysin, while 89% and 72% of the clinical and food isolates showed cytotoxin activity (Martins *et al.*, 2002). Seventy-three *A. veronii* biovar *sobria* strains isolated from diseased fish, the aquaculture environment and humans suffering from diarrhea, respectively, displayed cytotoxic activity and were also capable of hemolysin production (Rahman *et al.*, 2002). These isolates were obtained from different geographical locations, indicating the wide-spread occurrence of virulent strains. This also opens the

possibility that certain environmental compartments contain reservoirs of virulent *Aeromonas* strains capable of causing human infections.

Little is know about the effects of environmental factors on the production of virulence factors. The production of virulence factors is important in the assessment of the potential threat that *Aeromonas* spp. pose to human health. Temperature seems to be an important factor when considering the pathogenicity of an organism since the production of virulence factors at 37 °C is essential to cause a human infection. Production of hemolysin and cytotoxin by *A. hydrophila* decreased faster at 37 °C than at 28 °C, while cytotoxin was more unstable at 37 °C (Tsai *et al.*, 1997). This is indicative of a possible lack of virulence in humans, but the temperature-dependent activity of virulence factors can be strain-specific and dependent on other factors such as effective secretion systems (Chacon *et al.*, 2004; Tsai *et al.*, 1997).

Type III secretion systems (TTSS) have been identified as specific pumps which export virulence factors and allow their translocation to the cytosol of eukaryotic host cells. Seven open reading frames (ORFs) encoding inner-membrane channel protein homologues have been identified in *A. salmonicida* subsp. *salmonicida* (Burr *et al.*, 2002). These identified genes formed a *virA* analogue which is central to the function of TTSS in Gram-negative bacteria. The presence of different components of TTSS is not limited to *A. salmonicida* species, as they have also been detected in clinical *A. hydrophila*. *A. veronii* and *A. caviae* isolates with greater prevalence among the first two mentioned species (Chacon *et al.*, 2004; Vilches *et al.*, 2004).

It is known that members of the genus *Aeromonas* can cause a variety of diseases such as diarrhea in humans, highlighting the importance of the presence of virulence factors and secretion systems as indicative of *Aeromonas* spp. pathogenesis (Martins *et al.*, 2002; Sinha *et al.*, 2004; Takahashi *et al.*, 2005).

1.3.4. Antimicrobial resistance

Four categories of mechanisms providing resistance against antimicrobial agents currently exist, including the inactivation of the antibiotic through enzyme activity; mutations in the structural or regulatory genes of the targeted proteins; alteration in the membrane permeability to the antibiotic; and the secretion of the antimicrobial agent through efflux pumps (Gootz, 2005; Gootz, 2006; Hawkey, 2003). Many multi-drug resistant bacteria (MDR), including *Aeromonas* spp., display all four categories of resistance affecting the susceptibility to different antimicrobial agents (Gootz, 2006). A wide variety of structurally unrelated antibiotics can provide selective pressure to maintain these resistance genes in bacteria (Gootz, 2006). Furthermore, mutations within an enzyme-encoding gene can confer resistance to more than one class of unrelated antibiotics (Klugman & Levin, 2006; Robicsek *et al.*, 2006).

Extensive studies involving the assessment of antibiotic resistance among members of the *Aeromonadaceae* family have been conducted in different geographical and environmental settings (Schmidt *et al.*, 2001b). *Aeromonas* isolates from different sources are known to be intrinsically resistant to penicillins, but the prevalence of resistance seems to be higher in the clinical setting than among environmental isolates (Huys *et al.*, 2001; Morita *et al.*, 1994; Schmidt *et al.*, 2000; Son *et al.*, 1997). In conjunction with this intrinsic β-lactam resistance phenotype, resistance to the third-generation cephalosporins such as cefotaxime, ceftazidime and cefoxitin is more prevalent among clinical isolates, while susceptibility to carbapenems such as aztreonam is prevalent among isolates from both sources (Morita *et al.*, 1994; Palu *et al.*, 2006; Radu *et al.*, 2003). Variations in resistance to "new" β-lactam antibiotics, in particular imipenem, is observed in both clinical and environmental important isolates (Morita *et al.*, 1994).

Resistance to trimethoprim, chloramphenicol and erythromycin is wide-spread among environmental *Aeromonas* spp. isolates (Akinbowale *et al.*, 2006; Huddleston *et al.*, 2006; Ko *et al.*, 1996; Radu *et al.*, 2003; Toranzo *et al.*, 1984). Miranda and Castillo (1998) reported erythromycin resistance among *Aeromonas* spp. strains isolated from

aquatic sources, while susceptibility to chloramphenicol and trimethoprim prevailed. Susceptibility to quinolones, ciprofloxacin and ofloxacin, was observed among *Aeromonas* isolates obtained from environmental and clinical compartments, while resistance to nalidixic acid occurs in both settings (Akinbowale *et al.*, 2006; Huys *et al.*, 2001; Palu *et al.*, 2006; Vila *et al.*, 2002). Environmental isolates appeared to be more susceptible to gentamicin, kanamycin and amikacin, although kanamycin-resistant *Aeromonas* strains have been isolated from the clinical setting (Akinbowale *et al.*, 2006; Huys *et al.*, 2001; Ko *et al.*, 1996; Palu *et al.*, 2006). When assessing the prevalence of tetracycline resistance among environmental and clinical *Aeromonas* strains, it is clear that this resistance phenotype occur in both settings (Huys *et al.*, 2001). Palu *et al.* (2006) observed a higher incidence of tetracycline resistance among clinical isolates, although this antibiotic is frequently used in aquaculture practices and animal husbandry (Chopra & Roberts, 2001; Teuber, 2001). Emerging streptomycin resistance among clinical and environmental *Aeromonas* spp. isolates has also been observed (L'Abee-Lund & Sϕrum, 2000; Miranda & Castillo, 1998; Sinha *et al.*, 2004).

Resistance profiles for different *Aeromonas* spp. shows variation with respect to antibiotic resistance profiles and is not species-specific (Miranda & Castillo, 1998; Palu *et al.*, 2006). Identical resistance phenotypes have been observed for *A. hydrophila, A. veronii* biovar *sobria* and *A. caviae* strains isolated from an aquatic environment (Radu *et al.*, 2003). Morita *et al.* (1994) reported a higher degree of resistance to β-lactams among clinical and environmental *A. hydrophila* isolates than that displayed by *A. sobria* and *A. caviae* isolates from the same sources. Furthermore, *A. salmonicida* and *A. hydrophila* strains isolated from South African aquaculture systems displayed resistance to a wider variety of antibiotics than *A. veronii* biovar *sobria*, *A. encheleia*, *A. media* and *A. ichtiosoma* isolates obtained from the same niches (Jacobs & Chenia, 2007). The current literature provides evidence that interspecies differences among *Aeromonas* spp. isolated from specific environmental compartments do exist (Hatha *et al.*, 2005; Huddleston *et al.*, 2006; Overman & Janda, 1999). This complicates the comparison of the different resistance phenotypes displayed by *Aeromonas* spp. from a specific environmental setting to those isolated from other geographical areas. Gentamicin resistance has been identified in *Aeromonas* spp. isolated from diseased fish in India, while other *Aeromonas*

isolates isolated from Australian aquaculture systems and retail fish in Malaysia are susceptible to the antibiotic (Akinbowale *et al.*, 2006; Majumdar *et al.*, 2006; Palu *et al.*, 2006; Son *et al.*, 1997).

Differences in resistance phenotypes of clinical and environmental *Aeromonas* spp. might be due to the fact that earlier studies included clinical isolates which had previous exposure to antimicrobial agents resulting in the misinterpretation of antibiotic susceptibility data (Huys *et al.*, 2001; Ko *et al.*, 1996). Resistance profiles of environmental *Aeromonas* spp. isolates might differ as a result of the different levels of pollution and dumping of waste material in certain geographical areas or the different sampling sites, i.e., water and sediment (Goni-Urriza *et al.*, 2000; Huddleston *et al.*, 2006). The variety of antibiotics used differentially may also impact the development of certain resistance phenotypes.

It is clear when comparing the resistance profiles of clinical and environmental *Aeromonas* spp. isolates that no specific trends emerge (Brown *et al.*, 1997). The various MDR phenotypes displayed by the *Aeromonadaceae* family and variability in displayed resistance phenotypes, have major implications for the treatment of *Aeromonas*-associated infections (Vila *et al.*, 2002). The wide-spread occurrence of antibiotic resistance in *Aeromonas* spp. isolates from different environmental compartments and geographical regions is indicative of the possible dissemination of the resistance phenotypes between compartments. It is thus important to investigate the role of mobile genetic elements such as plasmids in the dissemination of resistance genes within a population and/or between unrelated organisms since mobile DNA elements are capable of mobilizing other structures such as integrons and transposons harbouring other resistance genes (Taylor *et al.*, 2004).

1.3.5. Role of plasmids in antimicrobial resistance and virulence

All bacteria display intrinsic resistance mechanisms such as membrane permeability and chromosomally-encoded efflux pumps which confer low levels of

resistance to a wide variety of unrelated antibiotics (Gootz, 2006). Subsequent acquisition of mobile resistance genes encoded by plasmids can lead to a highly resistant phenotype displayed by the host (Martinez-Martinez *et al.*, 2003). Mobile genetic elements such as plasmids in conjunction with transposons and integrons facilitate the spread of resistance genes among bacteria that are closely related, and between organisms occupying different ecological niches. This has led to the increasing number of MDR bacteria and the establishment of mobile gene reservoirs in various environmental settings (Rhodes *et al.*, 2000). Resistance to antimicrobial agents is facilitated by interspecies transfer through conjugation, transformation and transduction (Bruun *et al.*, 2003). For specific antimicrobial agents, the low level of resistance mediated by residential plasmids can serve as stepping-stones for the evolution of bacteria with inherited resistance to clinical concentrations of antibiotics (Klugman & Levin, 2006). The stepping-stone model suggests that the state of resistance to a particular antibiotic, susceptible, intermediate and resistant, can serve as the foundation for the mutation of susceptible strains to intermediate and intermediate strains to resistant (Klugman & Levin, 2006).

Development of antibiotics resistance in bacterial populations is mainly due to the transferability of resistance genes usually found on plasmids (Taylor *et al.*, 2004). Four *A. hydrophila* strains isolated from diseased fish contained a 21 kb plasmid encoding resistance to tetracycline. The resistance phenotype was lost when the organisms were cured of the plasmid, but resistance to tetracycline was obtained upon re-introduction of the plasmid into the *A. hydrophila* strains or into another host (Majumdar *et al.*, 2006). The conjugal transfer of large plasmids from *Aeromonas* spp., harbouring tetracycline resistance determinants to different recipients such as *E. coli* and *Yersinia ruckeri,* presents a problem since it is clear that gene exchange does occur in the environment (Bruun *et al.*, 2003). Different plasmid-encoded tetracycline resistance genes have been identified in *Aeromonas* spp. isolates with *tetA* being the dominant resistance gene (Rhodes *et al.*, 2000). Rhodes *et al.* (2000) identified the *tetA* resistance determinant in 58.8% of plasmids isolated from *Aeromonas* spp. strains obtained from both a clinical and environmental setting. *Aeromonas* spp. resistance plasmids (R-plasmids)

characterized by Adams *et al.* (1998) also contained the class A tetracycline resistance gene, although the plasmids were not identical.

Resistance to aminoglycosides is generally chromosomally-encoded, but plasmid-mediated resistance does occur in the genus *Aeromonas.* Aminoglycosides inhibit protein synthesis by binding to the 30S subunit of the bacterial ribosome where inhibition of translation of genetic material takes place (Vakulenko & Mobashery, 2003). Aminoglycoside resistance is predominantly spread by integrons present on conjugative plasmids. Gene cassettes frequently contain *aadA*, *aadB*, *aacA*, *aacC* and *ant(3")1a* genes conferring resistance to streptomycin, spectinomycin and other aminoglycosides (Hall & Collis, 1998; Schmidt *et al.*, 2001a). The linked streptomycin resistance genes, *strA-strB*, have been identified as part of the composite transposon (Tn*5393*) residing on the *A. salmonicida* subsp. *salmonicida* R-plasmid, pRAS2 (L'Abee-Lund & Sørum, 2000).

Over the past few decades, sulfonamide resistance genes (*sul*I and *sul*II) have been isolated from a wide variety of ecological niches including the aquaculture environment (L'Abee-Lund & Sørum, 2000). Not only was the *sul*I gene isolated from different environmental compartments, but also from unrelated organisms, frequently as part of the conserved 3'-end of integron structures (Collis & Hall, 1995). In *A. salmonicida* subsp. *salmonicida* and *A. salmonicida*, sulfonamide resistance determinants formed part of the R-plasmids pRAS2 and pRAS1, respectively (Casas *et al.*, 2005; L'Abee-Lund & Sørum, 2000; Sørum *et al.*, 2003). Casas *et al.* (2005) characterized plasmid-mediated sulfonamide resistance in nine atypical *A. salmonicida* strains and found the presence of the *sul*I gene in eight of the studied plasmids. Combined sulfonamide and trimethoprim resistance encoded by plasmids occurs among *Aeromonas* spp., mediated by the presence of integron structures bearing the 3'-end-located *sul*I and a trimethoprim resistance cassette (Casas *et al.*, 2005; Schmidt *et al.*, 2001b).

Plasmid-mediated β-lactam resistance is frequently associated with the presence of resistance genes conferring resistance to related and unrelated or synthetic antimicrobial agents (Jacoby *et al.*, 2006; Wang *et al.*, 2006). Plasmid-mediated class A

β-lactamase TEM24, isolated from *E. aerogenes*, could transfer the resistance phenotype to a susceptible *A. hydrophila* isolate, suggesting that plasmid-encoded class A determinants are capable of residing within members of the genus *Aeromonas* (Fosse *et al.*, 2004). Resistance to carbapenems such as imipenem is due to the presence of the group 3 metallo-β-lactamases and has previously been identified in *A. hydrophila*, which harboured the *cphA* gene conferring resistance to imipenem (Walsh *et al.*, 1998). A chromosomally-encoded metallo-β-lactamase, ImiS, isolated from *A. veronii* biovar *sobria* showed 98% homology to the plasmid-mediated *cphA* gene (Walsh *et al.*, 1998). This could be indicative of the common origin of plasmid-mediated metallo-β-lactamases in *Aeromonas* spp. isolates.

In *A. salmonicida* subsp. *salmonicida* isolated from diseased fish, type three secretion system (TTSS) genes were located on a 140 kb thermolabile plasmid and the loss of the plasmid was correlated with the loss of virulence (Stuber *et al.*, 2003b). Although the TTSS genes in *A. salmonicida* subsp. *salmonicida* were plasmid-encoded, in general there was no correlation between plasmid presence and virulence in environmental and clinical isolates (Brown *et al.*, 1997). However, Majumdar *et al.* (2006) reported that loss of a 21 kb plasmid in four *A. hydrophila* strains isolated from diseased catfish, resulted in the inability of those strains to cause UDS as well as the loss of resistance phenotypes (Majumdar *et al.*, 2006). In the same study, the conjugal transfer to other species such as *Flavobacterium psychrophilum* and *Pseudomonas putida* was unsuccessful showing that certain bacterial species are not receptive to incoming plasmids. The host-range and incompatibility of plasmids should also be considered as these factors and the already resident plasmids play a role in the increased/reduced dissemination of plasmids within a population.

1.4. Plasmids

Plasmids are exogenous DNA found in organisms representing the three domains of the living world namely *Archeae, Bacteria* and *Eukarya.* These extra-chromosomal DNA's can exist as linear or circular, single- or double-stranded fragments varying in

size and gene composition. Plasmids are very diverse in terms of size (1.5 - >600 kb), copy number and in relation to the different phenotypic characteristics they confer upon their hosts (Osborn *et al.*, 2000). The main function of plasmids is the ability to incorporate and deliver genes through recombination and transposition, thus favouring genetic exchange within a bacterial population. Plasmids rarely encode gene products that are essential for the growth and reproduction of the organism; instead they provide a selective advantage under certain environmental conditions (Brantl, 2004). Plasmids are selfish genetic elements in the sense that they constitute a burden for the bacterial host cell. They provide a competitive advantage in the presence of adverse environmental conditions or the presence of unusual/complex metabolic substrates and heavy metals. Plasmids are ultimately dependent on the host fitness in order to be efficiently transferred to new hosts and dependents on the selective advantages and/or disadvantages conferred by their phenotype on the host (van Elsas *et al.*, 2000).

1.4.1. Backbone genes

The plasmid replicon has three distinct characteristics: (i) each plasmid has an origin(s) of replication, (ii) a plasmid-encoded protein is generally required to initiated replication, and (iii) plasmid-mediated functions to control plasmid replication are present (del Solar *et al.*, 1998). The minimum requirement for a plasmid is the ability to replicate thus, housekeeping genes have essential functions which allows plasmids to replicate, to be maintained in the cell and to be transferred between different host cells (Couturier *et al.*, 1988; Osborn *et al.*, 2000).

1.4.2. Replication and copy number control

Two mechanisms of plasmid replication exist, namely theta and rolling-circle replication. These different strategies are employed to facilitate the assembly and attachment of the replisome protein complex to the origin of replication (*oriV*). These mechanisms include the opening of the DNA strands at the A + T-rich origin of

replication (*oriV*) in the case of theta replication and the introduction of single-strand DNA to generate the free 3'-OH end used as the primer to initiate replication when rolling-circle replication occurs (Espinosa *et al.*, 2000). Theta replication has been studied in Gram-negative bacteria with plasmids such as ColE, RP4 and RSF1010. In plasmids replicating through the theta mechanism of replication, DNA synthesis can start from several origins and can be either uni- or bi-directional (del Solar *et al.*, 1998). Rolling-circle replication is found to be common in Gram-positive bacteria, but plasmids isolated from Gram-negative bacteria and members of the *Archeae* kingdom have also been shown to replicated using this strategy (del Solar *et al.*, 1998).

Plasmids using the theta mechanism of replication require a plasmid-encoded Rep initiator protein such as members of the IncQ plasmid family, while others, i.e., ColE1-type plasmids, require the host DNA polymerase 1 during replication (del Solar *et al.*, 1998). These plasmids replicate either independently or dependently of host factors (Brantl, 2004; del Solar *et al.*, 1998; Toukdarian, 2004).

ColE1 plasmid replication requires the host-encoded DNA polymerase 1. Generally, the plasmid-encoded primer RNAII is essential for replication and the regulation of the copy number within the host (Snyder & Champness, 2003). Replication starts with the synthesis of a 550 nucleotide long primer (RNAII) by host-encoded RNA polymerase which undergoes conformational changes to ensure activity (Fig. 1.2).

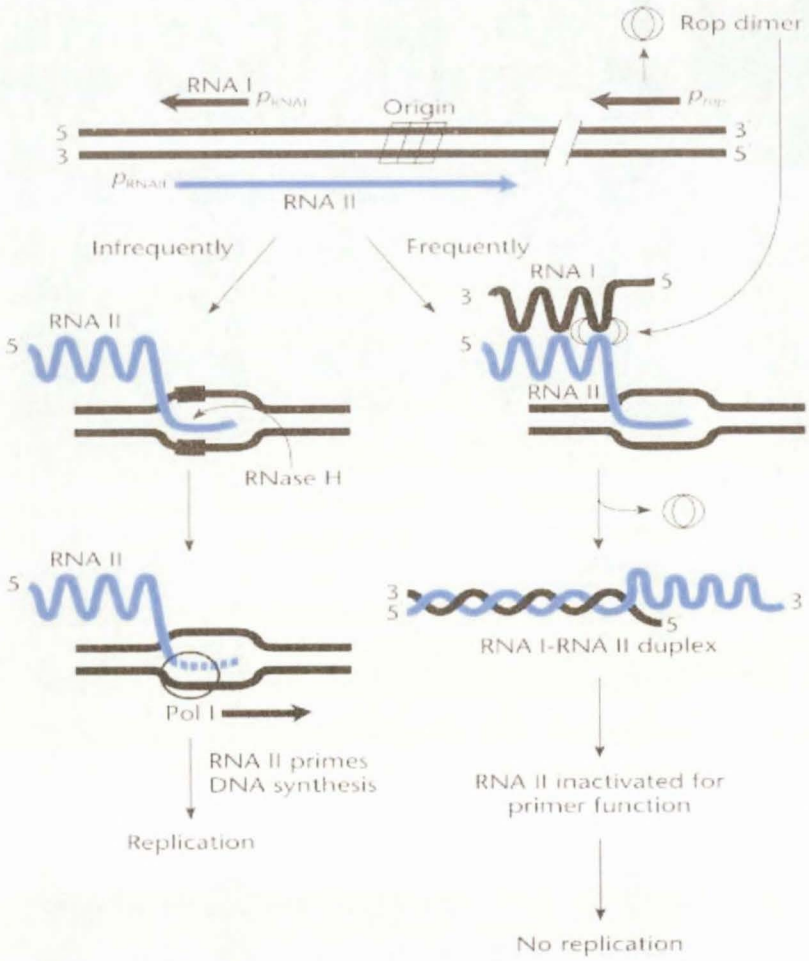

Figure 1.2. Replication and copy number control mechanism of the ColE1 plasmid family (Snyder & Champness, 2003).

A hybrid is formed between the active RNAII and the DNA at the origin of replication. Host-encoded RNaseH is responsible for the cleavage of the RNA-DNA hybrid that delivers the free 3'-OH end required by DNA polymerase 1. Replication is initiated at the free 3'-OH end with the synthesis of the leading strand (Brantl, 2004; del Solar *et al.*, 1998). DNA synthesis is continuous on one of the strands (leading strand), while it occurs discontinuously on the other strand (lagging strand) (del Solar *et al.*, 1998).

In general, plasmids that replicate independently of the host, encode the necessary factors required for replication. IncQ plasmids encode their own replication initiator

protein (RepC), helicase (RepA) and primase (RepB), needed to initiate replication. The origin of replication (*oriV*) contains an array of approximately 22 bp repeats (iterons) with an adjacent A + T-rich region and one or two binding sites for the initiator protein. Replication starts with the binding of the initiator protein RepC, to the iteron sequences in the origin of replication which leads to the melting of the DNA in the A + T rich region adjacent to the origin (Toukdarian, 2004). The RepA helicase is loaded onto the replication fork followed by the binding of the primase, RepB at one of the ssi (small palindromic sequences) sites located on complementary strands, and proceeds continuously in direction with the uncopied strand being displaced as a loop (del Solar *et al.*, 1998; Kruger *et al.*, 2004). The final product at the one ssi site is double-stranded, while the displaced strand is single-stranded. DNA synthesis occurs continuously at the previously unused ssi site and converts the single-stranded loop to double-stranded DNA (Toukdarian, 2004). The presence of two ssi sites, ssiA and ssiB, further extend the host-range of plasmids, since these sites can be utilized in a host-specific manner.

A number of control mechanisms have evolved to ensure that the resident plasmid is inherited by the daughter cells after cell division. Plasmids with a high copy-number should provide enough independent segregation units to ensure the stable maintenance of the plasmid during growth and cell division (Espinosa *et al.*, 2000).

Replication is controlled by the Rep protein concentration, where an increase in concentration leads to the initiation of replication followed by the inhibition through "handcuffing" (Chattoraj, 2000). The "handcuffing" model for replication control centers around 3 main features (Fig. 1.3). Firstly, binding of the Rep proteins to iterons is essential for replication and is needed to initiate the processes that follow (Kruger *et al.*, 2004). Secondly, the Rep-mediated coupling of the *ori*-region of two plasmids, blocks the initiation of replication in both plasmids, and thirdly, the reversal of the coupled plasmids which leads to the initiation of replication (Chattoraj, 2000).

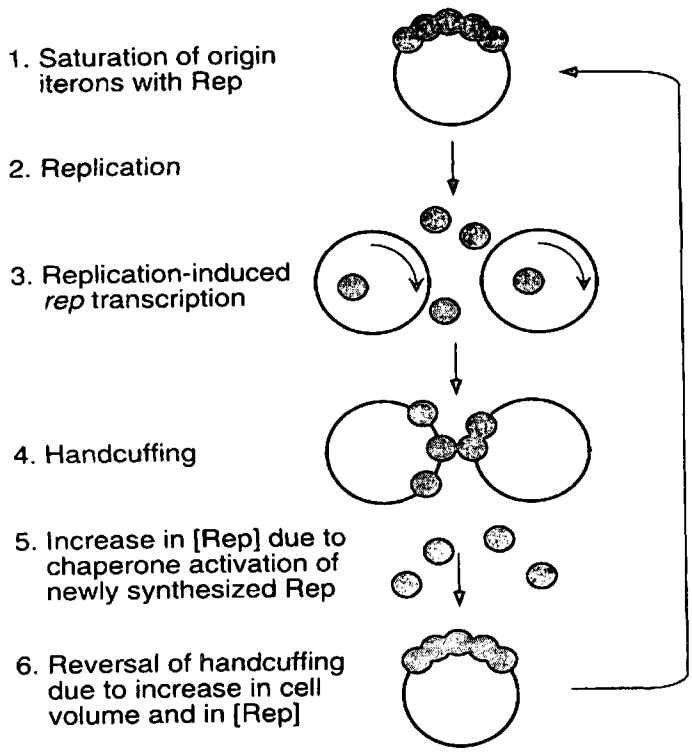

Figure 1.3. Schematic representation of the handcuffing model for the control of plasmid copy number illustrating the role of the Rep proteins in the initiation and control of replication. Step 1 shows the saturation of the origin of replication with bound Rep protein molecules followed by replication in step 2 and 3. The "handcuffing" of two plasmids is illustrated in step 4 followed by the reversal of the coupled plasmids due to the increased Rep concentration (Chattoraj, 2000).

Regulation of replication through the binding of antisense RNA is important in the stable inheritage of resident plasmids (Brantl, 2004; Snyder & Champness, 2003). Replication is controlled by the 10-nt long antisense RNA, RNAI (Fig. 1.2). The continuously transcribed RNAI consists of three stem-loops with an unstructured 5' tail (Brantl, 2004). The binding of RNAI to RNAII prevents the formation of the RNA-DNA hybrid (Brantl, 2004; del Solar *et al.*, 1998). If the stable RNA-DNA hybrid is not formed, the RNAII molecule cannot be processed by the RNase H and therefore, the availability of the free 3'-OH group is the rate limiting factor (del Solar & Espinosa, 2000).

The Rop dimer plays an important role in the control of replication (Fig. 1.2). The inhibitory effect of RNAI is further enhanced by the presence of the Rop protein

which facilitates the formation of the RNAI-RNAII complex and ultimately inhibits plasmid replication (del Solar & Espinosa, 2000).

1.4.3. Classification and incompatibility groupings of plasmids

Identification and classification of plasmids is essential and necessary for three reasons. These includes analyzing their distribution in nature and investigating the relationship to the host cells, discovering the genetic relatedness and evolution of plasmids and lastly, characterizing the transfer of genetic material (Francia *et al.*, 2004).

Traditionally, plasmids have been characterized and grouped based on their incompatibility, a property which is directly related to the replicon. The replicon is defined as the minimal DNA fragment harbouring the origin of replication (*oriV*) and which is capable of replication (Osborn *et al.*, 2000; Prescott *et al.*, 1999). Plasmids are termed incompatible if two different plasmids are incapable of co-residing within the same host and thus belong to the same incompatibility (Inc) group. Two plasmids with identical replicons are unable to reside within the same host due to the inability of the host to discriminate between the replication machinery (Osborn *et al.*, 2000). Plasmids of the same Inc group can be incompatible due to similar replication control mechanisms and/or similar partitioning functions. There may be hundreds of different Inc plasmid groups which allows the basic classification of plasmids (Snyder & Champness, 2003). Another approach to the classification of plasmids is the comparison of the plasmid-encoded replication proteins (Rep) (Francia *et al.*, 2004; Rawlings & Tietze, 2001). Characterization of *rep* genes have been shown to be useful in the establishment of evolutionary relatedness of plasmids (Rawlings & Tietze, 2001).

Plasmids are further classified as being self-transmissible or mobilizable. Self-transmissible plasmids encode all the factors needed for the movement between hosts, while mobilizable plasmids encode only certain factors and require the presence of a self-transmissible plasmid for their mobilization to another host organism (Prescott *et al.*, 1999). The host-range of a plasmid is indicative of the types of different bacteria in

which the plasmid can successfully replicate and plasmids are normally grouped as narrow- or broad-host-range plasmids (Snyder & Champness, 2003).

1.4.3.1. IncQ and IncQ-like plasmids

Plasmids belonging to the IncQ incompatibility group are characterized by their relative small size (5-14 kb), their ability to be mobilized by other self-transmissible plasmids, in particular by the IncP family of plasmids, and the broad-host range that they exhibit (Rawlings & Tietze, 2001). The promiscuous nature of the IncQ plasmid family is the result of these characteristics. The best characterized IncQ plasmid is undoubtedly RSF1010. This 8684 bp plasmid confers resistance to streptomycin and sulphonamide compounds and is capable of replication in almost all Gram-negative bacteria (Scherzinger *et al.*, 1991). Plasmids belonging to the IncQ family have been identified in diverse environmental and geographical settings such as a Norwegian marine compartment, a German pig farm, from a German wastewater treatment plant and from a South African bio-mining environment (Bonemann *et al.*, 2006; Gardner *et al.*, 2001; L'Abee-Lund & Sϕrum, 2002; Smalla *et al.*, 2000). Furthermore, IncQ plasmids have been isolated from different bacteria including *E. coli, A. salmonicida, Acidithiobacillus ferrooxidans* and *At. caldus* (Bonemann *et al.*, 2006; L'Abee-Lund & Sϕrum, 2002; Rawlings *et al.*, 1984; Smalla *et al.*, 2000). These plasmids carry a diversity of antibiotic resistance genes, and thus IncQ plasmids are important in the dissemination of antibiotic resistance genes.

Nucleotide sequences of the Rep family of proteins, responsible for the initiation of replication, are used to further classify the plasmids within this Inc group (Fig. 1.4) (Espinosa *et al.*, 2000; Rawlings & Tietze, 2001). This comparative strategy allows the identification of possible IncQ and IncQ-like plasmids in bacterial communities (Bonemann *et al.*, 2006; Smalla *et al.*, 2000).

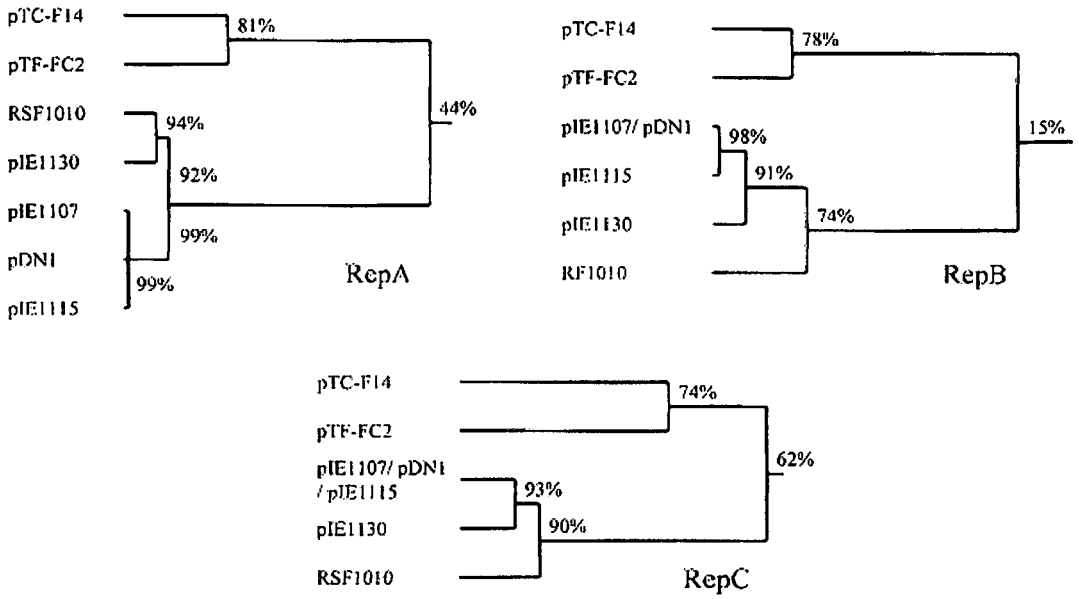

Figure 1.4. Phylogenetic relationship in terms of the percentage amino acid sequence identity between the Rep proteins of the IncQ and IncQ-like plasmid family (Rawlings & Tietze, 2001).

Although all IncQ plasmids have uniform replicons, i.e., *repA*, *repB* and *repC* genes as well as conserved sequences in the *oriV*, the mobilization regions differ among the IncQ and IncQ-like plasmids (Rawlings & Tietze, 2001). IncQ plasmids like RSF1010 form one of the groups which contains a three-*mob*-gene system (*mobACB*), while the mobilization region of the IncQ-like plasmids consists of a five-*mob*-gene system (*mobABCDE*) (Rohrer & Rawlings, 1992).

Different antibiotic resistance genes have been shown to be carried by IncQ plasmids. Other than the streptomycin (*strA-strB*) and sulphonamide (*sul*II) resistance genes encoded by plasmid RSF1010, tetracycline (*tetC*) and nalidixic acid (*qnrS2*) resistance determinants have been identified on plasmids pRAS3 and pGNB2, respectively (Bonemann *et al.*, 2006; Derbyshire & Willets, 1987; L'Abee-Lund & Sϕrum, 2002). Furthermore, the two IncQ-like plasmids pTC-F14 and pTF-FC2 are cryptic in nature and do not encoded any antibiotic resistance genes (Gardner *et al.*, 2001). This suggests a high level of diversity among members of the IncQ plasmid family.

1.4.3.2. ColE-type plasmids

The ColE family represents a group of closely related plasmids that replicate in *E. coli* with a relatively high copy number but differ from other plasmids in that they rely on host-encoded proteins for replication (Espinosa *et al.*, 2000). These mobilizable plasmids are widely used for the construction of bacterial cloning vectors and can be mobilized by plasmids belonging to different incompatibility groups including IncIα, IncFI, IncW and IncP members (Francia *et al.*, 2004). Both ColE1 and ColE2-type plasmids replicate using the theta mechanism of replication (del Solar *et al.*, 1998; Espinosa *et al.*, 2000). The minimum replicating unit of the ColE2-type plasmids consists of a primase, a short antisense RNA and a *cis* acting *oriV*, where the primase binds (Boyd *et al.*, 2003). The ColE1-type plasmids, however, do not require plasmid-encoded proteins and replication is mediated by two RNA molecules serving as primers for DNA synthesis. ColE2-type plasmids are often mutually compatible, thus allowing the presence of two plasmids harbouring the same replicon (Boyd *et al.*, 2003).

Boyd *et al.* (2003) characterized three small cryptic plasmids, pAsa1 (5424 bp), pAsa2 (5247 bp) and pAsa3 (5616 bp), isolated from *A. salmonicida* subsp. *salmonicida* strain A449 obtained from brown trout (Fig. 1.5). Plasmid pAsa2 harboured a ColE1-type replicon, while the other two plasmids appeared to belong to the ColE2 family of replicons. Since the replication strategies of the two different replicon types differ, they can be stably maintained in the same host. The prevalence of ColE plasmids in the environment is limited due to the dependence of these plasmids on host-encode factors to initiate replication (van Elsas *et al.*, 2000).

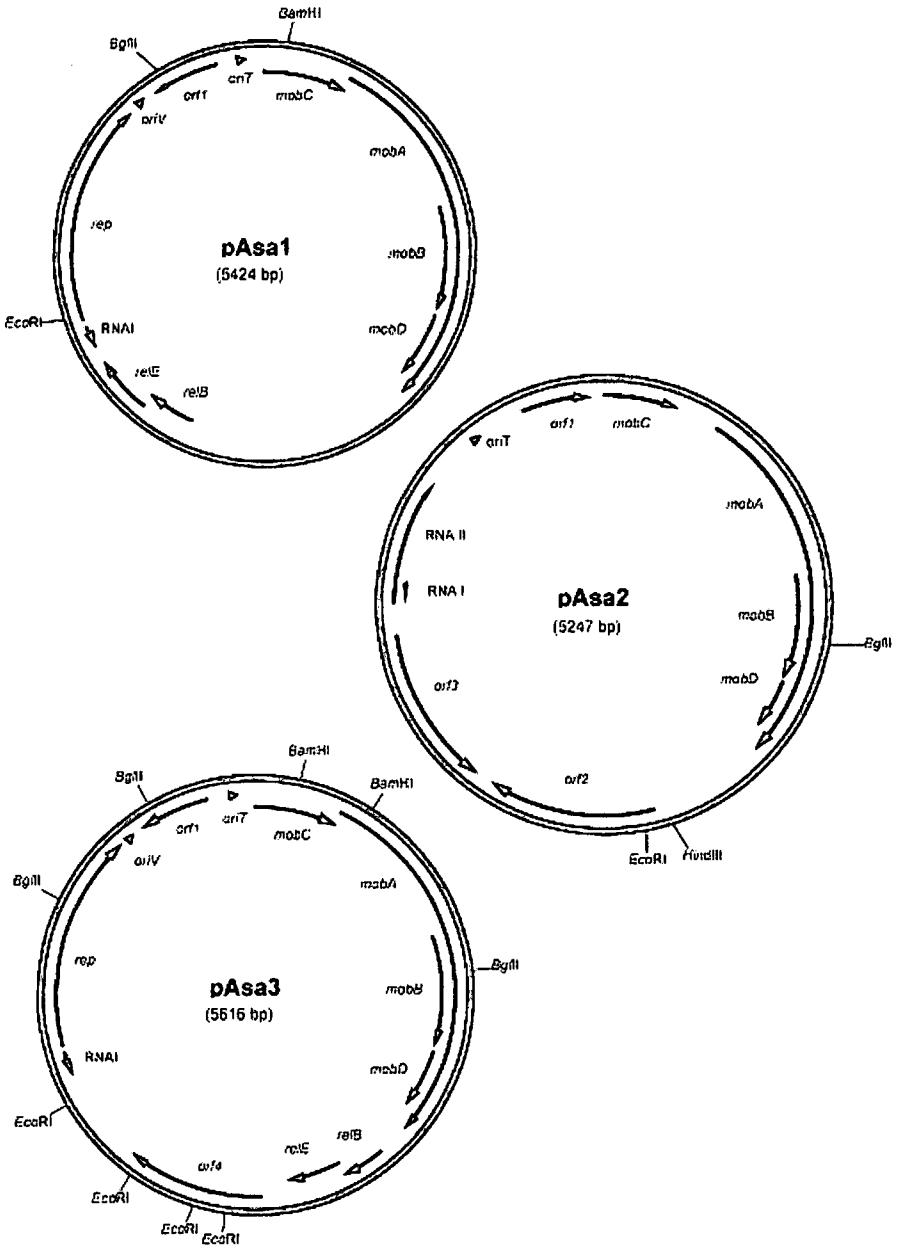

Figure 1.5. Restriction maps of three cryptic plasmids isolated from *A. salmonicida* subsp. *salmonicida* strain A449. Replicon types and sizes of plasmids are indicated (Boyd *et al.*, 2003).

1.4.4. Accessory genes

All bacteria contain intrinsic resistance mechanisms which tend to confer low levels of resistance to a wide variety of unrelated antibiotics, but subsequent acquisition of mobile resistance determinants may lead to strains displaying a highly resistant

phenotype (Osborn *et al.*, 2000). The possibilities for the acquisition of accessory functions such as antibiotic and heavy metal resistance, genes conferring resistance to radiation, transfer of DNA to higher eukaryotes and genes encoding enzymes for metabolic functions are endless (del Solar *et al.*, 1998; Dennis, 2005). These genes are transferred by a host of other mobile genetic elements such as gene cassettes, integrons, transposons and insertion sequences (IS) elements either into the host's chromosome or into independent replicons such as plasmids (Fig. 1.6) (Osborn *et al.*, 2000).

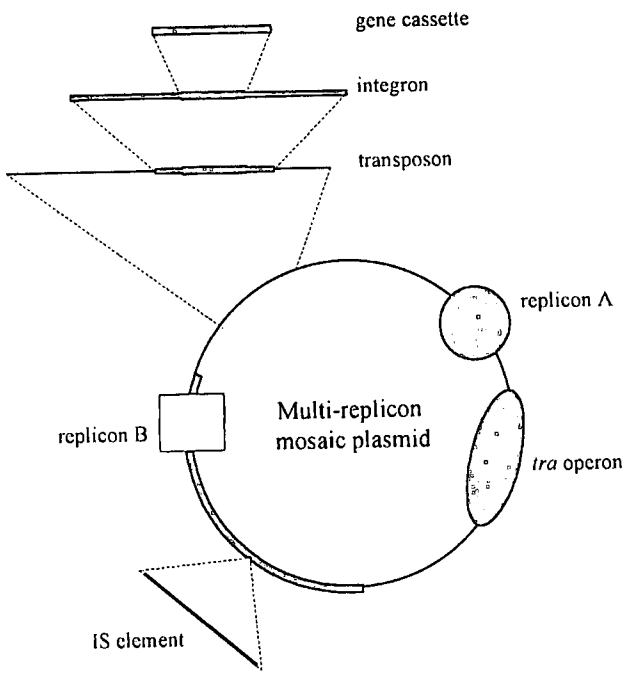

Figure 1.6. The mosaic structure of plasmids as vectors for the spread of accessory genes. Adapted from Osborn *et al.* (2000).

1.4.4.1. Transposons

Transposable elements are widespread in bacterial genomes and are capable of movement within the chromosome and between genomic and exogenous DNA's (Prescott *et al.*, 1999), in addition to carrying genes required for the process of transposition. For transposition to occur, the level of homology between the insertion sequences (IS) does not have to be high (Prescott *et al.*, 1999). This increases the rate at which genes move in a particular DNA segment or between different DNA fragments.

Other than the genes required for transposition, transposons may contain other genes including antibiotic resistance genes (Rice, 1998; Smalla *et al.*, 1993). This highlights the importance of transposons as facilitators for the spread of antibiotic resistance since these elements are capable of movement between the host's chromosomal DNA and other exogenous DNA such as plasmids.

Transposons are widely associated with resistance plasmids and are found in both Gram-positive and Gram-negative bacteria (L'Abee-Lund & Sørum, 2000; Rice, 1998). Tn*1721* and Tn*5393* are both present on R-plasmids isolated from the human pathogen *Salmonella enterica* (Pezzella *et al.*, 2004). The association of *tetA* with transposon Tn*1721* also occurs in other organisms including *A. salmonicida* (Casas *et al.*, 2005; Sørum *et al.*, 2003). Transposon Tn*5393* together with the linked streptomycin resistance genes, *strA-strB* have been identified as part of the conjugative R-plasmid pRAS2 isolated from *A. salmonicida* (L'Abee-Lund & Sørum, 2000). The kanamycin resistance determinant, *npt*II, has been identified and shown to be associated with transposon Tn*5* among *Aeromonas* spp. isolated from sewage material (Smalla *et al.*, 1993). The prevalence of this transposon was not limited to *Aeromonas* spp. but was detected in *E. coli* strains as well (Smalla *et al.*, 1993). Furthermore, the Tn*5* was detected in different environments including river water, soil and pig manure slurries. The potential for transposons associated with antibiotic resistance genes to exchange genetic material within a complex bacterial population and between different environmental compartments is great. The impact of these composite transposons is furthermore enhanced when associated with the presence of gene-capturing structures such as integrons (Pezzella *et al.*, 2004).

1.4.4.2. Integrons

Integrons as genetic elements are of great interest since these structures are capable of acquiring a wide variety of resistance gene cassettes through recombination (Collis & Hall, 1995; Hall & Collis, 1998). Structurally, integrons consist of an integrase (*intI*) gene encoding a site-specific recombinase and a recombination site (*attI*) (Fig.

1.7A). The recombinase mediates recombination between the primary site (*attI*) and the secondary site, the 59-base element which is located adjacent of the resistance gene cassette (Ploy *et al.*, 2000). The free circular gene cassette consists of an open reading frame encoding the different resistance genes with a unique downstream 59-base element (Fig 1.7B.) (Rowe-Magnus *et al.*, 2001).

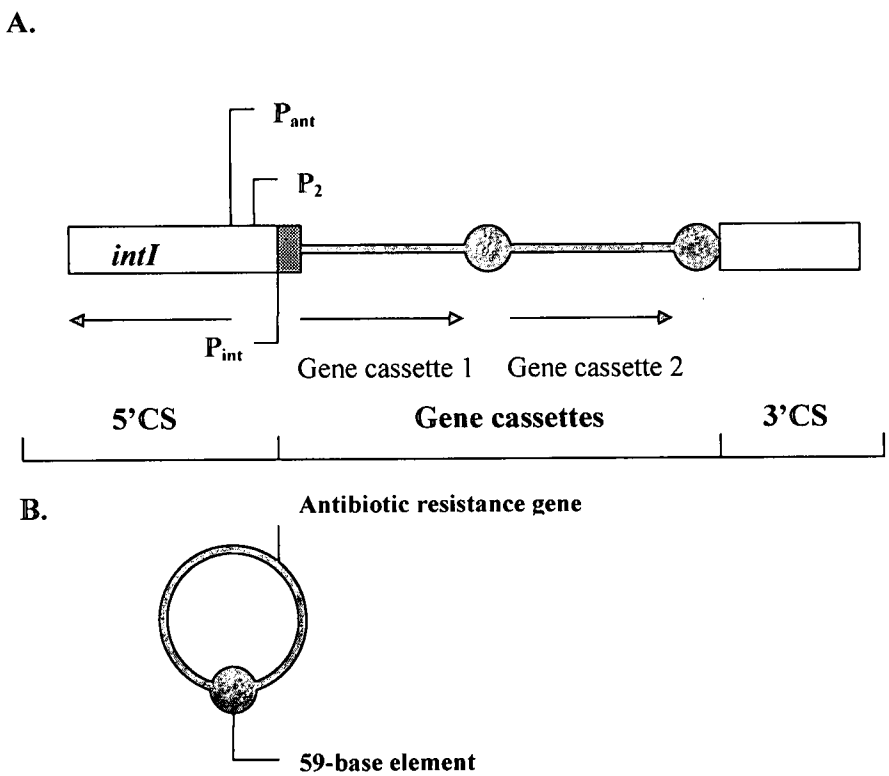

Figure 1.7. **(A)** Schematic representation of the integron structure, showing the inserted gene cassettes and the promoters responsible for transcription of the integrase gene (P_{int}), the inserted gene cassettes (P_{ant}) and the secondary promoter (P_2) (Collis & Hall, 1995). **(B)** The free circular antibiotic gene cassette with the downstream 59-base element (Hall & Collis, 1998).

Integration and excision of the exogenous DNA is catalyzed by the site-specific recombinase (*intI*) encoded by the integron. Insertion of genes in a cassette is in the same order when inserted into the integron with no limit to the amount or the composition of the gene cassettes. The promoter (P_{ant}) is responsible for the expression of the gene cassette. This promoter is not conserved as four variants have been identified (Collis & Hall, 1995). The orientation of the cassettes is critical for the expression of the genes

within the cassette together with the position of the genes (Hall & Collis, 1998). The level of gene expression is further influenced by the 59-base element that might serve as a transcriptional terminator (Collis & Hall, 1995). Together with the orientation of the genes within the integron, the strength of the P_{ant} plays an important role in the levels of resistance displayed, with up to 20-fold differences being observed in the level of gene expression (Collis & Hall, 1995).

Four classes of integrons based on the integrase genes, have been described to date. Class 1 integrons contain a conseved 3' region encoding the *qacEΔ1* and *sul*I genes and an open reading frame of unknown function. The *qacEΔ1* and *sul*I genes confer resistance to quaternary ammonium compounds and sulphonamides, respectively. Integrons belonging to the first class have been widely isolated from estuarine environments, aquaculture settings and different diseased fish species. Gram-negative bacteria including *Aeromonas* species isolated from aquatic environments, *Klebsiella pneumoniae, E. coli* and *Citrobacter freundii* isolated from a clinical setting, harboured an array of different gene cassettes encoding aminoglycoside, chloramphenicol and/or erythromycin resistance as part of integrons structures (Jones *et al.*, 1997; Rosser & Young, 1999; Schmidt *et al.*, 2001a; Sϕrum *et al.*, 2003). Class 2 integrons are normally embedded in the Tn7 family of transposons. Similar to the class 1 integrons, these integrons consist of an integrase gene followed by a gene cassette containing antibiotic resistance genes (Fluit & Schmitz, 2004). Class 3 integrons share the same basic structure as class 1 and 2 integrons, but the amino acid identity between the integrase genes of classes 2 and 3 with respect to class 1 integrase are only 45% and 60%, respectively (Hall & Collis, 1998). Integron structures, formerly grouped as class 4 integrons are now named *V. cholerae* super-integrons, which encode a wide variety of different functions and are located on bacterial genomes (Fluit & Schmitz, 2004).

The gene cassettes captured within the integron show diversity in terms of the number and type of resistance determinants (Schmidt *et al.*, 2001a). During the late nineties is was estimated that over 40 different antibiotic resistance gene cassettes existed in nature, but currently more than 90 have been described with more cassettes likely to be found in the future (Fluit & Schmitz, 2004; Hall & Collis, 1998; Recchia & Hall, 1995).

Class 1 integrons isolated from Gram-negative aquatic bacteria contain gene cassettes conferring resistance to trimethoprim (*dfr1a, dfrIIc, dfrV, dfrVII, dfrA16, dfrXII*), chloramphenicol (*catB3, catB5*), aminoglycosides *(aadA2, aacA4, aacC1, ant(3")1a*), β-lactams (*oxa2*) and erythromycin (*ereA*) (Jones *et al.*, 1997; Rosser & Young, 1999; Schmidt *et al.*, 2001b; Sϕrum *et al.*, 2003). Tennstedt *et al.* (2003) found eleven different antibiotic resistance gene cassettes inserted into class 1 integrons. These cassettes contained different combinations of genes conferring resistance to ampicillin (*oxa10, oxa1*), chloramphenicol (*catB2*), gentamicin (*aacC1*), trimethoprim (*dfrV, dfrVII*) and streptomycin and streptinomycin (*aadA1, aadA5, aadB*). The integron structure itself can also show variation in the presence of the conserved 3' end (*sul*I and *qacE*ΔI) and in some cases, integrons isolated from aquatic bacteria lacked inserted gene cassettes (Rosser & Young, 1999). These "empty" integrons still have the ability to acquire resistance gene cassettes from the gene reservoir with the onset of selective pressure.

Although integrons are not capable of mobilization, they are often associated the conjugative plasmids and thus the integron-encoded resistance genes are transferable from on organism to another (Schmidt *et al.*, 2001a; Walsh, 2006). Schmidt *et al.* (2001a) reported the prevalenc of co-transfer of class 1 integrons carrying antibiotic resistance cassettes together with large conjugative plasmids (13 – 150 kb) isolated from aquatic bacteria.

1.4.5. Plasmids and associated resistance genes in *Aeromonas* spp.

Four R-plasmids, pRAS1, pRAS2, pRAS3 and pRAS4, have been identified in the fish pathogen *A. salmonicida* subsp. *salmonicida* isolated from a Norwegian aquaculture system (L'Abee-Lund & Sϕrum, 2000; L'Abee-Lund & Sϕrum, 2002; Sϕrum *et al.*, 2003). Three of the above-mentioned have also been identified in less pathogenic strains of atypical *A. salmonicida* and will be the focus of this section. These plasmids differ with respect to size, antibiotic resistance genes and their mobility.

1.4.5.1. Plasmid pRAS1

The 45 kb conjugative plasmid, pRAS1 was isolated from an atypical *A. salmonicida* strain isolated from diseased Norwegian Atlantic salmon (Sørum *et al.*, 2003). This IncU plasmid transferred resistance to tetracycline, trimethoprim and sulphamethoxazole to *E. coli* following conjugation experiments indicating that these resistance determinants were plasmid-encoded (Sørum *et al.*, 2003). The region harbouring the resistance gene contained two genetic elements, i.e, In*4*-like integron and Tn*1721* regions (Fig. 1.8).

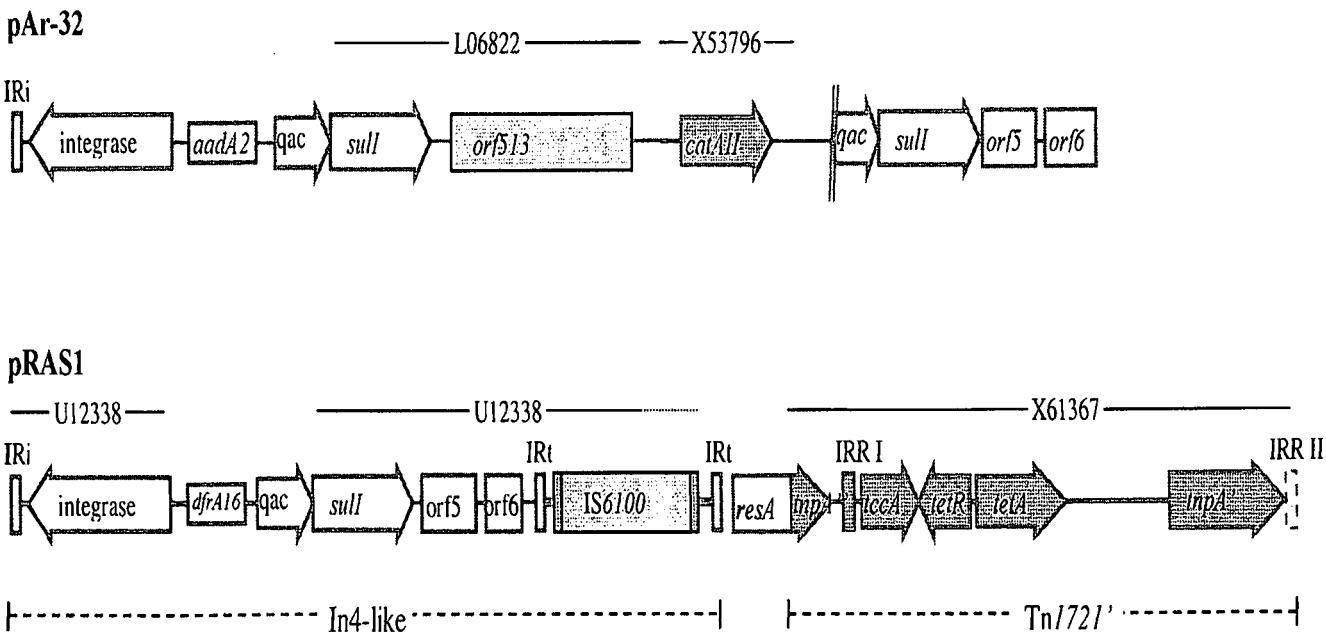

Figure 1.8. Comparison of the integron structures found on two resistance plasmids isolated from *A. salmonicida* spp. isolates (Sørum *et al.*, 2003).

The complete class 1 integron contained the *dfrA16* gene, conferring resistance to trimethoprim while the 3' conserved region harboured the *sul*I gene responsible for resistance to sulphonamides (Sørum *et al.*, 2003). The second region comprised the truncated Tn*1721* tetracycline-associated transposon. This self-transmissible plasmid was capable of transferring its resistance phenotype to both *E. coli* and *S. enterica* strains,

highlighting the capacity to move between different organisms and the exchange of resistance genes with other mobile genetic elements (Sϕrum *et al.*, 2003).

Comparison of the R-plasmid pAr-32 (pRA3), isolated from diseased biwamasu (*Oncorynchus rhodurus* f. *rhodurus*) and pRAS1 (Fig. 1.8) revealed similarities in the backbone genes and structure (Sϕrum *et al.*, 2003). Both plasmids harboured an IncU replicon resulting in the broad-host-range of these plasmids. However, the class 1 integron identified on pAr-32 was more complex containing the *catA2* and *aad2* genes, encoding resistance to chloramphenicol and streptomycin, respectively.

Characteristics of pRAS1 such as IncU incompatibility, the presence of the IS6100 insertion sequences, *sul*I gene, *tetA* gene, resistance to trimethoprim and the presence of a complete or truncated Tn*1721* have been used to identify pRAS-like plasmids isolated from aquatic environments (Casas *et al.*, 2005). Using these features of pRAS1, Casas *et al.* (2005) differentiated nine plasmids as being pRAS-like and non-pRAS-like. While both groups were able to transfer the tetracycline resistance phenotype to a recipient by conjugation, the 58 kb pRAS-like plasmids also conferred resistance to sulphonamides and trimethoprim, encoded by *sul*I and *dfrA16* genes, respectively. The larger size of the pRAS-like plasmids might be due to the presence of other accessory genes and/or other mobile genetic elements.

Characteristics similar to that of plasmid pRAS1 have led to the identification of several related plasmids. Seventeen *Aeromonas* spp. isolates, predominantly *A.veronii* biovar *sobria* and *A. hydrophila* obtained from aquaculture and hospital settings, respectively, contained plasmids encoding resistance to tetracycline and streptomycin with the resistance phenotype capable of being transferred to *E. coli* (Rhodes *et al.*, 2000). Results indicated that 10 of the plasmids carried the TetA resistance determinant while seven of the tetracycline resistance genes remained unidentified. Seven of the plasmids were identified as IncU plasmids, and this corresponded with the broad-host range exhibited during conjugation. Six of the identified IncU plasmids from *A. hydrophila* harboured the TetA determinant while the other TetA-encoding IncU plasmid was isolated from *A. caviae*. Rhodes *et al.* (2000) observed the spread of a single plasmid

within a population, and identified differences in the plasmid backbone genes despite similarities in the accessory genes content.

On comparison of the tetracycline resistance genes and the incompatibility grouping, similarities were observed between the IncU plasmids isolated and characterized by Rhodes *et al.* (2000), and plasmids pRAS1 and pASOT previously isolated by Adams *et al.* (1998). Differences in geographical areas and environmental settings of specimens or sampling and/or similarities in selective pressures in the different compartments, might explain the broad-host-range of the plasmids. Plasmid pIE402 isolated from an *E. coli* strain from a German pig farm was identical to pRAS1 (Tschape *et al.*, 1981). Although isolation occurred from an unrelated organism, this supports the fact that transfer of plasmids occurs between different organisms and within/between different environmental settings (Rhodes *et al.*, 2000).

Plasmids isolated from *Aeromonas* spp. have been compared to pRAS1 using restriction fragment length polymorphism analysis (Adams *et al.*, 1998; Rhodes *et al.*, 2000). With this comparative approach, it is possible to identify related plasmids and to assess the persistence of a certain plasmid within a population. Although all plasmids carried the same class of tetracycline resistance genes, the profiles generated were not similar. The TetA determinant was, however, located on a 5.4 kb *Eco*RI fragment as observed for pRAS1 (Adams *et al.*, 1998).

1.4.5.2. Plasmid pRAS2

Plasmid pRAS2 was also isolated from a Norwegian *A. salmonicida* subsp. *salmonicida* strain 1682/92, which had no previous streptomycin exposure (L'Abee-Lund & Sørum, 2000). The 48 kb conjugative plasmid harbours a *sulII* gene conferring resistance to sulphonamides and a *tet31* tetracycline resistance gene (Fig. 1.9).

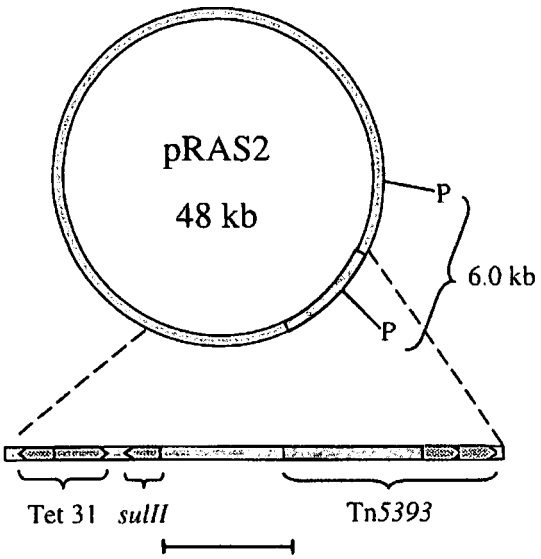

Figure 1.9. Plasmid map of pRAS2 indicating the resistance determinants *sul*II and Tet 31 located adjacent to the functional transposon Tn*5393*c. Tn*5393*c harbours the linked resistance genes *strA-strB* conferring high levels of streptomycin resistance (L'Abee-Lund & Sϕrum, 2000).

Plasmid pRAS2 contained the functional transposon, Tn*5393* harbouring the linked *strA-strB* genes which encoded high levels of streptomycin resistance (Fig. 1.10.). *strA-strB* genes are frequently identified in clinical isolates harbouring small, non-conjugative plasmids, while these genes are associated with Tn*5393* in plant pathogens (Sundin & Bender, 1995; Sundin & Bender, 1996). Insertion sequence IS*6100* is present in both plasmids pRAS1 and pRAS2, but has a regulatory effect in the expression of the streptomycin resistance in pRAS2. Resistance to tetracycline and sulphonamides was transferred to *E. coli* by conjugation and MICs remained constant, however, resistance to streptomycin decreased in the transconjugant (L'Abee-Lund & Sϕrum, 2000). When comparing the two R-plasmids isolated from members of the *Aeromonadaceae* family, the dominant differences were the sizes of the plasmids, the presence of different transposons, and the absence of an integron in pRAS2. Plasmid pRAS1 also carried a trimethoprim resistance determinant in addition to the *sul*I and *tetA* genes which is different in pRAS2. Both plasmids, pRAS1 and pRAS2, are conjugative and conferred the resistant phenotype to new host cells on transfer (L'Abee-Lund & Sϕrum, 2000; L'Abee-Lund & Sϕrum, 2002).

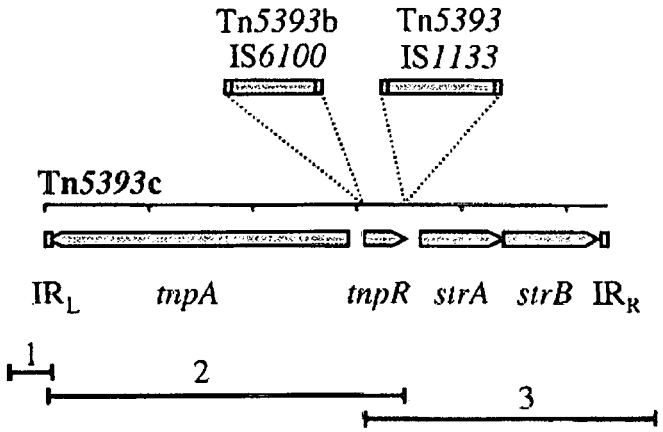

Figure 1.10. Genetic composition of the functional transposon Tn5393c harbouring the strA-strB resistance genes present on pRAS2. Insertion sequences IS6100 and IS1133 form part of the transposon isolated from *Xanthomonas campestris* pv. *vesicatoria* and *Erwinia amylovora*, respectively (L'Abee-Lund & Sϕrum, 2000).

1.4.5.3. Plasmid pRAS3

Two variants of the small, non-conjugative plasmid pRAS3, pRAS3.1 and pRAS3.2, have been isolated from *A. salmoncida* subsp. *salmonicida* and atypical *A. salmonicida,* respectively (L'Abee-Lund & Sϕrum, 2002). The two variants were not only isolated from different geographical regions, but also showed a slight difference in plasmid size. DNA sequencing revealed that pRAS3.1 harboured additional copies of repeated sequences, namely iterons, in the region between the *oriT* and the *mobA* gene, as well as in the *oriV* which accounts for the size difference of 11,851 kb and 11,823 kb calculated for pRAS3.1 and pRAS3.2, respectively (L'Abee-Lund & Sϕrum, 2002).

Nucleotide comparison and organization of the mobilization genes (*mobACDE*) and replication proteins (*repAB*), revealed a high percentage of nucleotide homology (Fig. 1.11) to that of the small, cryptic IncQ-like plasmid pTF-FC2 isolated from *At. ferro-oxidans* (L'Abee-Lund & Sϕrum, 2002). This suggested that these plasmids were related to members of the IncQ plasmid family.

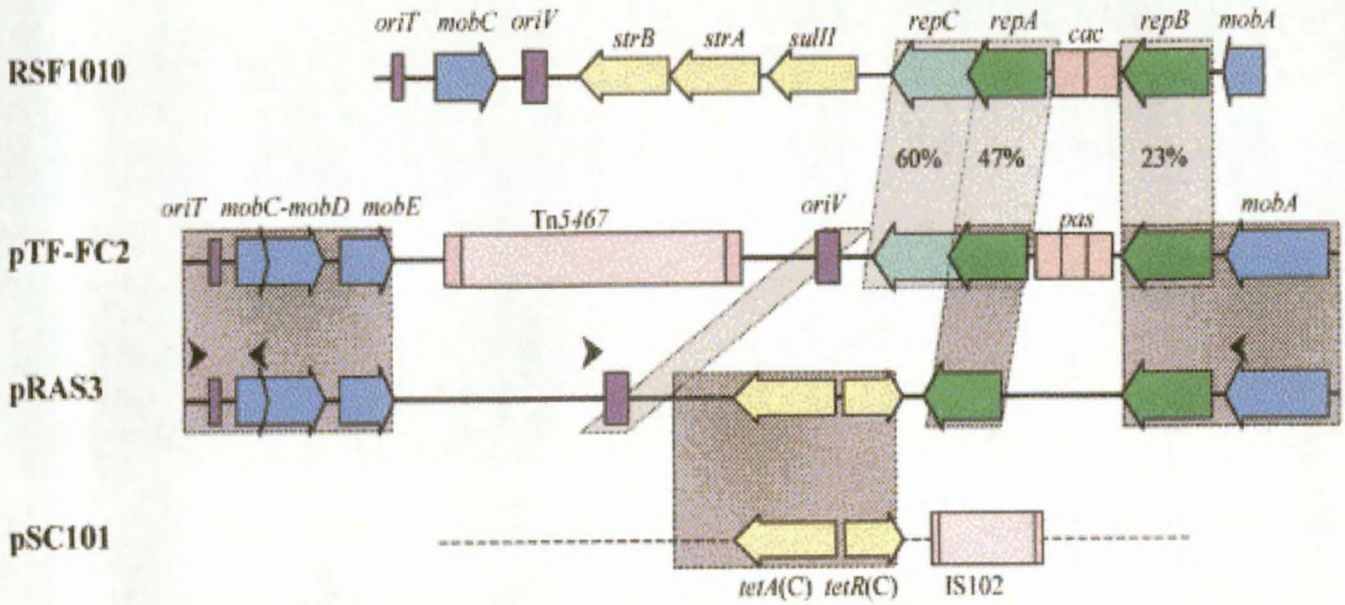

Figure 1.11. Comparison of mobilization and replication gene arrangment of pRAS3 and plasmids RSF1010 and pSC101, respectively. Replication proteins of RSF1010 and pRAS3 has a lesser degree of homology but were included to provide information related to the IncQ plasmids. The dark grey boxes indicate areas with a shared homology greater than 90% (L'Abee-Lund & Sϕrum, 2002).

The tetracycline resistance determinant *tetA(C)* showed 98.9% homology to that of R-plasmid, pSC101, originating from a *Salmonella* spp. (L'Abee-Lund & Sϕrum, 2002). Further sequence analysis of pRAS3.1 and pRAS3.2 revealed the presence of a *repC* gene, which is essential to replication. Nucleotide sequence similarity between the pRAS3 *repC* and those of the IncQ-like plasmids pTC-F14 and pTF-FC2 was shown to be low with only 29% and 37% identity, respectively (W. Loftie-Eaton, personal communication). This indicates that variations in backbone DNA of IncQ-like plasmids do exist.

The transfer of pRAS3 to a recipient was only possible in the presence of other self-transmissible plasmids. pRAS3.1 and pRAS3.2 are frequently found to co-exist with pRAS1 and pRAS2 due to the compatibility of the replicons and have been found capable of transfer in such cases (L'Abee-Lund & Sϕrum, 2000; L'Abee-Lund & Sϕrum, 2002).

The dissemination of self-transmissible R-plasmids between different geographical and environmental compartments is of great importance when viewing the current problem of emerging multi-drug resistant organisms in aquaculture (Taylor *et al.*, 2004; Teuber, 2001). However, non-conjugative plasmids should not be ignored. The presence of other mobile elements, i.e., transposons and integrons carrying antibiotic resistance cassettes on non-conjugative plasmids can still spread within an environmental compartment as the result of other plasmids enabling their mobilization. This further emphasizes the role of both self-transmissible and non-conjugative plasmids in the dissemination of accessory genes.

1.5. Aquatic environments: Reservoir for genetic exchange?

Different factors influence the transfer of plasmids in aquatic environments, namely the presence of nutrients, salinity and the presence of ions, pH, temperature, cell density and antimicrobial exposure (van Elsas *et al.*, 2000). Aquatic systems can be divided into distinct sub-habitats where biotic and abiotic factors differ. This provides selection for genetic exchange, since certain sub-habitats contain higher cell densities, more nutrients and more complex bacterial populations (van Elsas *et al.*, 2000). Several "hot spots" for the movement of genetic material have been identified in different environmental settings (Fig. 1.12). The normal bacterial flora is a common reservoir of drug resistance genes for bacterial pathogens in both human medicine and animal husbandry including fish farming, even in the absence of antibiotic exposure (Sørum and Sunde, 2001).

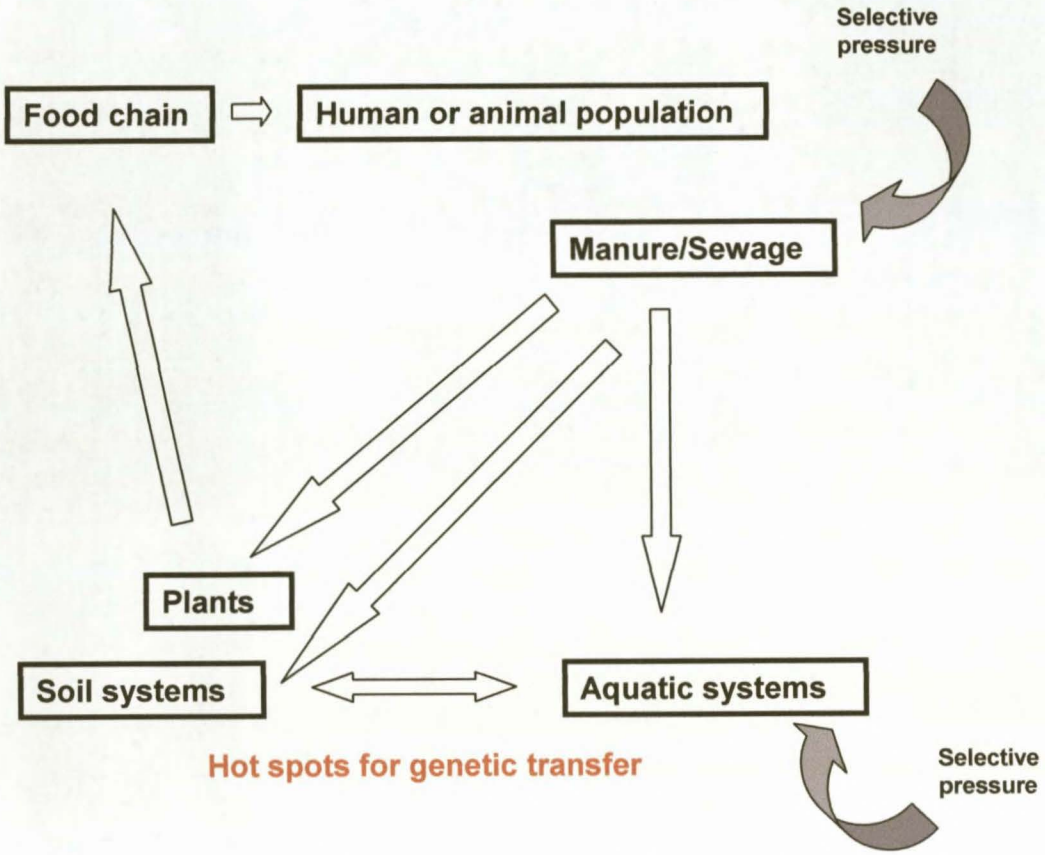

Figure 1.12. Environmental compartments are linked microbiologically. Selective pressures provided by the use and misuse of antibiotics create "hot spots" for transfer of genetic material and the subsequent spread of resistance genes. (Adapted from van Elsas *et al.* (2000).

Selective pressures applied to different environmental settings play important roles in the acquisition of certain resistance genes, since different types of antimicrobial agents are used in different compartments. This impacts the type of acquired resistance phenotype and in conjunction with the movement of micro-organisms between environmental compartments, increases the diversity of resistance determinants within the gene reservoir. Furthermore, the introduction of an antibiotic into a closed system affects not only the level of resistant pathogens, but also the level of resistant commensal bacteria. These naturally-occurring bacteria in one environmental compartment could be opportunistic human pathogens in a clinical setting and thus pose a threat to human health due to their high levels of acquired resistance.

The aquaculture setting has been identified as another potential reservoir of antibiotic resistance genes due to the use and misuse of antimicrobial agents as growth promoters as well as for preventing the outbreak of infectious diseases (Poirel *et al.*, 2005). Different aquatic organisms such as *Photobacterium, Vibrio, Pseudomonas, Salmonella, Alteromonas* and *Citrobacter* isolated from an aquatic environment were resistant to tetracycline which is frequently used in aquaculture practices (Furushita *et al.*, 2003). The presence of these resistance determinants among different genera illustrates the movement of genetic material between different organisms within a population. *Aeromonas* spp. isolated from four different Danish rainbow trout farms, showed no variations in terms of tetracycline resistance profiles, but an increase in resistance levels between species isolated from the ponds and outlet systems were evident (Schmidt *et al.*, 2000). This proves that different biotic and abiotic factors within the system influence the transfer of mobile genetic elements to other bacteria. More importantly however, is the release of these multi-resistance phenotypes into the environment leading to the contamination of other environmental compartments. River waters are the main recipient of sewage and urban effluent and are one of the major sources of water for human consumption. This may contribute to the maintenance and even spread of bacterial antibiotic resistance (Goni-Urriza *et al.*, 2000). Resistance plasmids isolated from a wastewater treatment plant, showed the presence of genes encoding resistance to tetracycline, erythromycin, cephalsporins, streptomycin, trimethoprim, amikacin, gentamicin, kanamycin, nalidixic acid and chloramphenicol (Szczepanowski *et al.*, 2004; Tennstedt *et al.*, 2003). Bacteria within the treatment plant originated from different environmental settings and would previously have been exposed to a diverse variety of different antimicrobial agents, resulting in the large variety of resistance genes.

The movement of resistance genes between the human and environmental compartments is evident (Rhodes *et al.*, 2000). The importance of integrons as vectors for the spread of antibiotic resistance genes was highlighted when similarities in terms of inserted gene cassettes between the environmental samples and clinical *E. coli* isolates were found. The trimethoprim/aminoglycoside resistance gene cassette identified in this study was also isolated from the human pathogen, *K. pneumoniae*. Integrons were also identified in bacteria from the final effluent of the wastewater treatment plant suggesting

that integron-containing bacteria are released into the environment and further facilitate the spread of resistance determinants (Rhodes *et al.*, 2000). Class 1 integrons present on multi-resistance plasmids isolated from a wastewater treatment plant, contained different gene cassettes with the *aadA* gene, conferring resistance to aminoglycosides, being most predominant (Tennstedt *et al.*, 2003).

Many aquaculture systems are treated with antimicrobial agents to ensure sufficient mass production and to prevent the outbreak of infectious diseases. The antimicrobial agents are incorporated in the fish feed or added to the tanks in disease situations. The treatment of fish in aquaculture systems selects for bacterial resistance phenotypes and may be a possible route for creation and transmission of resistant zoonotic pathogens (Teuber, 2001). In disease outbreak situations, when pathogens do not respond to initial therapy, fish are treated with higher dosage concentrations or other antimicrobial agents. Many of the microbes in aquaculture contain resistance genes and some of these microbes may be opportunistic human pathogens. Fish are handled by workers on a daily basis whether during breeding or during the preparation of fish products posing a potential threat to human health as the result of human infections arising from MDR strains. The impact of the misuse of antibiotics is a major concern, since it is clear that these substances are introduced into the environment on a larger scale than was previously thought.

1.6. Rationale for study and hypothesis

The presence of multiple-antibiotic resistant *Aeromonas* strains in fish farms and hatcheries prevents the effective control of infectious diseases. The high densities of fish kept in close proximity, is a major factor contributing to infectious diseases and inevitably leads to the spread of the resistant bacteria. The current literature provides a detailed account of the increased MDR phenotypes being presented by members of the genus *Aeromonas* and the presence of gene-capturing systems such as integrons and transposons associated with conjugative plasmids frequently identified among these bacteria. The recognition of *A. hydrophila* as an opportunistic human pathogen, the

prevalence of virulence factors and the variety of different R-plasmids, has urged the investigation of the location of resistance determinants and virulence factors, the mobility of R-plasmids and the determination of the host-range capabilities. Currently, there is limited information concerning the correlation between the presence of plasmids and the localization of antibiotic resistance genes. There is also limited information on the diversity of antibiotic resistance trends in *Aeromonas* spp. from South African aquaculture systems and the role of plasmids and other mobile genetic elements in the dissemination of resistant phenotypes. It is thus hypothesized that *Aeromonas* spp. plasmids from local aquaculture systems display diversity with regard to their plasmid profiles. It is further hypothesized that variation exists with respect to the types of plasmid replicons and the diversity of resistance genes harboured by these plasmids contributing to variable resistance profiles.

1.7. Objectives

a. To determine the prevalence and diversity of plasmids within *Aeromonas* spp. isolates.
b. To investigate whether the different antibiotic resistance phenotype from different *Aeromonas* spp. could be transferred to *Escherichia coli*.
c. To examine the diversity of accessory genes carried by *Aeromonas* spp. plasmids.
d. To investigate the replication potential of isolated *Aeromonas* spp. plasmids.
e. To identify the different replicon-types harboured by *Aeromonas* spp. plasmids.
f. To establish homology between the 14 kb IncQ-like plasmids and plasmid pRAS3.1 isolated from *Aeromonas salmonicida*.
g. To characterize a 27 kb plasmid isolated from *A. veronii* biovar *sobria*.

1.8. Aims of present study

a. To identify plasmid composition of *Aeromonas* spp. isolates from an aquaculture environment using the alkaline lysis method and pulsed-field gel electrophoresis.

b. To transform *Escherichia coli* DH5α with plasmids isolated from *Aeromonas* spp. isolates and to determine the carriage of antibiotic resistance genes by disk diffusion assays of transformants.

c. To identify the prevalence of the *tetA* gene, tetracycline-associated transposon Tn*1721*, *bla*-TEM gene, class 1 integron structures and *strA-strB* gene pair by Southern hybridization experiments.

d. To transform isolated plasmids into a *polA*$^-$ mutant *E. coli* strain, GW125a to determine the role of DNA polymerase in plasmid replication.

e. To establish the presence of replication (*repBC*) and mobilization (*mobABC*, *mobACDE*) genes using Southern hybridization experiments.

f. To identify possible IncU-type replicons.

g. To construct restriction endonuclease maps of 2 possible 14 kb IncQ-like plasmids.

h. To characterize a 27 kb *A. veronii* biovar *sobria* plasmid by restriction endonuclease mapping, Southern hybridization experiments, and sequencing analysis.

Chapter 2

Investigation of the plasmid content of *Aeromonas* spp. isolates from South African aquaculture systems

2.1. Introduction

The genus *Aeromonas* is ubiquitous to aquatic environments and members have been isolated from different fish species, reptiles and amphibians (Hatha *et al.*, 2005; Munro & Hastings, 1993; Robersts, 1993). Currently, species of the genus *Aeromonas* are readily isolated from food products and water sources, implicating them as possible threats to human health since some *Aeromonas* spp. isolates are known to cause human illness (Bin Kingombe *et al.*, 2004; Conzalez-Rodrigues *et al.*, 2002; Daskalov, 2005; Massa *et al.*, 2001; Palu *et al.*, 2006).

The plasmid content of opportunistic pathogens such as *Aeromonas* spp. have been studied for a number of reasons. Firstly, the presence of plasmids has been directly implicated in the acquisition of antibiotic resistance determinants and subsequently the spread of these genes within a specific population (Adams *et al.*, 1998; Sϕrum *et al.*, 2003). Secondly, the presence of specific virulence factors has been showed to be plasmid-mediated in *Aeromonas* spp., in particular in *A. salmonicida,* an important fish pathogen (Stuber *et al.*, 2003b). Lastly, resistance to various heavy metals and metalloids such as mercury and arsenite have been shown to be plasmid-mediated among *environmental A. hydrophila* and *A. encheleia* isolates (Huddleston *et al.*, 2006). Prevalence of plasmids among *Aeromonas* spp. isolates is very diverse and does not appear to be species-specific nor linked to specific environmental compartments (Casas *et al.*, 2005; Chang & Bolton, 1987; Huddleston *et al.*, 2006; Kruse & Sϕrum, 1994). Brown *et al.*(1997) investigated the plasmid content of both clinical and environmental *Aeromonas* spp. including *A. hydrophila* and *A. veronii* biovar *sobria* isolates, and observed a prevalence of plasmids in 40% and 12% of environmental and clinical *A. veronii* biovar *sobria* isolates, respectively. Thirteen and 11% of *A. hydrophila* strains

isolated from clinical and environmental settings possessed plasmids (Brown *et al.*, 1997). Radu *et al.* (2003) detected plasmids in 1.7%, 10% and 45% of food-borne *A. caviae, A. hydrophila* and *A. veronii* biovar *sobria*, respectively.

Plasmids have been detected in all 4 phenospecies of the *Aeromonas* genus, namely *A. hydrophila, A. salmonicida, A. sobria* and *A. caviae* (Boyd *et al.*, 2003; Brown *et al.*, 1997). Variations in the number and size of resident plasmids among the 4 phenotypes have been reported. Plasmids ranging from 2.3 – 15.7 kb were detected in nearly 60% of *Aeromonas* spp. isolated from fish aimed for human consumption in Malaysia (Radu *et al.*, 2003). A third of *A. hydrophila* strains isolated from cultured tilapia fish, showed the presence of detectable plasmids of 3 to 63.4 kb (Son *et al.*, 1997). Larger plasmids ranging from 85 to 140 kb have been isolated from *A. salmonicida* and *A. caviae* strains isolated from clinical settings, while small (5 kb) cryptic plasmids have also been found (Boyd *et al.*, 2003; Rhodes *et al.*, 2004; Stuber *et al.*, 2003b). Plasmids isolated from *A. salmonicida* subsp. *salmonicida* and atypical *A. salmonicida* strains displayed variation in the number and size of harboured plasmids, with a greater prevalence among atypical strains (Boyd *et al.*, 2003). Plasmids isolated from *A. salmonicida* subsp. *salmonicida* and atypical *A. salmonicida* showed variation with respect to the number (1 to 4) and sizes (52 to 105 MDa) of detected plasmids (Sørum *et al.*, 1993; Sørum *et al.*, 2001). Although some studies have examined the prevalence of plasmids amongst *Aeromonas* spp. isolates, limited information is available on the prevalence and diversity of plasmids amongst *Aeromonas* spp. isolated from South African aquaculture systems. The aim of this study was to investigate the incidence of plasmids among members of the genus *Aeromonas*, isolated from various fish species currently raised in aquaculture settings.

2.2. Materials and Methods

2.2.1. Bacterial strains and growth conditions

Thirty-seven *Aeromonas* spp. isolates were cultured from koi (*Cyprinus carpio*), trout (*Oncorhynchus mykiss*) and tilapia (*Oreochromis mossambicus*) fish farmed in

South African aquaculture systems. Two (AE1, AE3), 6 (AE2, AE4-AE8) and 29 (AE9-AE37) isolates were obtained from koi, trout and tilapia fish farmed in South African aquaculture systems, respectively (Jacobs & Chenia, 2006). *Aeromonas* spp. isolates were previously identified by the production of honey-yellow colonies on selective starch-ampicillin plates (SA), and assigned to species based on the *aroA* PCR-RFLP profiles (Table 2.1).

Table 2.1. Characterization of *Aeromonas* spp. isolates with respect to *aroA* PCR-RFLP species identification, fish host, presence of integrons and tetracycline resistance genes (Jacobs & Chenia, 2007).

Isolate	Fish Host	Identification	Integrase Type	Tet Determinant/s
AE1	Koi	*A. hydrophila* G2	Int 1	B+D/H
AE2	Trout	*A. hydrophila* G2	Int 1	A+B+D/H+E
AE3	Koi	*A. hydrophila* G2	Int2	B
AE4	Trout	*A. salmonicida*	Int 2	A
AE5	Trout	*A. hydrophila G2*	Int 3	-
AE6	Trout	*A. sobria* G1	-	A
AE7	Trout	*A. encheleia*	-	A
AE8	Trout	*A. sobria* G1	Int 3	-
AE9	Tilapia	*A. icthiosomia*	Int 2	A+E
AE10	Tilapia	*A. sobria* G2	Int1	A+E
AE11	Tilapia	*A. icthiosomia*	-	A+E
AE12	Tilapia	*A. sobria* G2	Int 1	A+E
AE13	Tilapia	*A. sobria* G2	Int 2 + 3	A+E
AE14	Tilapia	*A. encheleia*	Int 1	B+D/H
AE15	Tilapia	*A. encheleia*	-	A+E
AE16	Tilapia	*A. encheleia*	-	A+E
AE17	Tilapia	*A. sobria* G2	Int 1 + 2	A
AE18	Tilapia	*A. media*	Int 2	A
AE19	Tilapia	*A. sobria* G2	-	-
AE20	Tilapia	*A. hydrophila* G2	-	-
AE21	Tilapia	*A. sobria* G1	-	-
AE22	Tilapia	*A. sobria* G1	-	A
AE23	Tilapia	*A. sobria* G2	Int 1	A+E
AE24	Tilapia	*A. sobria* G1	Int 1 + 2	A
AE25	Tilapia	*A. sobria* G2	Int 1 + 2	A+E
AE26	Tilapia	*A. encheleia*	Int 1	-
AE27	Tilapia	*A. sobria* G1	Int 1 + 2	-
AE28	Tilapia	*A. ichtiosomia*	Int 1	-
AE29	Tilapia	*A. hydrophila* G2	Int 1	-
AE30	Tilapia	*A. hydrophila* G1	Int 1	-
AE31	Tilapia	*A. sobria* G1	Int 3	A+E
AE32	Tilapia	*A. salmonicida*	Int 1 + 2	-
AE33	Tilapia	*A. hydrophila* G1	Int 1	A+E
AE34	Tilapia	*A. hydrophila* G1	-	A+E
AE35	Tilapia	*A. hydrophila* G2	Int 1	A+E
AE36	Tilapia	*A. hydrophila* G1	Int 1	A+E
AE37	Tilapia	*A. sobria* G2	Int 1	A+E

Isolates were grouped into 6 species i.e. *A. hydrophila* (AE1, AE2, AE3, AE5, AE20, AE29, AE30, AE33, AE34, AE35, AE36), *A. sobria* (AE6, AE8, AE10, AE12, AE13, AE17, AE19, AE21, AE22, AE23, AE24, AE25, AE27, AE31, AE37), *A. media* (AE18), *A. salmonicida* (AE4, AE32), *A. encheleia* (AE7, AE14, AE15, AE16, AE26) and *A. ichtiosomia* (AE9, AE12, AE28). *A. hydrophila* and *A. sobria* isolates were divided into 2 groups, G1 and G2, based on the *aroA* PCR-RFLP profile variations (Jacobs & Chenia, 2006). Isolates have also been characterized with respect to their antimicrobial susceptibility patterns, presence of class 1, 2 and 3 integrons and the type of tetracycline (*tet*) resistance determinant (Table 2.1) (Jacobs & Chenia, 2006). The 37 *Aeromonas* spp. isolates were maintained on Luria Bertani (LA) agar plates (Biolab Diagnostics, Guateng, South Africa) at room temperature (21 °C ±2) under aerobic conditions. For long term storage, isolates were stored at -80°C in LB broth containing 20% glycerol.

2.2.2. Plasmid isolations

Plasmid DNA was isolated using the modified alkaline lysis protocol (Birnboim & Doly, 1979). Overnight LB cultures (5 ml) were harvested, washed and resuspended in 100 µl solution 1 (25 mM Tris HCl, 50 mM glucose, 10 mM EDTA, pH 8.0) and incubated at room temperature for 5 min. Cells were lysed by the addition of 200 µl solution 2 (0.2 N NaOH, 1% SDS) and incubated on ice for 5 min. Hundred and fifty microliters of solution 3 (3 M sodium acetate, pH 4.8) was added and chromosomal DNA, SDS-protein complexes and cell debris were removed through centrifugation. DNA was precipitated by the addition of 2 volumes of absolute ethanol and incubation at room temperature for 1 h. Precipitated DNA was collected through centrifugation at 12000 rpm for 10 min after which the pellet was resuspended in 200 µl TE buffer (10 mM TRIS HCl, 1 mM EDTA, pH 8.0). Four hundred microliters of absolute ethanol and 60 µl of solution 3 were added followed by a 1 h incubation period at room temperature. Plasmid DNA was pelleted by centrifugation and washed with 70% ethanol. Pellets were resuspended in 20 µl TE buffer and stored at -20 °C.

Plasmid DNA samples were subjected to flat-bed gel electrophoresis in 1% agarose gels (Hispanagar, Spain) for 2 hours at 60V. Ethidium-bromide staining followed to allow visualization using UV light and molecular size calculation was performed using UviPhotoMW software (UVItec, Cambridge, UK). A 30 kb plasmid, pUCBM21 in *E. coli* JM105 was included for control purposes, while phage lambda DNA digested with *Eco*RI and *Hin*dIII was used for sizing of plasmids.

2.2.3. Pulsed-field gel electrophoresis

Large plasmids (>50 kb) were isolated using a modified protocol described by Barton *et al.* (1995) and Gardner *et al.* (2001). Study isolates were grown overnight in LB broth (Biolab Diagnostics, Gauteng, South Africa). Harvested cells were washed twice in SET buffer (25% sucrose, 2 mM EDTA, 50 mM Tris, pH 8). The optical density at 600 nm was adjusted to 1.2 to ensure sufficient cell density. Bacterial cells were set in an equal volume of 2% low-melting point (LMP) agarose (Boehringer Mannheim, Germany). Proteinase K at a concentration of 1 mg/ml was added and agarose plugs incubated at 37 °C for 1 h. In order to lyse the cells, the plugs were incubated in ESP buffer (0.5 M EDTA [pH 8], 1% sodium lauryl sarcosine, 1 mg/ml proteinase K) for 16 h at 50 °C. This step was repeated, after which the proteinase K was inactivated by incubation of the plugs in TE buffer containing 5 mM Prefabloc (Roche Molecular Biochemicals, Germany) for 1 h. Plugs were when washed in TE buffer to remove any residual Prefabloc and incubated in TE buffer overnight. Soaking of the plugs in 10 mM Tris (pH 7.5) was followed by the digestion with S1 nuclease (Roche) in Barton buffer [50 mM NaCl, 30 mM sodium acetate (pH4.5), 5 mM $ZnSO_4$] at 37 °C for 45 min (Barton *et al.*, 1995). The reaction was stopped by soaking the plugs in ES solution (0.5 M EDTA [pH 8], 1% sodium lauryl sarcosine), followed by a final wash step in TE buffer.

Electrophoresis was performed using a GeneLine™ transverse alternating field electrophoresis system (Beckman Instruments, Inc, USA). DNA fragments were separated in a 1% agarose (Hispanagar) gels at 150 mA and 12 °C for 16 h with a pulse

interval of 13 s. For sizing of isolated plasmids, concatemeric phage lambda was included during gel electrophoresis.

2.3. Results

2.3.1. Detection of small plasmids through standard alkaline lysis

Seventeen of the 37 (46%) *Aeromonas* spp. isolates, originating from different fish species, harboured plasmids ranging from 1 to 35.0 kb (Fig. 2.1).

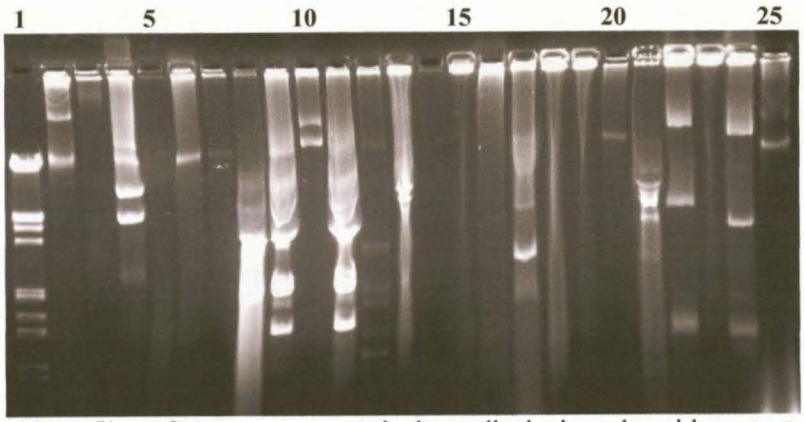

Figure 2.1. Plasmid profiles of *Aeromonas* spp. isolates displaying plasmid content diversity. Lane 1: lambda phage DNA digested with *Eco*RI and *Hin*dIII; lane 2: pUCBM21 (30 kb); lanes 3-25: AE1, AE2, AE3, AE4, AE8, A9, AE11, AE12, AE13, AE15, AE16, AE18, AE19, AE22, AE24, AE25, AE29, AE31, AE33, AE34, AE35, AE36, AE37. Multiple plasmids were observed for isolates AE2, AE9, AE11, AE12, AE13, AE15, AE16, AE24, AE33, AE34 and AE36, while no plasmids were detected for isolates AE1, AE18, AE19, AE22, AE29 and AE35.

Of the 17 plasmid-containing isolates, two belonged to the *A. hydrophila* G2 complex, three to the *A. hydrophila* G1 cluster, three to the *A. sobria* G1 complex, while four isolates belonged to the *A. sobria* G2 cluster (Table 2.2).

Table 2.2. The seventeen *Aeromonas* spp. isolates obtained from different aquacultural fish species harboring plasmids varying in size and number of plasmids present.

		Birnboim & Doly		Pulsed-field gel electrophoresis		
Isolate	**Species**	**Number of plasmids**	**Plasmid size (kb)**	**Number of plasmids**	**Plasmid size (kb)**	**Total plasmid content**
AE1	*A. hydrophila* G2	ND*	-	1	200	1
AE2	*A. hydrophila* G2	6	26,6; 26,2; 19,3; 12,1; 7,8; 4,7	3	100; 130; 150	9
AE3	*A. hydrophila* G2	4	26,2; 23.9; 20,6; 12,4	ND	-	4
AE4	*A. salmonicida*	4	25,6; 23.9; 12,3; 12,4	ND	-	4
AE8	*A. sobria* G1	1	23,5	ND	-	1
AE9	*A. icthiosomia*	9	23,5; 18,5; 14,4; 11,1; 9,3; 6,3; 5,0; 4,2; 1	ND	-	9
AE11	*A. icthiosomia*	9	23,5; 18,5; 14,4; 11,1; 9,3; 6,3; 5,0; 4,2; 1	ND	-	9
AE12	*A. sobria* G2	9	23,5; 18,5; 14,4; 11,1; 9,3; 6,3; 5,0; 4,2; 1	ND	-	9
AE13	*A. sobria* G2	9	23,5; 18,5; 14,4; 11,1; 9,3; 6,3; 5,0; 4,2; 1	ND	-	9
AE15	*A. encheleia*	2	35,0; 23,0	ND	-	2
AE16	*A. encheleia*	3	23,0; 7,1; 5,5	2	200; 150	5
AE19	*A. sobria* G2	ND	-	1	100	1
AE20	*A. hydrophila* G2	ND	-	1	75	1
AE24	*A. sobria* G1	1	22,6	ND	-	1
AE25	*A. sobria* G2	2	16,8; 4,9	ND	-	2
AE31	*A. sobria* G1	7	28,8; 23,0; 14.0; 12,8; 4,8; 1,9; 1,2	1	75	8
AE33	*A. hydrophila* G1	5	22,1; 10, 9; 7,0; 4,4; 3,5	2	100; 130	5
AE34	*A. hydrophila* G1	3	22,1; 7,0; 3,5;	ND	-	3
AE36	*A. hydrophila* G1	3	21,3; 14.0; 5,1	ND	-	3
AE37	*A. sobria* G2	1	26,6	ND	-	1

*ND - no plasmids detected using either the alkaline lysis (Birnboim & Doly, 1979) or S1 nuclease pulsed-field gel electrophoresis (Barton *et al.*, 1995) plasmid isolation methods.

Plasmids were also detected in two *A. icthiosomia* and two *A. encheleia* strains, respectively. Only one of the two *A. salmonicida* strains harboured plasmids detectable using the Birnboim and Doly method. Plasmids isolated from the study population showed variations with respect to the number and size of plasmids (Table 2.2). Single plasmids (23.5, 22.6 and 26.6 kb) were detected in 18% (3/17) of plasmid-containing isolates, i.e., two *A. sobria* G1 and one *A. sobria* G2 isolates, respectively. Multiple plasmids were detected in 14 (82%) of the plasmid-containing *Aeromonas* spp. isolates and included strains belonging to all six identified species (Tabel 2.2).

Isolates AE9 and AE11 (*A. icthiosomia*), and AE12 and AE13 (*A. sobria* G2) harboured a total of 9 plasmids, all of which were identical in size. Variations in the band intensities of isolated plasmids may be indicative of differences in copy number of the respective plasmids.

2.3.2. Detection of large plasmids

Large plasmids ranging from 75 to 200 kb in size were detected in seven of the 37 (19%) *Aeromonas* spp. isolates (Fig. 2.2 A-I and Table 2.2).

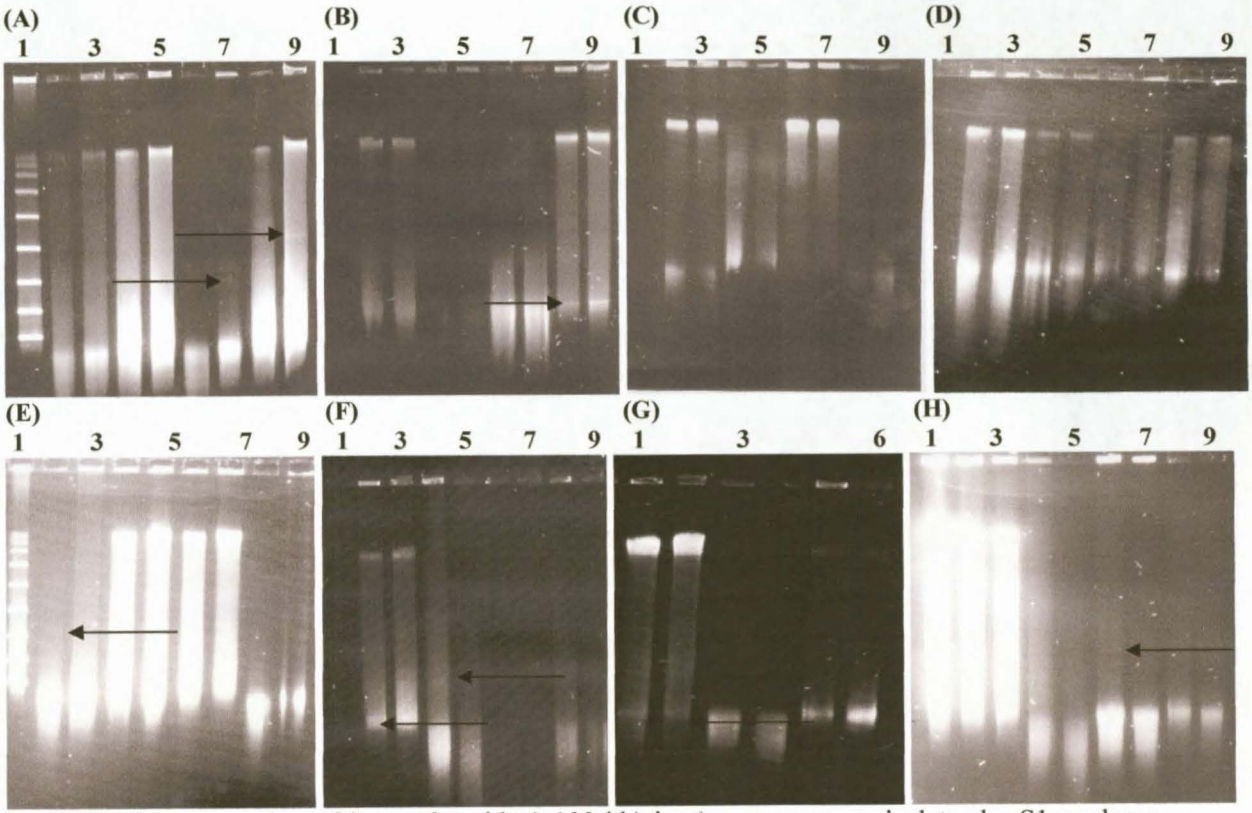

Figure 2.2. Detection of large plasmids (>100 kb) in *Aeromonas* spp. isolates by S1 nuclease restriction of agarose imbedded cells. Plasmids are indicated by the arrows. **(A)** Lane 1: concatemeric phage lambda DNA (Roche Molecular Biochemicals, Germany); lanes 2-9: AE5-S1, AE5, AE4-S1, AE4, AE2-S1, AE2, AE1-S1, AE1. **(B)** Lanes 2-9: AE35, AE35-S1, AE14, AE14-S1, AE36, AE36-S1, AE37, AE37-S1. **(C)** Lanes 2-9: AE21, AE21-S1, AE4, AE4-S1, AE30, AE30-S1, AE29, AE29-S1. **(D)** Lanes 2-9: AE22, AE22-S1, AE24, AE24-S1, AE25, AE25-S1, AE33, AE33-S1. **(E)** Lanes 1-9: concatemeric phage lambda DNA, AE19, AE19-S1, AE18, AE18-S1, AE13, AE13-S1, AE9, AE9-S1. **(F)** Lanes 2-9: AE20, AE20-S1, AE16, AE16-S1, AE11, AE11-S1, AE10, AE10-S1. **(G)** Lanes 1-6: AE31, AE31-S1, AE32, AE32-S1, AE27, AE27-S1. **(H)** Lanes 1-9: AE34, AE7, AE7-S1, AE28, AE28-S1, AE33, AE33-S1, AE26, AE26-S1.

Isolates AE2, AE16 and AE33 harboured multiple large plasmids, while four isolates (AE1, AE19, AE20, and AE31) contained only a single large plasmid (Fig. 2.2A, F and H). Isolates AE1, AE19, AE20 and AE31 contained single plasmids of 200 kb, 100 kb and 75 kb, respectively. No correlation between specific *Aeromonas* spp. and the presence of large detectable plasmids was evident as plasmids were detected in *A. hydrophila* G1 and G2, *A. sobria* G1 and G2, and *A. encheleia* isolates. Plasmid profiling using the S1-nuclease assay revealed plasmid diversity within the *Aeromonas* spp. isolates with respect to size as well as number of plasmids present. No specific plasmid profile appeared to be common among all the studied isolates.

2.4. Discussion

Aeromonas spp. are frequently isolated from aquatic environments and form part of the bacterial population present in aquaculture settings (Munro & Hastings, 1993; Robersts, 1993). Aquaculture enterprises suffer major losses due to the outbreak of infectious diseases caused by members of the *Aeromonadaceae* family. Worldwide, *Aeromonas* spp. have been isolated from various fish species and have emerged as important pathogens in both aquaculture environments and clinical settings (Hatha *et al.*, 2005). The primary pathogen, *A. salmonicida*, is the causative agent of furunculosis in salmonids, while *A. hydrophila, A. sobria* and *A. caviae* are responsible for secondary infections such as haemorrahagic septicaemia in compromised fish exposed to stress factors such as handling and high bacterial loads (Munro & Hastings, 1993; Robersts, 1993).

The incidence of plasmids among 46% of the study isolates is in agreement with that reported by Radu *et al.* (2003), who observed that 56.6% of isolated *Aeromonas* spp. harboured plasmids ranging from 2.3 to 15.7 kb in size. Cryptic plasmids (2.3 – 15 kb) which mediated no apparent advantages to the host, have also been identified among members of the *Aeromonadaceae* family (Brown *et al.*, 1997; Radu *et al.*, 2003). This correlates with the presence of small plasmids (1.9 – 14.4 kb) observed among 76.5% of the plasmid-containing *Aeromonas* spp. isolates. The prevalence of smaller-sized plasmids (2.3 – 63.4 kb) among members of the genus *Aeromonas* is reported to vary from a low frequency (16%) to a more significant prevalence of 100% (Brown *et al.*, 1997; Chang & Bolton, 1987; Majumdar *et al.*, 2006; Radu *et al.*, 2003; Son *et al.*, 1997). The prevalence plasmid ranging from 21.1 to 35 kb observed in 94.1% of the *Aeromonas* spp. isolates reported in this study is supported by the above-mentioned findings. The presence of plasmids ranging from 21 to 63.4 kb have been implicated in the virulence of pathogenic *Aeromonas* spp. isolates as well as the antibiotic resistance phenotypes displayed (Chang & Bolton, 1987; Majumdar *et al.*, 2006; Son *et al.*, 1997). Plasmids larger than 20 kb have been isolated from different *Aeromonas* spp., including *A. hydrophila, A. salmonicida* and *A. sobria* (L'Abee-Lund & Sϕrum, 2000; Majumdar *et al.*, 2006; Sϕrum *et al.*, 2003; Son *et al.*, 1997). The presence of detectable plasmids is

not species-specific, but a higher incidence of plasmids have been identified in *A. sobria* and *A. hydrophila* strains (Chang & Bolton, 1987; Radu *et al.*, 2003). In keeping with previous reports, 41% and 30% of the plasmid-containing *Aeromonas* spp. isolates were *A. sobria* and *A. hydrophila* strains, respectively.

The presence of multiple plasmids, displayed variation with respect to the number and size of detected plasmids, have been identified in *A. hydrophila*, *A. veronii* bv sobria, *A. salmonicida* and *A. caviae* strains obtained from both clinical and environmental compartments (Akinbowale *et al.*, 2006; Brown *et al.*, 1997; L'Abee-Lund & Sϕrum, 2002; Son *et al.*, 1997). In keeping with work done by the above-mentioned authors, multiple plasmids were also identified among different *Aeromonas* spp. isolates, although no correlation could be made due to the variations with respect to size and number. The occurrence of multiple plasmids among Gram-negative bacteria, frequently isolated from the aquacultural environment, have been reported for *Edwardsiella* spp., *Vibrio* spp. and *Flavobacterium* spp. (Akinbowale *et al.*, 2006). These isolated plasmids also displayed differences in terms of the number (1 – 7) and sizes (3 – 51 kb) of resident plasmids (Akinbowale *et al.*, 2006).

The two methods used to identify plasmids among the studied *Aeromonas* spp. isolates allowed the detection of plasmids ranging from 1 to 250 kb. The S1 nuclease assay proved to be powerful tool in accessing the true large plasmid content of the *Aeromonas* spp. isolates. The isolation of large plasmids (100 to 200 kb) from *A. hydrophila* G1 (AE33) is in keeping with previous reports of the presence of 140 and 160 kb plasmids in *A. salmonicida* subsp. *salmonicida* (Stuber *et al.*, 2003b). In the present study, larger plasmids were detected in two *A. hydrophila* isolates, which supports the finding of Son *et al.* (1997), who isolated 45.3 and 63.4 kb plasmids from *A. hydrophila* strains. The presence of two large plasmids in *A. encheleia* is the first report of this species harbouring any plasmids. This might be due to the fact that only clinically and environmentally important species such as *A. hydrophila* and *A. salmonicida* are investigated. The presence of multiple plasmids larger than 50 kb did not seem to be species-specific as these plasmids were isolated from *A. hydrophila* G1 and G2 complex, *A. encheleia*, and *A. sobria* G2 and G1 complexes species (Table 2.2). Isolates belonging

to the *A. hydrophila* G2 complex showed differences in terms of the number of large plasmids present in the cell, with isolates displaying both single and multiple large plasmids. When considering the presence of plasmids detected using both methods of isolation, no species-specific trends emerged.

The diversity of plasmid profiles indicated by the differences in the number of detectable plasmids, the different *Aeromonas* spp. and the different fish species from which the organisms were isolated from. Variation in plasmid content, i.e., number of detected plasmids, plasmid sizes and plasmid band intensities have been reported previously (Akinbowale *et al.*, 2006; Chang & Bolton, 1987; Majumdar *et al.*, 2006; Radu *et al.*, 2003; Son *et al.*, 1997). Both environmental and clinical *Aeromonas* spp. isolates exhibited differences in the number and size of resident plasmids (Brown *et al.*, 1997; Burr *et al.*, 2002; Stuber *et al.*, 2003b). The high level of plasmid diversity reported here is in correlation with the aboved-mentioned studies.

The plasmid content of the South African isolated *Aeromonas* spp. strains appeared to be diverse with respect to the number and size of detectable plasmids. No specific correlation could be made between the species and the presence of a particular plasmid profile, although identical plasmid profiles were observed for four isolates belonging to two different spp. The two plasmid isolation methods also proved to be useful in detecting both large and smaller plasmids. Further investigation into the presence of antibiotic resistance genes, possible plasmid-encoded virulence factors, heavy metal resistance genes and other mobile genetic elements together with the ability of these isolated plasmids to be transferred to other hosts is necessary to establish the importance of these plasmids in the acquisition of different genes and the role of plasmids in providing a competitive advantage to their hosts. Having identified the *Aeromonas* spp. isolates displaying diverse plasmid profiles; the role of these plasmids in antibiotic resistance was investigated. This entailed identifying the ability of plasmids to be transferred to alternative bacterial hosts, and to identify phenotypic characteristics endowed to the new host cell by plasmid acquisition.

Chapter 3

Characterization of plasmid-mediated antibiotic resistance genotypes in *Aeromonas* spp. isolates

3.1. Introduction

Aeromonas spp. causes diseases such as furunculosis and atypical furnculosis among different fish species which leads to large annual economic losses in the aquacultural setting (Munro & Hastings, 1993). Control of disease outbreaks in aquaculture is mainly through the use of antibiotics in animal feed or the addition of antimicrobial agents to the fish tanks. In the clinical setting, *Aeromonas* spp. are increasingly recognized as opportunistic human pathogens causing various infections, including gastroenteritis (Janda, 1991). Again, antibiotics play an important role in the treatment of such diseases.

The development of antimicrobial resistance among bacteria has been attributed to the use and misuse of antibiotics commonly used in human medicine and veterinary practices (Chopra & Roberts, 2001; Teuber, 2001). These antimicrobial agents include penicillins, cephalosporins, tetracyclines, chloramphenicols, aminoglycosides, streptinomycin, lincosamides, macrolides, sulphonamides, trimethoprim and quinolones (Prescott, 2000). This use and misuse of antibiotics in environmental settings has led to the increased occurrence of MDR *Aeromonas* spp. which are both fish pathogens and opportunistic human pathogens. An increase in single and multiple antimicrobial resistance phenotypes among members of this genus has been observed; including resistance to tetracycline, which is frequently used in aquaculture systems (Huys *et al.*, 2001; Miranda & Castillo, 1998). MDR phenotypes displayed by bacteria are often the result of the acquisition of resistance determinants present on mobile genetic elements such as conjugative plasmids in conjunction with transposons and integrons (Tennstedt *et al.*, 2003).

Plasmids have the ability to acquire antibiotic resistance determinants from a variety of sources and to constantly evolve new resistance genes as observed with the recent development of plasmid-mediated resistance to fluoroquinolones (Taylor *et al.*, 2004; Tran & Jacoby, 2002). In aquaculture systems, mobile genetic elements encoding antibiotic resistance genes, virulence factors, secretion system components and heavy metal resistance, have the capacity to be transferred to related and unrelated bacterial species (Huddleston *et al.*, 2006; Toranzo *et al.*, 1984).

R-plasmids, isolated from different *Aeromonas* spp. obtained from different environmental settings, are associated with the increased antibiotic resistance phenotypes observed in the genus. Chang and Bolton (1987) isolated a 110-MDa conjugative R-plasmid encoding resistance to β-lactam antibiotics, aminoglycosides and trimethoprim from a clinical *A. hydrophila* strain. Plasmids were also isolated from clinical *A. sobria* spp. isolates, but the resistance phenotype could not be transferred to a new host (Chang & Bolton, 1987). Two *A. hydrophila* and an *A. caviae* strain obtained from human faeces harboured plasmids of 3 and 15 kb in size, which transferred the tetracycline resistance phenotype to *E. coli* following transformation experiments (Palu *et al.*, 2006). Limited information regarding the prevalence of R-plasmids in clinical *Aeromonas* spp. exists since these organisms are only regarded as potential human pathogens.

The pathogenesis and increased MDR phenotypes displayed by environmental *Aeromonas* spp., however, has led to the investigation of the mobility of resistance determinants and virulence factors especially in the aquacultural setting. A diversity of R-plasmids encoding resistance to a number of different antibiotics and antimicrobial agents have been isolated from different fish species and geographical settings (Adams *et al.*, 1998; Bruun *et al.*, 2003; Casas *et al.*, 2005; Kruse & Sϕrum, 1994; L'Abee-Lund & Sϕrum, 2000; L'Abee-Lund & Sϕrum, 2002; Sandaa & Enger, 1994; Schmidt *et al.*, 2001b; Sϕrum *et al.*, 2003; Toranzo *et al.*, 1984). Furthermore, R-plasmids have been identified in both the primary fish pathogen, *A. salmonicida* as well as the secondary pathogen, *A. hydrophila* (Casas *et al.*, 2005; L'Abee-Lund & Sϕrum, 2000; L'Abee-Lund & Sϕrum, 2002; Majumdar *et al.*, 2006). A number of these R-plasmids have been found to carry other mobile genetic elements such as integrons and transposons (Rosser &

Young, 1999; Schmidt *et al.*, 2001a; Schmidt *et al.*, 2001b). Plasmid involved in virulence have also been identified in the fish pathogen *A. salmonicida* subsp. *salmonicida* (Stuber *et al.*, 2003b).

It is important to assess the antibiotic resistance profiles and plasmid content of *Aeromonas* spp. isolated from fish species cultured in aquaculture systems, since the fish are ultimately aimed for human consumption. Although the presence of plasmids and the displayed antibiotic resistance and/or virulence phenotype do not always coincide, the possibility that these plasmids could acquire resistance genes exists (Brown *et al.*, 1997; Messi *et al.*, 2004; Radu *et al.*, 2003). Environmental bacteria are a common reservoir of resistance genes, facilitating the transfer of resistance genes among related and unrelated organisms. The presence of potential bacterial pathogens displaying a MDR phenotype in different environmental compartments is a major threat not only in regards to the outbreak of infectious diseases, but also the spread of R-plasmids between occupants of a specific environment and their movement between other environmental settings. In the present study, 46% of the *Aeromonas* spp. isolates from a diversity of aquacultural fish species carried single or multiple plasmids. In order to investigate the plasmid diversity and the antibiotic resistance phenotypes they confer, plasmids were transformed into *E. coli* DH5α. The antibiotic resistance gene content was investigated by PCR assays and Southern hybridization experiments.

3.2. Materials and Methods

3.2.1. Transformation of isolated *Aeromonas* plasmids

Plasmids detected using the alkaline lysis method (Chapter 2, section 2.3.1.) were transformed into plasmid-free *Escherichia coli* DH5α cells (Table 3.1). Competence of the DH5α cells was increased through the addition of $CaCl_2$ (Ausubel *et al.*, 1998). Plasmid DNA was added to 100 µl of competent DH5α cells and the mixture was incubated on ice for 30 min. Heat shock at 42 °C for 1 min and incubation on ice for 15 min followed. Cells were allowed to recover in 1 ml LB broth at 37 °C for approximately

1 h. Tetracycline (30 µg/ml), ampicillin (100 µg/ml) and/or chloramphenicol (50 µg/ml), respectively, were incorporated into LA plates and used as the selective pressure to screen for positive transformants. On average, 32 colonies were selected and subjected to plasmid isolations to identify positive transformants.

Table 3.1. Bacterial strains used for general transformations and conjugation experiments.

Bacterial strains	Description	Reference or source
Strains		
DH5α	F⁻ø80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1*hsd*R17 (r_K^-, m_K^+) *pho*A *sup*E44 λ⁻*thi*-1 *rel*A1, Nal^S	Promega Corp, Madison

3.2.2. Antibiotic susceptibility testing of transformants

Transformants were subjected to susceptibility testing against 17 different antibiotics using the disk diffusion method (Bauer *et al.*, 1966). The following MASTDISCS™ (Mast Diagnostics, UK) antibiotic disks were used: amikacin (30 µg), amoxycillin (10 µg), ampicillin (30 µg), tetracycline (30 µg), nalidixic acid (30 µg), erythromycin (15 µg), augmentin (30 µg), gentamicin (10 µg), imipenem (10 µg), trimethoprim (1.25 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), cefuroxime (30 µg), sulphamethoxazole (25 µg), ofloxacin (5 µg), chloramphenicol (30 µg), azithromycin (15 µg), ceftazidime (30 µg), cefoxitin (30 µg) and piperacillin-tazobactam (110 µg) Overnight cultures of the transformants were standardized equivalent to a 0.5 McFarland standard and streaked onto Mueller-Hinton (MH) (Biolab Diagnostics) agar plates using sterile swabs, after which three antibiotic discs were placed on the agar at equal distances from each other. Plasmid-free *E. coli* DH5α was used as a control to establish the transferred resistance phenotype. Plates were incubated at 26 °C for 18-24 h. Inhibitory zone diameters were measured and resistance profiles (resistant, intermediate, and/or susceptible) assigned according to NCCLS breakpoints (NCCLS, 2002).

MAR index values (a/b, where 'a' represents the number of antibiotics the isolate was resistant to and 'b' represent the total number of antibiotics the isolate was tested against) were calculated for all transformants. A MAR index value of higher than 0.2

suggested that the isolate had been exposed to high-risk sources of contamination originating from humans or animals where antibiotics are often used. When antibiotic use was rare, a MAR index value less than or equal to 0.2 was observed (Krumperman, 1985). Resistance profiles and MAR indices of transformants were compared to wild-type *Aeromonas* spp. isolates to determine the percentage of antibiotic resistance determinants transferred by the plasmids.

3.2.3. Southern Blotting

3.2.3.1. DNA transfer and hybridization

To establish if the antibiotic resistance genes identified by Jacobs and Chenia (2007) were plasmid-located, total DNA preparations digested with *Hin*dIII and the total plasmid content of the 17 plasmid-containing *Aeromonas* spp. isolates were separated by agarose gel electrophoresis. Additionally, to investigate the presence of these resistance genes on the transformed plasmids, plasmid DNA was isolated from 23 *E. coli* DH5α transformants containing *Aeromonas* spp. plasmids (Birnboim & Doly, 1979) and separated on 1% agarose gels by electrophoresis. In both instances, DNA was transferred to Hybond-N^+ membranes (Amersham Biosciences) by overnight capillary blotting using 20 × SSC (3 M NaCl, 75 mM sodium citrate, pH 7) (Ausubel *et al.*, 1998). Following overnight transfer, membranes were briefly dried, subjected to UV cross-linking for 3 – 5 min and stored at 4 °C until further use. Prior to hybridization experiments, membranes were briefly rinsed in 2 × SSC (1 × SSC in 0.15 M NaCl plus 0.015 M sodium citrate, pH 7). For pre-hybridization, membranes were placed in DIG-EASY HYB (Roche Diagnostics) for a minimum of 1 h at 42 °C. Digoxigenin (DIG)-labeled probes were denatured for 10 min at 100 °C and added to the pre-hybridization solution. Hybridization was allowed to take place for 16 h at 42 °C (high-stringency) or 37°C (low-stringency). After hybridization, membranes were washed twice, for 5 min at room temperature in low-stringency wash solution (2 × SSC containing 0.1% [wt/vol] SDS). This was followed by two high-stringency washes (0.5 × SSC containing 0.1% SDS) for 15 min each at 65 °C.

3.2.3.2. Construction of probes

DIG-labeled probes were prepared by PCR amplifications and endonuclease restrictions of plasmid DNA, respectively. Prior to all labeling reactions, DNA fragments were purified with the GFX^{TM}PCR DNA and gel band purification kit (Amersham Biosciences) to remove any residual contaminants.

3.2.3.2.1. Tetracycline resistance determinant, TetA gene

A 293-bp tetracycline resistance gene (*tetA*) probe was obtained by PCR amplification of the transformed plasmid, p37T1, isolated from *Aeromonas* spp. isolate, AE37, using the TetABC primer set described by Schnabel and Jones (1999) (Table 3.2). *Hae*III digests of the PCR product did not yield the predicted 130 bp and 75 bp fragments corresponding to the *tetA* resistance determinant (Jacobs & Chenia, 2007; Schnabel & Jones, 1999). Jacobs and Chenia (2007) confirmed the presence of *tetA* by PCR amplification with the primer sets and subsequent restriction digests described by Furushita *et al.* (2003).

Table 3.2. Primers used for detection of specific genes used as probes during Southern blotting experiments.

Gene	Primer	Sequence	Size (bp)	Reference
Integrase 1 (*int*1)	IntA IntB	ATC ATC GTC GTA GAG ACG TCG G GTC AAG GTT CTG GAC CAG TTG C	892	(Reyes *et al.*, 2003)
Tn*1721*	TAF TetAR3	GTA ATT CTG AGC ACT GTC GC GGC ATA GGC CTA TCG TTT CCA	1200	(Pezzella *et al.*, 2004)
TetABC	Tet1-F Tet1-R	GCY RTV GGS ATH GGC YTK RTY ATG C ACM GCM CCW GTV GCB CCK GTG AT	293	(Schnabel & Jones, 1999)
TetDEH	Tet2-F Tet2-R	GCB ATK GGD MTY GGB MTN ATY ATG C ACV GCD CCD GTB GCR CCN GTR AT	239	(Schnabel & Jones, 1999)
bla-TEM	TEM-F TEM-R	AAG AAA CGC TAC TCG CCT GC CCA CTC AAC CCA TCC TAC CC	503	(Bert *et al.*, 2002)

The 25 µl PCR reaction mixtures included 100 ng template plasmid DNA, 200 µM of each dNTP (Roche), 50 pmol of each primer, 1 mM $MgCl_2$ and 1 U Super-Therm *Taq* DNA polymerase (JMR Holdings, Kent, UK) together with 1 × reaction buffer. PCR cycling parameters consisted of 35 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min in a PCRSprint thermal cycler (Hybaid). An initial denaturing step at 94 °C for 5 min and an elongation step at 72 °C for 10 min were included to ensure sufficient denaturation and elongation, respectively. Five µl of the PCR reaction mixture was subjected to electrophoresis in a 1% agarose gel, stained in ethidium bromide and visualized by UV transillumination. The O'GeneRuler™ 100 bp DNA Ladder (Fermentas, Canada) was included for accurate sizing of amplicons during all PCR reactions, as well as fragment sizes being calculated by UviPhotoMW software.

3.2.3.2.2. Tetracycline-associated transposon Tn1721

Screening of the *Aeromonas* spp. isolates indicated a high prevalence of the *tetA* resistance gene (Jacobs & Chenia, 2007), which is frequently associated with Tn*1721* amongst *Aeromonas* spp. (Rhodes *et al.*, 2000). To investigate the prevalence of Tn*1721*, a DNA probe consisting of the truncated version of Tn*1721*, lacking the left flanking region was constructed. The 1200 bp Tn*1721* probe was obtained by PCR amplification from plasmid p37T1 using the primer set described by Pezzella *et al.* (2004) (Table 3.2). PCR reactions mixtures were as described previously in section 3.2.3.2.1. PCR cycling parameters consisted of 35 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1.5 min in a PCRSprint thermal cycler (Hybaid). An initial denaturing step at 94 °C for 2 min and an elongation step at 72 °C for 10 min were included to ensure sufficient denaturation and elongation, respectively. Fragments were separated, visualized, sized and documented as described previously in section 3.2.3.2.1.

3.2.3.2.3. Class 1 integrase gene

The 892 bp class 1 integrase gene used as probe during DNA-DNA hybridizations was generated by PCR using the *Int*A-*Int*B primer set (Table 3.2) (Reyes *et al.*, 2003). Genomic DNA isolated from *Aeromonas* spp. isolate, AE37, was shown to contain a class 1 integron and was used as template for the PCR reactions (Jacobs & Chenia, 2007). PCR reaction mixtures were prepared as described previously in section 3.2.3.2.1. PCR cycling parameters consisted of 35 cycles of 94 °C for 30 s, 58 °C for 1 min and 72 °C for 1.5 min in a PCRSprint thermal cycler (Hybaid). As previously mentioned, an initial denaturing step at 94 °C for 3 min and an elongation step at 72 °C for 10 min were included to ensure sufficient denaturation and elongation, respectively. Fragments were separated, visualized, sized and documented as described in section 3.2.3.2.

3.2.3.2.4. β-lactamase (TEM) gene

The 506 bp TEM gene encoding β-lactam resistance was obtained by PCR amplification of genomic DNA isolated from *Aeromonas* spp. isolate AE37 using primers designed by Bert *et al.* (2002) (Table 3.2). PCR reaction mixtures were prepared as described previously in section 3.2.3.2.1. PCR cycling parameters consisted of 35 cycles of 94°C for 30 s; 55°C for 30 s and 72°C for 30 s in a PCRSprint thermal cycler (Hybaid). An initial denaturing step at 94°C for 3 min and an elongation step at 72°C for 10 min were included to ensure sufficient denaturation and elongation, respectively. Fragments were separated, visualized, sized and documented as described in section 3.2.3.2.

3.2.3.2.5. strA-strB gene

The linked streptomycin resistance determinant, *strA-strB* (1721 bp) present on the IncQ plasmid RSF1010 (Fig. 3.1) was obtained following double digests with restriction endonucleases *Eco*RI and *Eco*RV (Roche, Germany; Fermentas, Canada) at 37 °C for 3 h. Twenty µl of the restriction mixture was subjected to electrophoresis in a 1%

gel after which the required 1712 bp DNA band was excised and subsequently purified as described previously in section 3.2.3.2.

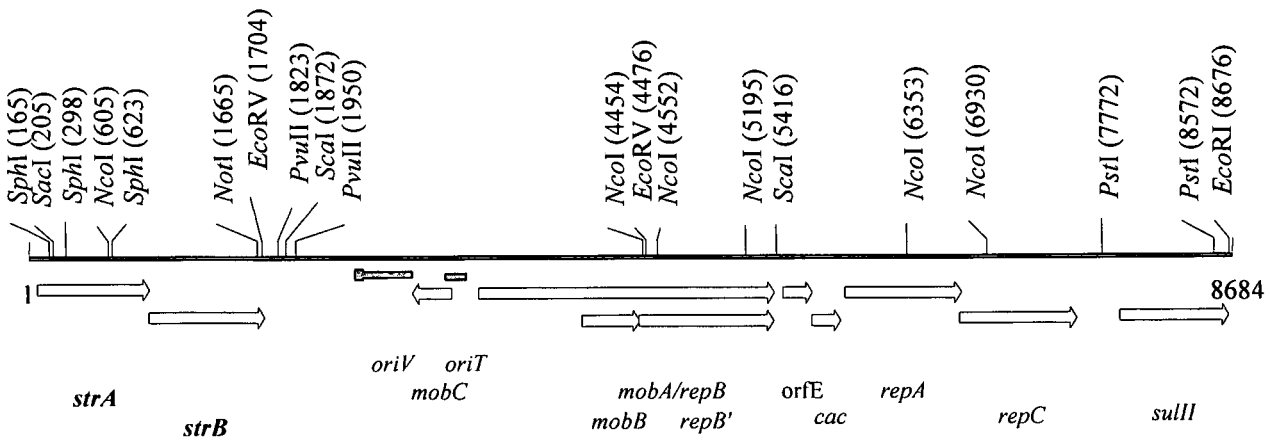

Figure 3.1. Restriction map of the IncQ plasmid, RSF1010, depicting the positions at which restriction enzymes *Eco*RI and *Eco*RV cleave the plasmid for generation of the *strA-strB* probe.

3.2.3.3. Labeling of probes

The respective purified PCR products (*tetA*, Tn*1721*, *intI*, *bla*-TEM) and fragments (*strA-strB*) together with double-distilled water to a final volume of 16 µl were added to an eppendorf tube. DNA was denatured by boiling in a water bath for 10 min and snap-cooled in a beaker of ice and ethanol. Two µl 10 × hexanucleotide primer mix (Roche), 2 µl 10 x dNTP's labeling mix (Roche, Germany) and 1 µl Klenow fragment (Roche) were added on ice. DNA was labeled overnight at 37 °C after which the reaction was stopped by boiling the labeling mixture for 10 min.

3.2.3.4. Detection

Following hybridization and stringency washes, membrane were briefly rinsed in washing buffer [maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), 0.3% (v/v) Tween 20] and incubated for 30 min in blocking solution (10 g non-fat milk powder dissolved in 190 ml maleic acid buffer). Membranes were incubated in antibody solution

(dilute anti-digoxigenin-AP 1:10 000 [75mU/ml] in blocking solution) for 30 min, followed by 2 washing steps (15 min each) in washing buffer. Membranes were equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 5 min. Membranes were placed DNA side facing up on a hybridization bag and 500 µl CSPD (Roche Biochemicals, Germany) was applied to the membrane. The second sheet of the hybridization bag was used to cover the membrane and to evenly distribute the CSPD without the formation of air bubbles. After 5 min at room temperature, excess liquid was squeezed out and the hybridization bag sealed. Membranes were exposed to X-ray film for 1 – 2 h. All detection steps were performed at room temperature.

3.2.3.5. Stripping and reprobing of DNA blots

Membranes were thoroughly rinsed in double distilled water, followed by two wash steps at 37 °C in 0.2 M NaOH containing 0.1% SDS for 15 min each to remove the DIG-labeled probe. Membranes were then rinsed in 2 × SSC. After the membranes were stripped, they were stored in maleic acid buffer or 2 × SSC at 4 °C until used again. Pre-hybridization and hybridization followed as described in section 3.2.3.1.

3.2.4. Analysis of plasmid-mediated antibiotic resistance genes by PCR amplification

To confirm Southern hybridization results, 23 transformed *Aeromonas* spp. plasmids were screened for the presence of the above-mentioned antibiotic resistance genes by PCR amplifications using reaction mixtures described in section 3.2.3.2.1. and primer sets described in Table 3.2.

3.3. Results

3.3.1. Replication in *E. coli* DH5α

A total of 23 plasmids, ranging in size from 1 – 27 kb, present in 17 plasmid-containing *Aeromonas* spp. isolates were successfully transformed into *E. coli* DH5α and were capable of replication in the new host (Fig. 3.2 and Table 3.3).

Table 3.3. Characterization of transformants with respect to the number and molecular weight of detected plasmids.

***Aeromonas* spp. isolates**	**Plasmid code**	**Number of plasmids**	**Sizes (kb)**
AE2	p2T1	1	26.2
AE2	p2T2	1	26.2
AE3	p3A5	1	23.9
AE4	p4T1	1	25.6
AE4	p4C1	1	23.9
AE8	p8C5	1	23.5
AE9	p9C1	1	23.5
AE11	p11T1	2	23.5 ; 5.0
AE11	p11T14	3	23.5 ; 6.8 ; 1
AE12	p12T1	2	18.5 ; 5.0
AE13	p13T1	2	23,5 ; 6.8
AE13	p13C1	1	23.5
AE15	p15C1	1	23.0
AE16	p16C1	1	23.0
AE24	p24C1	1	22.6
AE25	p25T1	2	16.8 ; 4.9
AE31	p31T1	2	28.8 ; 14.0
AE31	p31T2	1	14.0
AE31	p31T5	4	14.0 ; 4.8 ; 1.9 ; 1.2
AE33	p33C1	1	22.1
AE34	p34C2	1	22.1
AE36	p36T2	2	14.0 ; 5.1
AE37	p37T1	1	26.6

Of these, 60% (14/23) were transformed as single plasmids, while multiple plasmids from eight isolates (p11T1, p11T14, p12T1, p13T1, p25T1, p31T1, p31T5 and p36T2) were co-transferred (Fig. 3.2). The transfer of multiple plasmids is indicative of differences in the plasmid replicons that allow the co-transfer and residence of plasmids. For selection purposes, the concentrations of tetracycline, ampicillin and chloramphenicol were increased to 30 µg/ml, 100 µg/ml and µg/ml, respectively. Of the plasmids transformed, 56.5% (13/23) and 39.1% (9/23) were obtained by tetracycline and chloramphenicol selection, respectively. The use of ampicillin as a selective pressure did not prove useful, since only 4.3% (1/23) of the positive transformants showed resistance to this antibiotic.

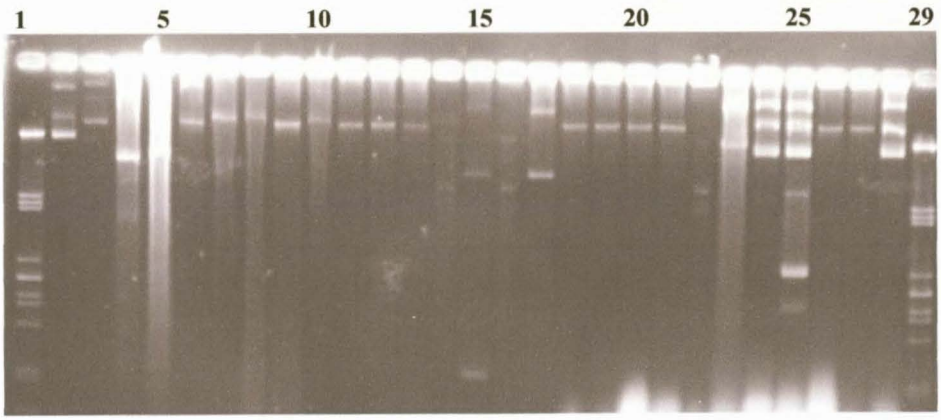

Figure 3.2. Plasmid DNA profiles of tetracycline-, ampicillin- and chloramphenicol-resistant *E. coli* transformants. Lanes 1 and 29 : *Eco*RI and *Hin*dIII digest of λ DNA ; lanes 2-28 : pTC-F14, pTF-FC2, pRAS3.1, pRAS3.2, p37T1, p2T1, p2T2, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13T1, p13C1, p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2.

3.3.2. Plasmid-mediated antibiotic resistance phenotypes of transformants

The dominant resistance phenotype transferred by plasmids isolated from the *Aeromonas* spp. isolates to *E. coli* DH5α was resistance to ampicillin, amoxycillin, augmentin, cefuroxime and ceftriaxone. Forty-three percent of the transferred plasmids displayed this resistance phenotype (Table 3.4). Only a single plasmid, p37T1 (isolated

from AE37), conferred resistance to the carbapenem, imipenem, to the *E. coli* host (Fig. 3.3).

Only transformants displaying inhibition by clavulanic acid (resistance to augmentin) and resistance to cefuroxime and/or ceftriaxone were subjected to testing against extended-spectrum-β-lactams such as ceftazidime, cefoxitin and piperacillin-tazobactam. Ten plasmids (p4T1, p4C1, p9C1, p12T1, p13T1, p13C1, p16C1, p24C1, p25T1 and p36T2), which displayed this resistance phenotype were tested for extended-spectrum-β-lactamase (ESβL) activity. Four transformants (40%) harbouring plasmids p4C1, p9C1, p16C1 and p24C1, encoded resistance to cefoxitin, while none of the transformants displayed plasmid-mediated resistance to piperacillin-tazobactam and ceftazidime (Fig. 3.3).

Sixty percent of the transformants which were susceptible to cefoxitin and resistant to penicillins and 2nd and 3rd generation cephalosporins indicated the presence of a plasmid-mediated ESβL. The remaining 40% of the transformed plasmids which conferred resistance to cefoxitin may harbour a possible *ampC* gene. All of the *E. coli* transformants displayed resistance to oxacillin which is the result of the intrinsic resistance phenotype of *E. coli* DH5α (Table 3.4).

Table 3.4. Comparison of the resistance profiles of wild-type *Aeromonas* spp. isolates with respect to the resistance profiles exhibited by the *E. coli* DH5α transformed with *Aeromor* spp. plasmids.

Wild-type *Aeromonas* spp. isolates	Resistance profile of wild-type isolate	MAR index * value of wild-type isolate	Plasmids transferred (plasmid size in kb)	Resistance profile of transformants	MAR index * value of transformants
AE2	AP, A, AUG, OX, E, ATH, T[#]	0.41	p2T2 (26.2)	AP, A, AUG, OX, ATH, E, T	0.41
			p2T1 (26.2)	AP, A, AUG, CRO, CXM, OX, E, T, C, TS	0.59
AE3	AP, A, AUG, CXM, OX, E, ATH, T[#]	0.47	p3A5 (23.9)	A, AP, AUG, CRO, CXM, OX, C, E, ATH, TS, NA	0.65
AE4	AP, A, AUG, CXM, CRO, FOX, OX, E, ATH, C [∂]	0.50	p4T1 (25.6)	OX, T, E	0.15
			p4C1 (23.9)	AP, A, AUG, CRO, CXM, OX, FOX, TS, E, C, NA	0.55
AE8	AP, A, AUG, OX, E [#]	0.29	p8C5 (23.5)	AP, A, AUG, CRO, CXM, OX, TS, E, C, NA,	0.59
AE9	AP, A, AUG, OX, FOX, NA, T [∂]	0.35	p9C1 (23.5)	AP, A, AUG, CRO, CXM, OX, TZP, C, TS, E, ATH, NA	0.60
AE11	AP, A, AUG, OX, NA, T [#]	0.35	p11T1 (23.5 ; 5)	A, OX, T, E, NA	0.29
			p11T14 (23.5 ; 6.8 ; 1)	A, OX, T, E, NA	0.29
AE12	AP, A, AUG, OX, NA, T, E [∂]	0.35	p12T1 (18.5 ; 5)	A, OX, T, E, NA	0.25
AE13	AP, A, AUG, OX, NA, T [∂]	0.30	p13C1 (23.5)	AP, A, AUG, CRO, CXM, OX, C, TS, E, NA,	0.50
			p13T1 (23.5 ; 6.8)	A, OX, T, E, NA	0.25
AE15	AP, A, AUG, OX, NA, T [#]	0.35	p15C1 (23)	AP, A, AUG, CRO, CXM, OX, C, TS, E, NA, T	0.65
AE16	AP, A, AUG, FOX, OX, T [#]	0.30	p16C1 (23)	AP, A, AUG, CRO, CXM, OX, FOX, C, TS, E, NA, T	0.60
AE24	AP, A, AUG, FOX, OX, NA, E, T [∂]	0.40	p24C1 (22.6)	AP, A, AUG, CRO, CXM, OX, FOX, C, TS, E, OFX, NA, T	0.65
AE25	AP, A, AUG, OX, FOX, T [∂]	0.30	p25T1 (16.8 ; 4.9)	AP, A, ATH, CRO, CXM, OX, C, E, NA, T	0.50
AE31	AP, A, AUG, OX, CIP, E, TS, T [#]	0.47	p31T1 (28.8 ; 14)	OX, T, E, NA	0.24
			p31T2 (14)	OX, T, E, NA	0.24
			p31T5 (14 ; 4.8 ; 1.9 ; 1.2)	OX, T, E, NA	0.24
AE33	AP, A, AUG, OX, TS, NA, T [#]	0.41	p33C1 (22.1)	AP, A, AUG, CRO, CXM, OX, C, E, NA, TS, T,	0.65
AE34	AP, A, AUG, OX, E, NA, T[#]	0.41	p34C2 (22.1)	AP, A, AUG, CRO, CXM, OX, TS, E, C, OFX, NA,	0.65
AE36	AP, A, AUG, OX, FOX, E, NA, T [∂]	0.40	p36T2 (14 ; 5.1)	OX, T, E, NA	0.20
AE37	AP, A, AUG, OX, E, C, TS, NA, T, W, SXT [◇]	0.58	p37T1 (26.6)	A, AP, AUG, CRO, CXM, OX, IMI, E, T, NA, GM, AK, W, SXT	0.68
DH5α	OX[∂]	0.05	Plasmid-free	-	-

Antibiotic abbreviations: (A) amoxycillin, (AP) ampicillin, (AUG) augmentin, (CXM) cefuroxime, (CRO) ceftriaxone, (FOX) cefoxitin, (TZP) piperacillin-tazobactam, (IMI) imipenem, (E) erythromycin, (ATH) azithromycin, (C) chloramphenicol, (T) tetracycline, (STX) sulphamethoxazole, (TS) cotrimoxazole, (NA) nalidixic acid, (CIP) ciprofloxacin, (OFX) ofloxacin, (GM) gentamicin, (AK) amikacin, (W) trimethoprim

* MAR index (a/b), where 'a' represent the number of antibiotics the isolates was resistant to and 'b' the total number of antibiotics the isolate was tested against

[#] Tested against 17 antibiotics; [◇] Tested against 19 antibiotics; [∂] Tested against 20 antibiotics

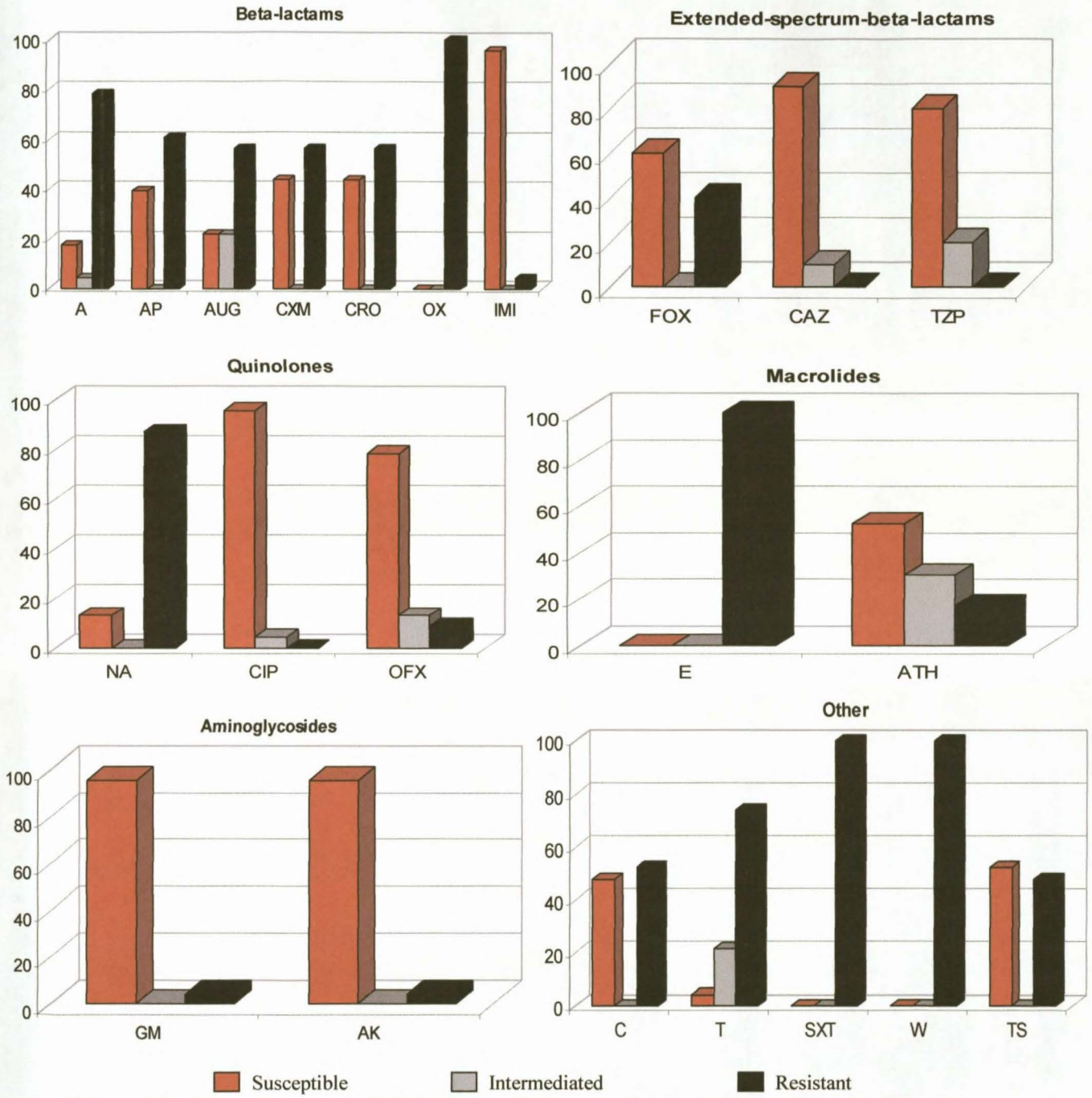

Figure 3.3. Percentage susceptibility, intermediate and resistant phenotypes displayed by the transformants. The X-axis indicates the type of antibiotic, while the Y-axis represents the percentage of the phenotype displayed. Antibiotic abbreviations: (A) amoxycillin, (AP) ampicillin, (AUG) augmentin, (CXM) cefuroxime, (CRO) ceftriaxone, (OX) oxacillin, (IMI) imipenem, (FOX) cefoxitin, (CAZ) ceftazidime, (TZP) piperacillin-tazobactam, (NA) nalidixic acid, (CIP) ciprofloxacin, (OFX) ofloxacin, (E) erythromycin, (ATH) azithromycin, (GM) gentamicin, (AK) amikacin (C) chloramphenicol, (T) tetracycline, (STX) sulphamethoxazole, (W) trimethoprim, (TS) cotrimoxazole.

Resistance to quinolone compounds such as ciprofloxacin, ofloxacin and nalidixic acid following transformation was varied. Resistance to nalidixic acid and ofloxacin was transferred by 87% and 8.7% of plasmids, respectively, while resistance to ciprofloxacin was shown to be chromosomally-encoded and not transferred to the new host (Table 3.5). An interesting pattern was also observed with respect to the quinolone antibiotics. Four of the 23 transformed plasmids (17.4%) did not confer resistance to nalidixic acid, i.e., p2T1, p2T2, p4T1 and p37T1, while 34.8% of the transformed plasmids conferred resistance to nalidixic acid only, i.e., p11T1, p11T14, p12T1, p13T1, p31T1, p31T2, p31T5 and p36T2. Decreased susceptibility (decreased zone diameters) to the 2nd and 3rd generation quinolones, ofloxacin and ciprofloxacin, was observed in 26.1% and 21.7% of the transformed plasmids, respectively (data not shown). Plasmids that displayed a decreased susceptibility to ofloxacin and resistance to nalidixic acid included p3A5, p9C1, p15C1, p25T1, p33C1 and p34C2, while plasmids p4C1, p8C5, p13C1, p16C1 and p24C1 displayed a decreased susceptibility to ciprofloxacin and resistance to nalidixic acid.

Resistance to the macrolide, erythromycin, was transferred by 100% of the transformed plasmids, while resistance to azithromycin was less frequent and was only displayed following the transformation with four *Aeromonas* plasmids, i.e., p2T2, p3A5, p9C1 and p25T1 (Fig. 3.3 and Table 3.4). Aminoglycoside resistance did not appear to be plasmid-mediated with only plasmid, p37T1 conferring resistance to amikacin and gentamicin to its new host (Fig. 3.3 and Table 3.4). Resistance to tetracycline and chloramphenicol was observed in 73.9% and 52.2% of the transformants, respectively (Fig. 3.3 and Table 3.4).

Resistance to cotrimoxazole appeared to be plasmid-mediated in 52.2% of the transformants, i.e., p2T1, p3A5, p4C1, p8C5, p9C1, p13C1, p15C1, p16C1, p24C1, p33C1, p34C2 and p37T1 (Table 3.4). Plasmid p37T1 transferred resistance to trimethoprim and sulphamethoxazole which are usually combined to form cotrimoxazole. The transferred resistance to cotrimoxazole may indicate the presence of possible integrons structures since the conserved 3'-end, encoding the *sulI* gene and a trimethoprim resistance gene (*dhfr*), confer resistance to this compound.

Antibiotic groupings displayed by wild-type plasmid-containing *Aeromonas* spp. isolates, comprised of 6 groups with resistance to ampicillin, amoxycillin, augmentin and oxacillin being displayed by all isolates forming the major group. In comparison, transformants displayed resistance to a wider range of antibiotics (Table 3.4). Two major groups were apparent. The first group displayed resistance to amoxycillin, ampicillin, augmentin, cefuroxime, ceftriaxone, erythromycin, chloramphenicol, cotrimoxazole, nalidixic acid, tetracycline and/or cefoxitin, and included transformants harbouring plasmids p3A5, p4C1, p8C5, p13C1, p15C1, p16C1, p33C1 and p34C2. Resistance to erythromycin, tetracycline and nalidixic acid, displayed by transformants containing plasmids p11T1, p11T14, p31T1, p31T2, p31T5 and p36T2, formed the second group. The co-transfer of specific resistance phenotypes was observed among all transformants and a number of trends were observed (Table 3.5).

The transformant MAR index values ranged from 0.15 - 0.68 (Table 3.4.). Of the 95.7% (22/23) of the transformants displaying index values of > 0.2, 56.5% (13/23) of the transformants had MAR indices > 0.5. MAR indices of > 0.6 were recorded for 34.8% (8/23) of the transformants. In comparison, wild-type *Aeromonas* spp. MAR indices ranged from 0.29 – 0.58. Although similar resistance profiles were displayed by p31T1, p31T2, p31T5 and p36T2, differences in the MAR indices were due to the variation in the number of antibiotics against which susceptibility was tested, i.e., additional cephalosporins tested.

Table 3.5. Grouping of transformed *Aeromonas* spp. plasmids based on resistance phenotypes transferred to *E. coli* DH5α.

Resistance phenotype transferred	% transformants displaying resistance phenotype	*Aeromonas* plasmids transferred
AP, T, E, NA	17.4%	p11T1, p11T14, p12T1, p13T1
T, E, NA	17.4%	p31T1, p31T2, p31T5, p36T2
A, AP, AUG, CRO, CXM, C, TS, NA	34.8%	p3A5, p4C1, p8C5, p9C1, p13C1, p15C1, p33C1, p34C2
AP, A, AUG, CRO, CXM, T	17.4%	p2T1, p15C1, p33C1, p37T1
TS, C	47.8%	p2T1, p3A5, p4C1, p8C5, p9C1, p13C1, p15C1, p16C1, p24C1, p33C1, p34C2
E, NA	87%	p3A5, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13T1, p13C1, p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p36T2, p37T1
C, TS, NA	43.5%	p3A5, p4C1, p8C5, p9C1, p13C1, p15C1, p16C1, p24C1, p33C1, p34C2
AP, A, AUG, CRO, CXM, NA	47.8%	p3A5, p4C1, p8C5, p9C1, p13C1, p15C1, p16C1, p24C1, p33C1, p34C2, p37T1

3.3.3. Identification of the TetA resistance determinant

A high prevalence of tetracycline resistance was observed for the 37 wild-type *Aeromonas* spp. isolates with *tetA*, *tetB*, *tetD/H*, *tetE*, *tetDEH* genes present in 62.1%, 10.8%, 10.8%, 43.2% and 2.7% of the studied *Aeromonas* spp. isolates, respectively (Jacobs & Chenia, 2007). Since the *tetA* gene was the predominant resistance determinant, the presence of this gene on detected plasmids was evaluated by Southern hybridization experiments with the *tetA* probe. Screening of the total DNA and total plasmid content of the plasmid-containing *Aeromonas* spp. isolates revealed that *tetA* gene was plasmid-encoded in 47.1% (8/17) of *Aeromonas* spp. isolates, i.e., AE2, AE9, AE12, AE13, AE16, AE25, AE33 and AE37 (Figs. 3.4 and Fig. 3.5). Positive hybridization signals were also observed with the total DNA content of 52.9% of screened isolates, i.e., AE2, AE4, AE9, AE12, AE16, AE25, AE31, AE33 and AE37.

Isolates AE2, AE9, AE12, AE16, AE25, AE33 and AE37 provided positive hybridization signals with both total DNA and total plasmid content.

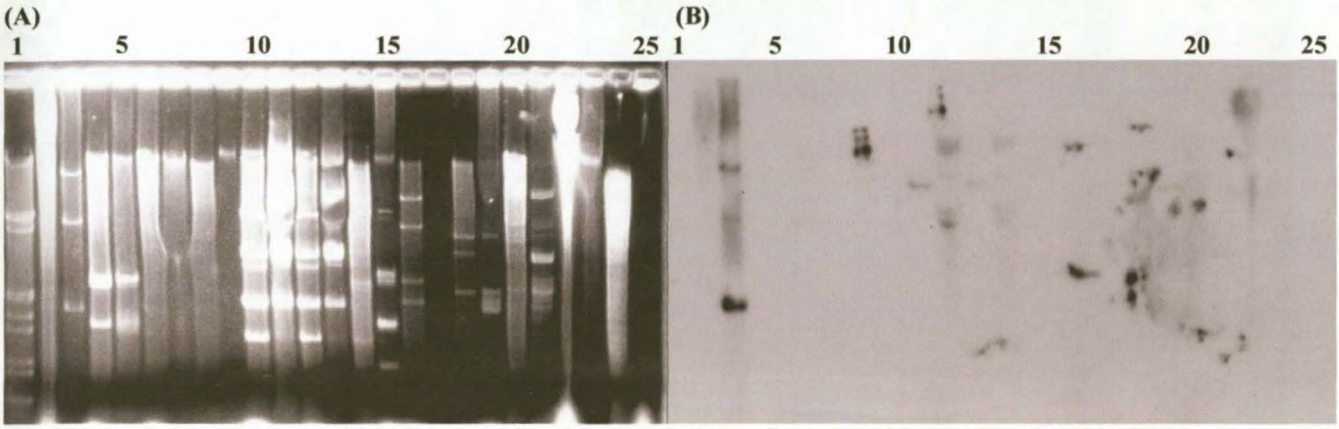

Figure 3.4. Agarose gel electrophoresis of the total DNA digested with *Hin*dIII and detected plasmids of 17 plasmid-containing *Aeromonas* spp. isolates. Total DNA preparations are indicated by (t) and plasmid DNA by (p). **(A)** Lane 1: λ phage DNA digested with *Eco*RI and *Hin*dIII; lanes 2 – 25: AE37(t), AE37(p) digested with *Sal*I, AE3(t), AE3(p), AE4(t), AE4(p), AE8(t), AE8(p), AE9(t), AE9(p), AE12(t), AE12(p), AE15(t), AE15(p), AE16(t), AE16(p), AE24(t), AE24(p), AE25(t), AE25(p), AE31(t), AE31(p), AE34(t), AE34(p). **(B)** Southern hybridization with the *tetA* probe for the localization of tetracycline resistance gene, *tetA*.

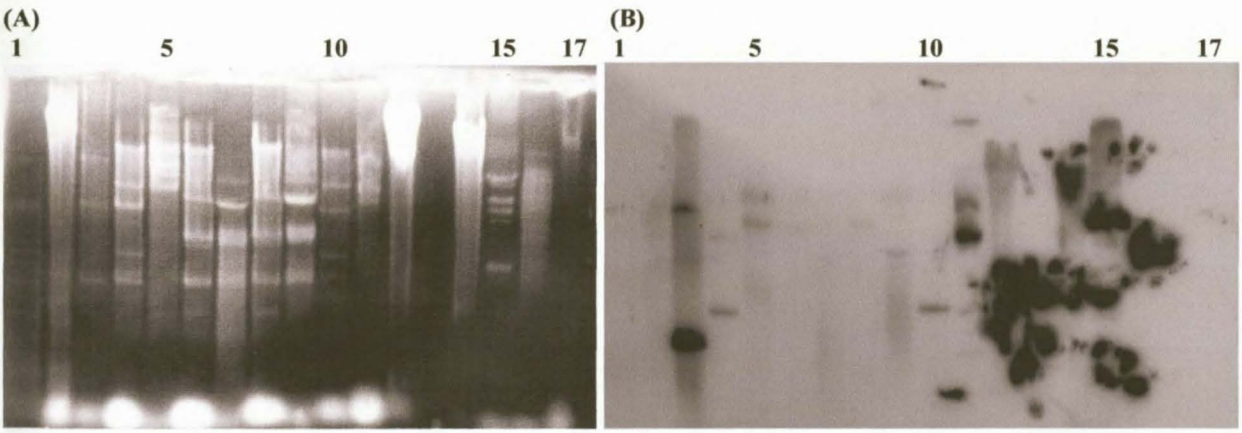

Figure 3.5. Agarose gel electrophoresis of the total DNA digested with *Hin*dIII and detected plasmids of 17 plasmid-containing *Aeromonas* spp. isolates. Total DNA preparations are indicated by (t) and plasmid DNA by (p). **(A)** Lane 1: λ phage DNA digested with *Eco*RI and *Hin*dIII; lanes 2 – 18: AE37(t), AE37(p) digested with *Sal*I, AE2(t), AE2(p), AE11(t), AE11(p), AE13(t), AE13(p), AE16(t), AE16(p), AE30(t), AE30(p), AE33(t), AE33(p), AE34(t), AE34(p). **(B)** Southern hybridization with the *tetA* probe for the localization of tetracycline resistance gene, *tetA*.

Screening of transformed plasmids with *tetA* probe allowed the identification of five plasmid preparation, p31T1, p31T2, p31T5, p36T2 and p37T1, which gave a positive

hybridization signal (Fig. 3.6B). The lower frequency obtained with the transformed plasmids was the result of the majority of the *Aeromonas* spp. plasmids remaining untransformed.

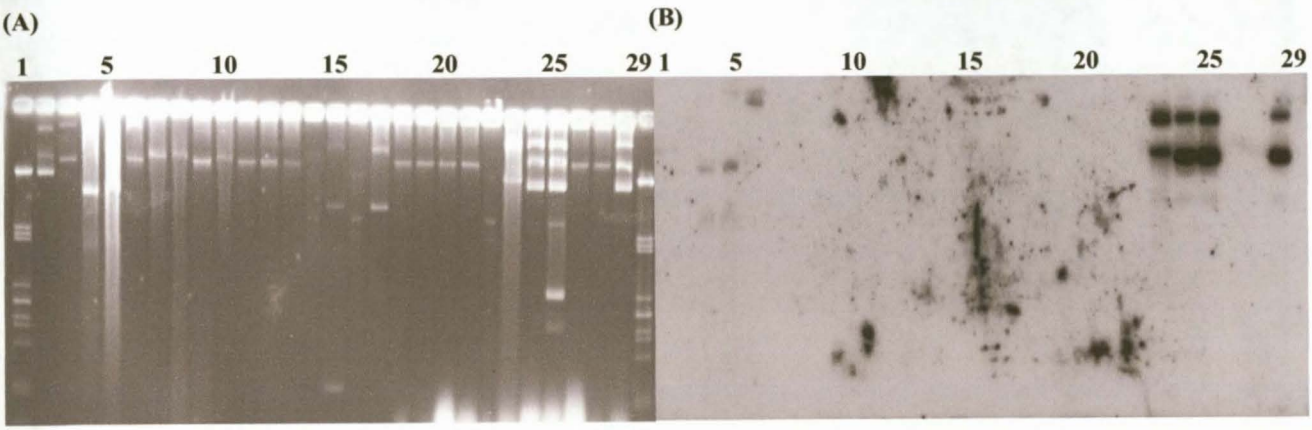

Figure 3.6. Plasmid DNA profiles of *E. coli* DH5α transformed with *Aeromonas* spp. plasmids. **(A)** Lanes 1 and 29 : DNA molecular-weight marker (*Eco*RI and *Hind*III digests of λ DNA); lanes 2-28: pTC-F14, pTF-FC2, pRAS3.1, pRAS3.2, p37T1, p2T1, p2T2, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13T1, p13C1, p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2. **(B)** Southern hybridization with the *tetA* probe.

In order to confirm hybridization results, PCR amplifications were carried out with the Tet-1 primer set (Table3.2). A positive *tetABC* amplicon was obtained for 34.8% (8/23) of the transformed *Aeromonas* spp. plasmids, i.e., p11T14, p12T1, p13T1, p31T1, p31T2, p31T5, p36T2 and p37T1 (Fig. 3.7). A single *tetDEH* amplicon was identified in p25T1 (data not shown). A total of 60% of the tetracycline-resistant transformants were identified as either harbouring a *tetABC* (53.3%) or *tetDEH* (6.7%) type resistance determinant.

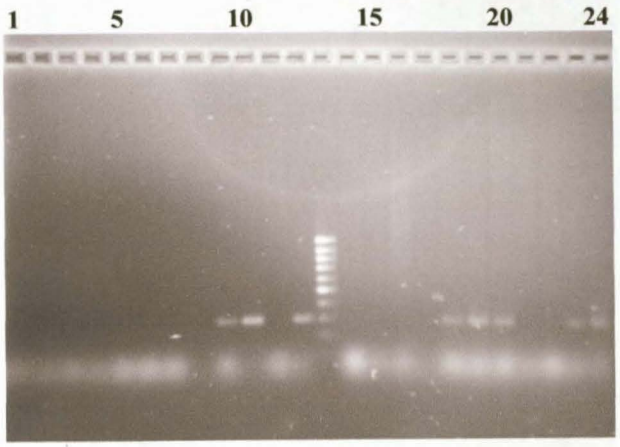

Figure 3.7. *tetABC* amplicons (300 bp) obtained following amplification of 23 *E. coli* DH5α transformants containing *Aeromonas* spp. plasmids with Tet-1 primer set (Schnabel & Jones, 1999). Lanes 1 – 12: p2T1, p2T1, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13C1, p13T1; lane 13: O'GeneRuler™ 100 bp Ladder (Fermentas, Canada); lanes 14 - 24: p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2, p37T1.

Thus, using both Southern hybridizations and PCR analysis, it was possible to identify the *tetA* gene on 8 transformed plasmids. The differences between the results obtained by Southern hybridizations and PCR amplifications highlighted the sensitivity of PCR analysis compared to probing with the *tetA* probe. A total of 14 plasmids were identified that harbour the *tetA* gene.

3.3.4. Prevalence of the tetracycline-associated transposon Tn*1721*

Screening of the total DNA and plasmid content of the 17 plasmid-containing *Aeromonas* isolates with the Tn*1721* probe revealed positive hybridization signals with both total DNA and plasmid preparations of 52.9% (9/17) of the isolates, i.e., AE2, AE9, AE12, AE16, AE24, AE25, AE31, AE33 and AE37. Positive signals with the total DNA (no plasmid hybridization) were obtained for isolates AE4 and AE8. Multiple positive signals were observed for 66.7% of isolates which possessed more than one plasmid or different conformational forms of a single plasmid (Figs. 3.8 and Fig. 3.9). The differences observed in intensity of hybridization signals may be an indication of varying levels of homology or the presence of more than one copy of the transposon.

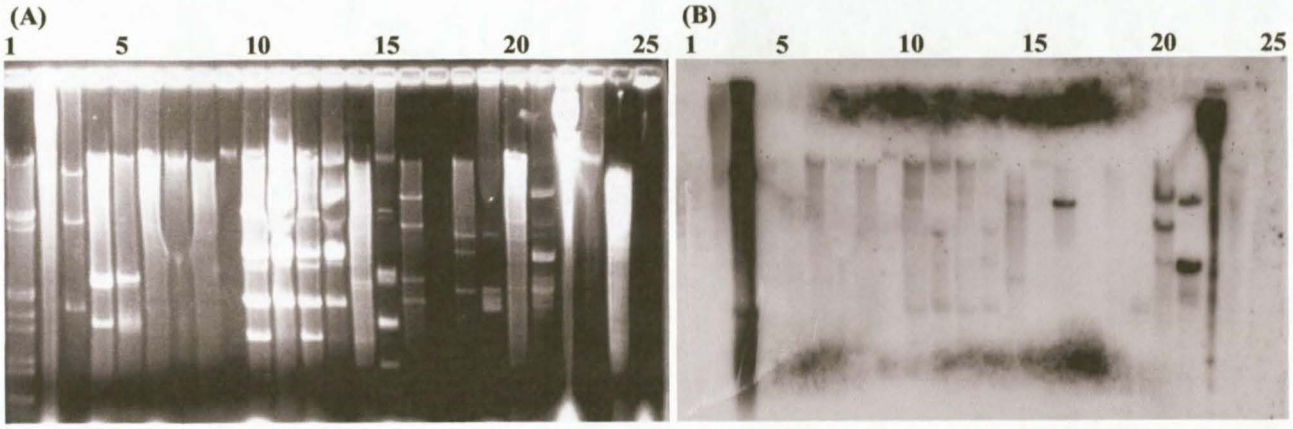

Figure 3.8. Agarose gel electrophoresis of the total DNA digested with *Hin*dIII and detected plasmids of 17 plasmid-containing *Aeromonas* spp. isolates. Total DNA preparations are indicated by (t) and plasmid DNA by (p). **(A)** Lane 1: λ phage DNA digested with *Eco*RI and *Hin*dIII; lanes 2 – 25: AE37(t), AE37(p) digested with *Sal*I, AE3(t), AE3(p), AE4(t), AE4(p), AE8(t), AE8(p), AE9(t), AE9(p), AE12(t), AE12(p), AE15(t), AE15(p), AE16(t), AE16(p), AE24(t), AE24(p), AE25(t), AE25(p), AE31(t), AE31(p), AE34(t), AE34(p). **(B)** Southern hybridization with the Tn*1721* probe for the localization of the tetracycline-associated transposon, Tn*1721*.

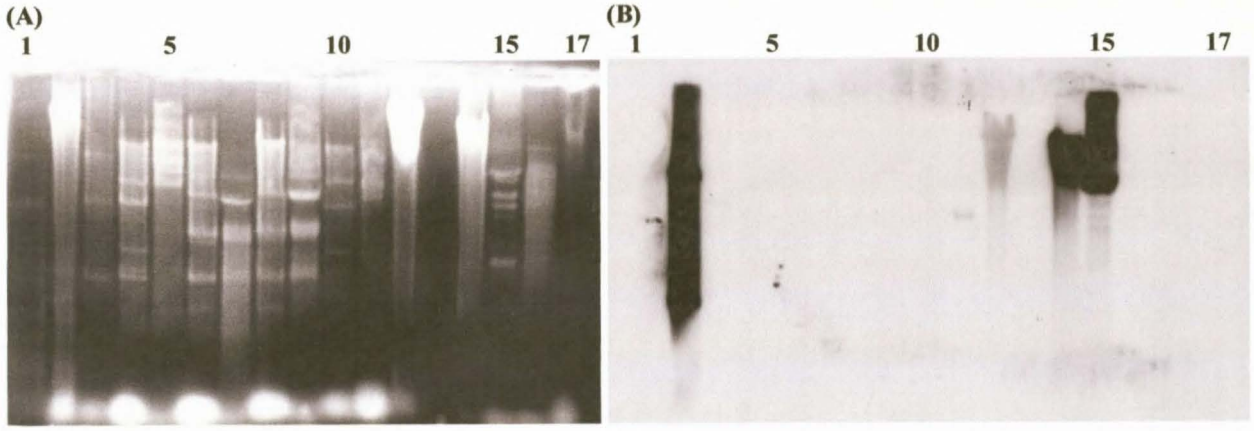

Figure 3.9. Agarose gel electrophoresis of the total DNA digested with *Hin*dIII and detected plasmids of 17 plasmid-containing *Aeromonas* spp. isolates. Total DNA preparations are indicated by (t) and plasmid DNA by (p). **(A)** Lane 1: λ phage DNA digested with *Eco*RI and *Hin*dIII; lanes 2 – 17: AE37(t), AE37(p) digested with *Sal*I, AE2(t), AE2(p), AE11(t), AE11(p), AE13(t), AE13(p), AE16(t), AE16(p), AE30(t), AE30(p), AE33(t), AE33(p), AE34(t), AE34(p). **(B)** Southern hybridization with the Tn*1721* probe for the localization of the tetracycline-associated transposon, Tn*1721*.

Screening of the transformed plasmids with Tn*1721* allowed the identification of five plasmid preparations, i.e., p31T1, p31T2, p31T5 and p36T2 (Fig 3.10B) positive for the transposon. Differences in the signal intensity might be due to the varied levels of homology or the presence of multiple copies of Tn*1721*. Plasmid p37T1 served as the positive control for both experiments, as the probe had been amplified from this plasmid.

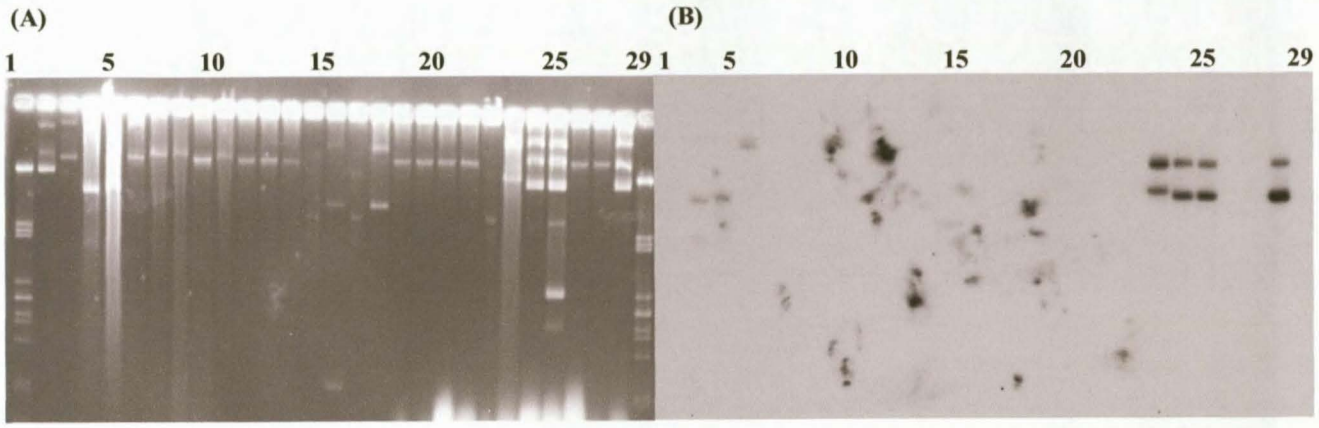

Figure 3.10. Plasmid profiles of *E. coli* DH5α transformed with *Aeromonas* spp. plasmids. **(A)** Lanes 1 and 29 : DNA molecular-weight markers (*Eco*RI and *Hin*dIII digests of λ DNA) : lanes 2-28 : pTC-F14, pTF-FC2, pRAS3.1, pRAS3.2, p37T1, p2T1, p2T2, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14 , p12T1, p13T1, p13C1, p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2. **(B)** Southern hybridization with the Tn*1721* probe.

A higher prevalence of Tn*1721* was observed among the total plasmid content of wild-type *Aeromonas* spp. isolates (52.9%) that among the transformed plasmids (21.7%). Five of the transformants displayed positive hybridization signals with both *tetA* and Tn*1721* probes, confirming the association of Tn*1721* with TetA. PCR analysis also confirmed the presence of Tn*1721* among 21.7% (5/23) of the transformed *Aeromonas* spp. plasmids, i.e., p31T1, p31T2, p31T5, p36T2 and p37T1 (Fig. 3.11).

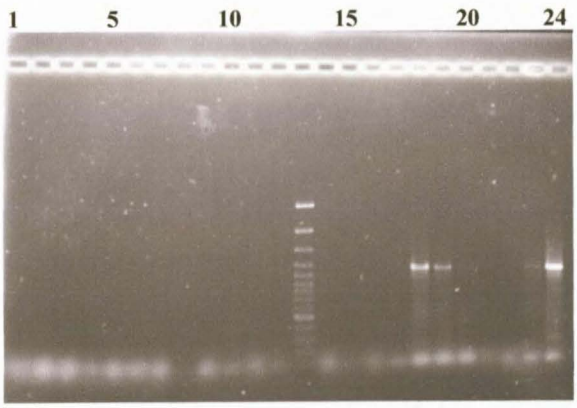

Figure 3.11. Tn*1721* amplicons (1200 bp) obtained following amplification of 23 *E. coli* DH5α transformant containing *Aeromonas* spp plasmids with the TAF-TetAR3 primer set (Pezzella *et al.*, 2004). Lanes 1 – 12: p2T1, p2T1, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13C1, p13T1; lane 13: O'GeneRuler™ 100 bp Ladder (Fermentas, Canada); lanes 14 – 24: p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2, p37T1.

3.3.5. Incidence of class 1 integrase gene, *int*I

Jacobs and Chenia (2007) identified the presence of class 1 integrase genes among 51.4 % of the *Aeromonas* spp. isolates. To establish the location of these integrons, i.e., chromosomally-encoded or plasmid-mediated, Southern hybridization experiments were undertaken. Screening of total DNA and plasmids preparations of the 17 plasmid-containing *Aeromonas* spp. isolates revealed positive hybridization signals with the total DNA isolated from two isolates, i.e., AE25 and AE37 (Figs. 3.12 and Fig. 3.13). No positive signals were observed for the detected plasmids.

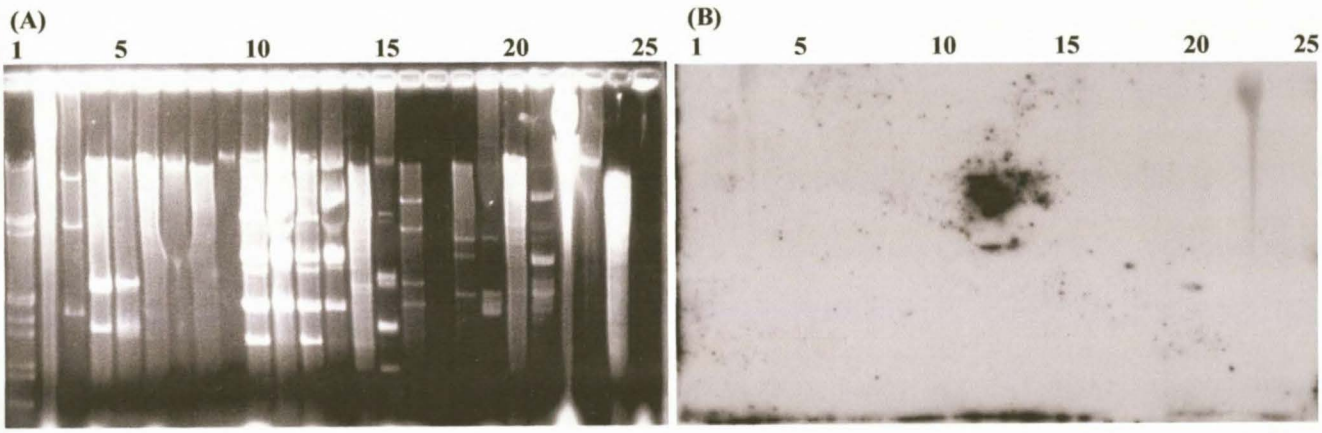

Figure 3.12. Agarose gel electrophoresis of the total DNA digested with *Hin*dIII and detected plasmids of 17 plasmid-containing *Aeromonas* spp. isolates. Total DNA preparations are indicated by (t) and plasmid DNA by (p). **(A)** Lane 1: λ phage DNA digested with *Eco*RI and *Hin*dIII; lanes 2 – 25: AE37(t), AE37(p) digested with *Sal*I, AE3(t), AE3(p), AE4(t), AE4(p), AE8(t), AE8(p), AE9(t), AE9(p), AE12(t), AE12(p), AE15(t), AE15(p), AE16(t), AE16(p), AE24(t), AE24(p), AE25(t), AE25(p), AE31(t), AE31(p), AE34(t), AE34(p). **(B)** Southern hybridization with the *int*I probe for the localization of the class 1 integrase gene.

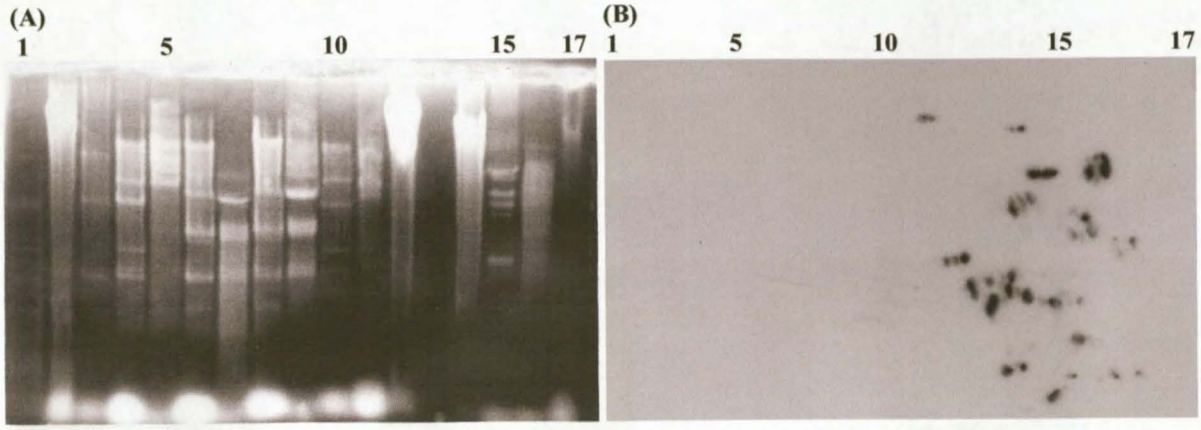

Figure 3.13. Agarose gel electrophoresis of the total DNA digested with *Hin*dIII and detected plasmids of 17 plasmid-containing *Aeromonas* spp. isolates. Total DNA preparations are indicated by (t) and plasmid DNA by (p). **(A)** Lane 1: λ phage DNA digested with *Eco*RI and *Hin*dIII; lanes 2 – 18: AE37(t), AE37(p) digested with *Sal*I, AE2(t), AE2(p), AE11(t), AE11(p), AE13(t), AE13(p), AE16(t), AE16(p), AE30(t), AE30(p), AE33(t), AE33(p), AE34(t), AE34(p). **(B)** No positive hybridization signals were obtained during Southern hybridization with the *int*I probe.

No positive hybridization signal with the *intI* probe was obtained for transformed plasmids (Fig. 3.14B). Futhermore, PCR amplifications with the IntA-IntB primer set did not result in the identification of transformed plasmids carrying the class 1 integrase gene.

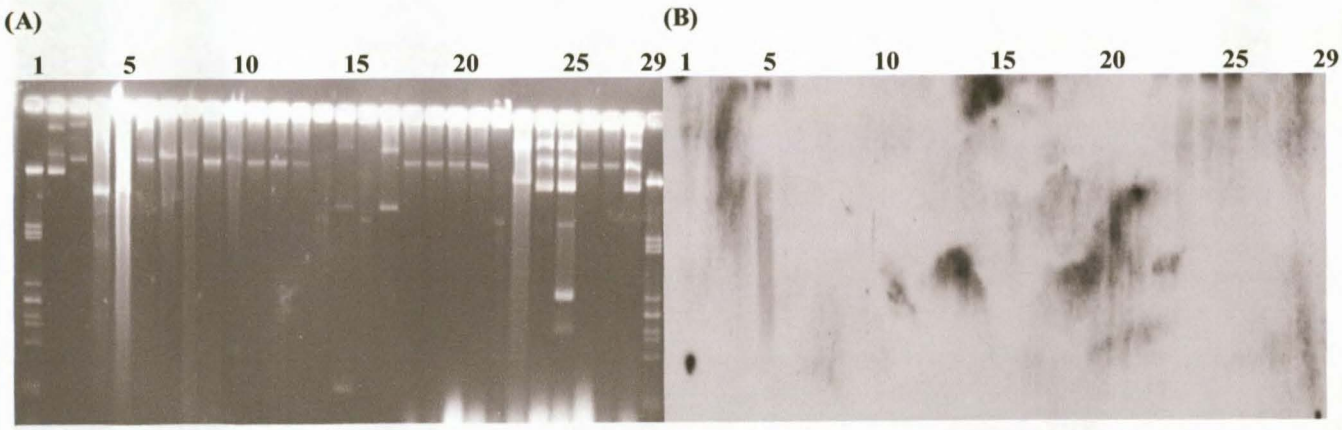

Figure 3.14. Plasmid DNA profiles of 23 *E. coli* DH5α transformants. **(A)** Lanes 1 and 29 : DNA molecular-weight markers (*Eco*RI and *Hin*dIII digests of λ DNA) : lanes 2-28 : pTC-F14, pTF-FC2, pRAS3.1, pRAS3.2, p37T1, p2T1, p2T2, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13T1, p13C1, p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2. **(B)** Southern hybridization with the *int*I probe for the localization of the class 1 integrase gene on 23 transformed plasmids.

PCR amplification of the *intI* gene was also unsuccessful, suggesting that the structures were chromosomally-encoded or present on other plasmids that were not transformed to *E. coli* DH5α.

3.3.6. Identification of TEM-1 type β-lactamase gene

Antibiotic susceptibility testing of the wild-type *Aeromonas* spp. isolates as well as transformants revealed high levels of resistance to β-lactam agents (Table 3.4). Integron-associated β-lactamase genes, *pseI* and *oxa2a* were detected in 29.7% (11/37) and 5.4% (2/37) of the *Aeromonas* spp. isolates, respectively (Jacobs & Chenia, 2007). In order to assess the prevalence of plasmid-mediated *bla*-TEM genes, Southern hybridization experiments were undertaken.

Screening of the total DNA and total plasmid content of the 17 *Aeromonas* spp. isolates with the *bla*-TEM probe revealed positive hybridization signals with total DNA preparations of isolates AE9, AE12 and AE37 (Fig. 3.15).

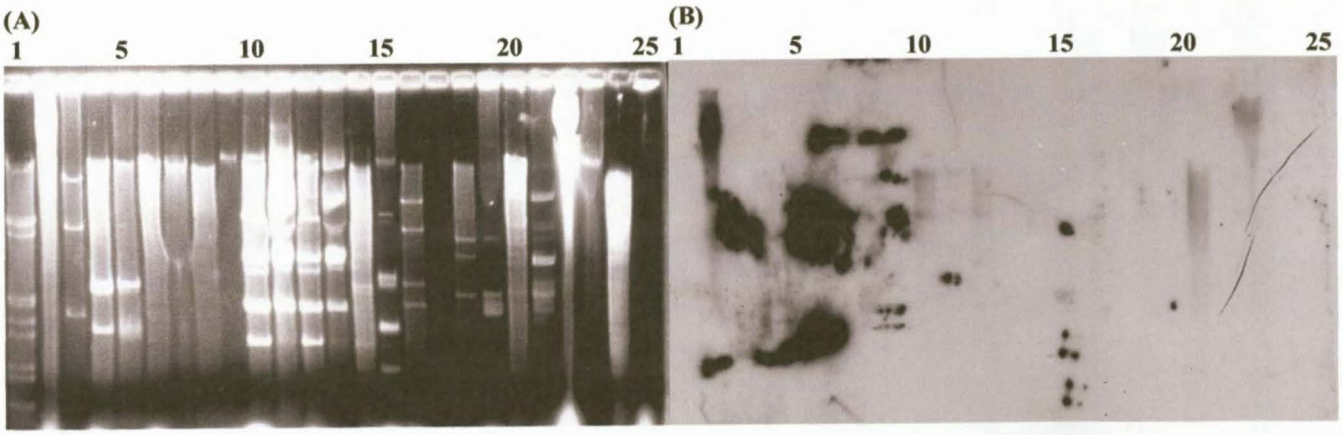

Figure 3.15. Agarose gel electrophoresis of the total DNA digested with *Hin*dIII and detected plasmids of 17 plasmid-containing *Aeromonas* spp. isolates. Total DNA preparations are indicated by (t) and plasmid DNA by (p). **(A)** Lane 1: λ phage DNA digested with *Eco*RI and *Hin*dIII; lanes 2 – 25: AE37(t), AE37(p) digested with *Sal*I, AE3(t), AE3(p), AE4(t), AE4(p), AE8(t), AE8(p), AE9(t), AE9(p), AE12(t), AE12(p), AE15(t), AE15(p), AE16(t), AE16(p), AE24(t), AE24(p), AE25(t), AE25(p), AE31(t), AE31(p), AE34(t), AE34(p). **(B)** Southern hybridization with the *bla*-TEM probe for the localization of the TEM-1 type β-lactamase gene.

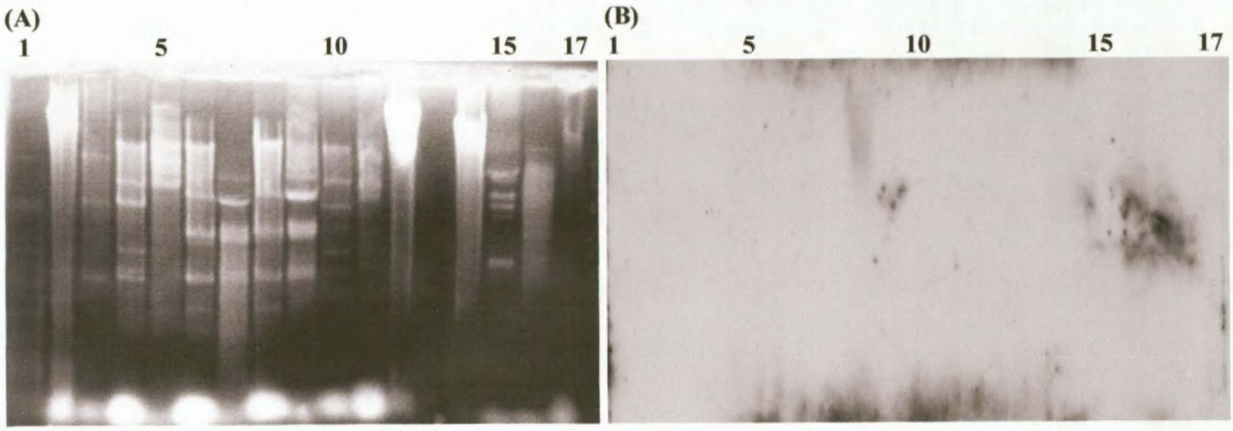

Figure 3.16. Agarose gel electrophoresis of the total DNA digested with *Hin*dIII and detected plasmids of 17 plasmid-containing *Aeromonas* spp. isolates. Total DNA preparations are indicated by (t) and plasmid DNA by (p). **(A)** Lane 1: λ phage DNA digested with *Eco*RI and *Hin*dIII; lanes 2 – 18: AE37(t), AE37(p) digested with *Sal*I, AE2(t), AE2(p), AE11(t), AE11(p), AE13(t), AE13(p), AE16(t), AE16(p), AE30(t), AE30(p), AE33(t), AE33(p), AE34(t), AE34(p). **(B)** No positive hybridization signals were observed using the *bla*-TEM probe.

A positive hybridization signal was also observed with the 5 kb plasmid isolated from AE9. This plasmid was not transformed to *E. coli* DH5α. Southern hybridization experiments did not result in the identification of *bla*-TEM genes present on the transformed *Aeromonas* plasmids. However, PCR analysis revealed the presence of the β-lactamase gene in 21.7% (5/23) of the *E. coli* transformants (p31T1, p31T2, p31T5, p36T2, p37T1). Thus, PCR together with Southern hybridization experiments identified a total of 6 *Aeromonas* spp. plasmids harbouring the TEM-1 type of β-lactamase.

3.3.7. Prevalence of the linked *strA-strB* genes among *Aeromonas* plasmids

The linked *strA-strB* streptomycin resistance gene, which has been identified as part of the R-plasmid pRAS2, confers high levels of resistance to streptomycin. The prevalence of this gene, isolated from plasmid RSF1010, was investigated in the total *Aeromonas* study population and the respective plasmids by Southern hybridization.

Screening of the total DNA and plasmid content of the 17 plasmid-containing *Aeromonas* spp. isolates with the *strA-strB* probe revealed positive hybridization signals with the total DNA of 23.5% (4/17) of the plasmid-containing *Aeromonas* spp. isolates

i.e., AE2, AE25, AE31 and AE37 (Fig. 3.17 and Fig. 3.18). Positive signals were also observed for plasmids isolated from AE25, AE31 and AE37 (Fig. 3.17 and Fig. 3.18).

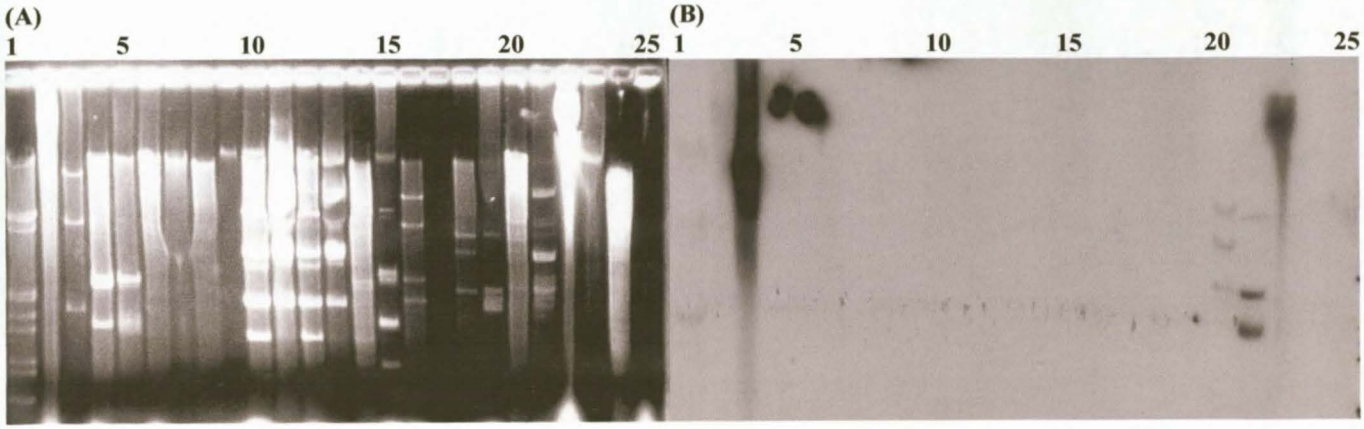

Figure 3.17. Agarose gel electrophoresis of the total DNA digested with *Hin*dIII and detected plasmids of 17 plasmid-containing *Aeromonas* spp. isolates. Total DNA preparations are indicated by (t) and plasmid DNA by (p). **(A)** Lane 1: λ phage DNA digested with *Eco*RI and *Hin*dIII; lanes 2 – 25: AE37(t), AE37(p) digested with *Sal*I, AE3(t), AE3(p), AE4(t), AE4(p), AE8(t), AE8(p), AE9(t), AE9(p), AE12(t), AE12(p), AE15(t), AE15(p), AE16(t), AE16(p), AE24(t), AE24(p), AE25(t), AE25(p), AE31(t), AE31(p), AE34(t), AE34(p). **(B)** Positive hybridization with the *strA-strB* probe observed for the smaller plasmids (2-3 kb) isolated from isolate AE25.

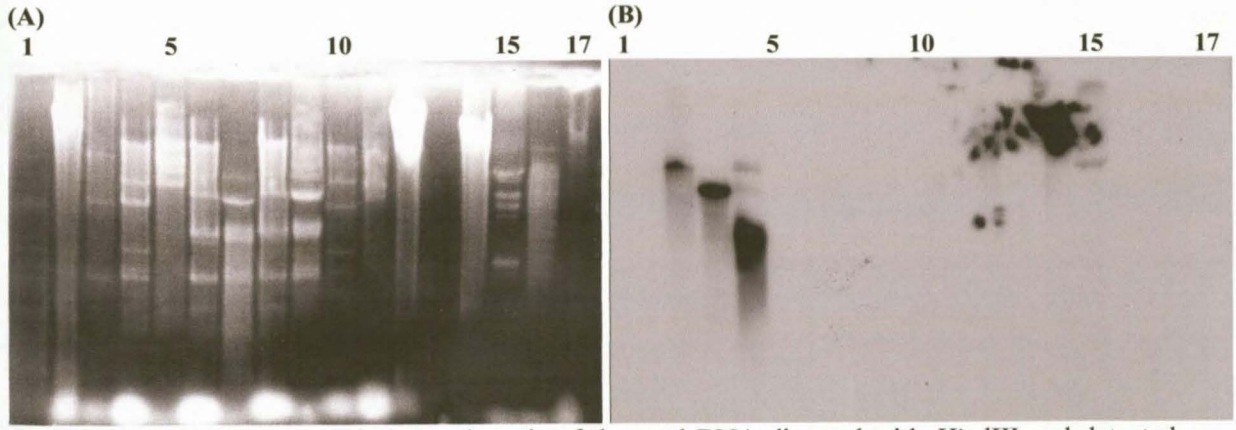

Figure 3.18. Agarose gel electrophoresis of the total DNA digested with *Hin*dIII and detected plasmids of 17 plasmid-containing *Aeromonas* spp. isolates. Total DNA preparations are indicated by (t) and plasmid DNA by (p). **(A)** Lane 1: λ phage DNA digested with *Eco*RI and *Hin*dIII; lanes 2 – 18: AE37(t), AE37(p) digested with *Sal*I, AE2(t), AE2(p), AE11(t), AE11(p), AE13(t), AE13(p), AE16(t), AE16(p), AE30(t), AE30(p), AE33(t), AE33(p), AE34(t), AE34(p). **(B)** Hybridization with the *strA-strB* probe for the localization of the streptomycin resistance gene, *strA-strB*. Strong signals were obtained for plasmids isolated from *Aeromonas* ssp. isolates AE33 and AE37.

Screening the 23 transformants containing *Aeromonas* spp. plasmids with the *strA-strB* probe allowed the identification of a single positive hybridization signal for p37T1 (which was later confirmed by sequence analysis) (Fig. 3.19B).

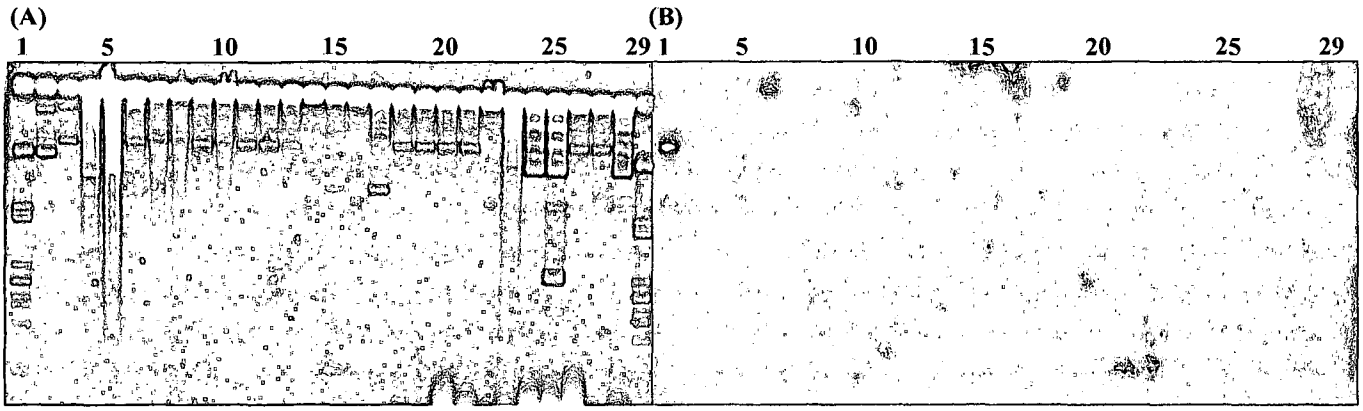

Figure 3.19. Plasmid DNA profiles of 23 *E. coli* transformants containing *Aeromonas* spp. plasmids. **(A)** Lanes 1 and 29: DNA molecular-weight markers (*Eco*RI and *Hin*dIII digests of λ DNA) : lanes 2-28: pTC-F14, pTF-FC2, pRAS3.1, pRAS3.2, p37T1, p2T1, p2T2, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13T1, p13C1, p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2. (B) Southern hybridization experiments with the *strA-strB* probe yielded no positive signals other than p37T1.

3.4. Discussion

Exogenous DNA such as plasmids in conjunction with other mobile genetic elements, i.e., transposons and integrons, have been implicated in the dissemination of various resistance determinants in and among different environmental compartments (Rhodes *et al.*, 2000). Certain plasmids are capable of replication in various host cells thus enabling the proliferation of these plasmids in a specific environment. Studies involving the investigation of bacterial plasmids often requires the transfer of these plasmids to laboratory strains to ensure optimum conditions to successfully study different aspects such as plasmid-mediated antibiotic resistance genes. R-plasmids are readily introduced into laboratory *E. coli* strains either through transformation and/or conjugation (Bruun *et al.*, 2003; L'Abee-Lund & Sørum, 2002; Palu *et al.*, 2006; Schmidt *et al.*, 2001a; Son *et al.*, 1997). Upon the introduction of the plasmid into the new host, the resistance phenotype encoded by the plasmid can be observed in the transformant or transconjugant without the influence of the host strain's chromosomally-encoded

antibiotic resistance phenotype (Bruun *et al.*, 2003; Son *et al.*, 1997). Successful transformation of plasmids isolated from all 17 plasmid-containing environmental *Aeromonas* spp. into general *E. coli* laboratory strain, DH5α, was not unexpected. Plasmids isolated from *Aeromonas* spp. have been transferred to unrelated bacterial species in simulated natural microenvironments and under laboratory conditions (Kruse & Sørum, 1994). The co-transfer of smaller cryptic plasmids was also observed, since the presence of these plasmids had not effect on the antibiotic susceptibility of the transformants (Table 3.4). This is indicative of the important role that these DNA elements play with regard to genetic exchange and the dissemination of different accessory genes within specific environmental compartments.

The β-lactam resistant phenotype displayed by all of the plasmid-containing *Aeromonas* spp. isolates was due in part to plasmid-mediated β-lactamase activity, since resistance to penicillins and the earlier cephalosporins was observed in the transformants. Chromosomally- and plasmid-mediated β-lactamases have been reported in members of the *Aeromonadaceae* family (Fosse *et al.*, 2004; Rossolini *et al.*, 1995; Walsh *et al.*, 1998) and the findings of the present study and that of Jacobs and Chenia (2007) correlate with these reports. Although the transferred β-lactamase activity was not extensively studied on a molecular level, results indicate that resistance to different β-lactam antibiotics is the dominant resistance phenotype transferred by the *Aeromonas* plasmids. Plasmid-encoded resistance determinants identified in *Aeromonas* spp. include *oxa*, and bla_{TEM} (Tennstedt *et al.*, 2003). More recent studies identified a plasmid-encoded *ampC* gene, bla_{CMY-9}, which showed 78% nucleotide identity to the chromosomally-encoded CefH of *A. hydrophila* (Doi *et al.*, 2002; Walsh *et al.*, 1998). This gene encodes resistance to broad-spectrum cephalosporins such as ceftazidime and was isolated from a clinical *E. coli* (Doi *et al.*, 2002). This raises the question as to the possibility that aquatic aeromonads serve as a reservoir of resistance genes which become mobilized through plasmids and spread to other environmental compartments. In support of this statement, Fosse *et al.* (2004) reported the stable maintenance of a plasmid-mediated bla_{TEM-24} gene in a clinical *A. hydrophila* isolate. This β-lactamase gene originated from an *Enterobacter aerogenes* strain and transferred the resistance phenotype to *A. hydrophila* after conjugation (Fosse *et al.*, 2004). The low incidence (21.7%) of the

TEM-1 type resistance gene among the transformed plasmids in the present study was unexpected. It is possible that the β-lactamase gene responsible for the resistance phenotype displayed by the transformants could belong to another TEM-type of β-lactamase or an ESβL. Resistance to carbapenems was shown to be predominantly chromosomally-encoded with only a single transformant (p37T1) displaying an imipenem resistance phenotype. This is in agreement with general findings, since evidence of the wide-spread occurrence of carbapenem resistance among *Aeromonas* spp. exists, but limited information concerning plasmid-mediated carbapenem resistance has been reported (Rossolini *et al.*, 1995; Walsh *et al.*, 1998).

Jacobs and Chenia (2007) identified the TetA resistance determinant in 62.2% of the studied *Aeromonas* spp. isolates, of which 60.9% were shown to be plasmid-encoded by Southern hybridizations and PCR analysis. Other studies have demonstrated the frequent transfer of tetracycline resistance genes as part of R-plasmids present in *A. salmonicida* and *A. salmonicida* subsp. *salmonicida* isolates (Casas *et al.*, 2005; L'Abee-Lund & Sørum, 2000; L'Abee-Lund & Sørum, 2002; Sørum *et al.*, 2003). Plasmid-mediated tetracycline resistance among *Aeromonas* spp. isolates is diverse, with *tetA, tetB, tetC, tetD, tetE* and *tet31* genes being identified (Adams *et al.*, 1998; Chopra & Roberts, 2001; Nawaz *et al.*, 2006; Schmidt *et al.*, 2001b). R-plasmids pRAS1, pRAS2 and pRAS3, all contain tetracycline resistance genes that have been successfully transferred to laboratory strains, which displayed the resistance phenotype following acquisition of the plasmid (L'Abee-Lund & Sørum, 2000; L'Abee-Lund & Sørum, 2002; Sørum *et al.*, 2003). In the aquaculture setting, the transfer of tetracycline resistance determinants A and D is the dominant transferred antibiotic resistance phenotype due to the frequent use of this agent to prevent disease outbreaks (Bruun *et al.*, 2003). In the present study, the presence of a plasmid-mediated TetA gene was detected in 37.8% of the plasmid-containing *Aeromonas* spp. isolates, suggesting that plasmids play an important role in the spread of tetracycline resistance genes in aquaculture settings. This is in keeping with findings reported by Schmidt *et al.* (2001a), who identified the presence of plasmid-borne *tetA* genes in 30% of tetracycline-resistant aeromonads obtained from an aquaculture setting. Transfer of the tetracycline resistance phenotype in 73.9% of transformants containing *Aeromonas* spp. plasmids is in correlation with

findings by Adams *et al.* (1998), who reported the transfer of the tetracycline resistance phenotype in 66% of plasmid-containing *A. salmonicida* strains. The lower frequency of plasmid-encoded *tetA* observed among the transformants may be due to many of the multiple *Aeromonas* spp. plasmids that were not transformed into *E. coli* DH5α. Association of the *tetA* tetracycline resistance gene and transposon Tn*1721*, among the transformed *Aeromonas* spp. plasmids is supported by findings indicating the prevalence and association of Tn*1721* and TetA resistance determinants in aquatic and other environments (Adams *et al.*, 1998; Rhodes *et al.*, 2000).

A low prevalence of the linked streptomycin resistance gene, *strA-strB*, was observed although, linked *strA-strB* genes have been identified as part of R-plasmids isolated from manure slurries and clinical settings in Taiwan (Chen *et al.*, 2006; Smalla *et al.*, 2000). The genes isolated in both settings were similar in arrangement to those of the IncQ plasmid RSF1010 (Smalla *et al.*, 2000). L'Abée-Lund and Sørum (2000) also identified this coupled resistance gene in the R-plasmid pRAS2, although it is normally isolated in clinical important bacteria or as part of large conjugative plasmids in plant pathogenic bacteria. Plasmid p37T1, which provided a positive hybridization signal with the *strA-strB* probe, displayed plasmid-mediated resistance to amikacin and gentamicin suggesting the presence of chromosomally-encoded aminoglycoside resistance genes including those associated with integrons The presence of gene-capturing structures such as integrons and transposons on R-plasmids increases the potential of plasmids as facilitators for horizontal gene transfer and the expansion of micro-organisms' expressed resistance phenotypes (Fluit & Schmitz, 2004; Rice, 1998).

Gene-capturing systems such as integrons play an important role in the acquisition of a wide variety of different antibiotic resistance determinants (Collis & Hall, 1995; Hall & Collis, 1998). The presence of possible chloramphenicol resistance genes on 52.2% of the transformed *Aeromonas* spp. plasmids is not surprising, since these genes frequently form part of integron structures of *Aeromonas* plasmids (Schmidt *et al.*, 2001b). Resistance to florfenicol, a chloramphenicol derivative, has been found to be plasmid-mediated in environmental *E. coli* and *Pasteurella multocida* isolates, which further supports the possibility of plasmid-mediated chloramphenicol resistance determinants

(Cloeckaert *et al.*, 2000; Kehrenberg & Schwarz, 2005). Schmidt *et al.* (2001a) identified class 1 integrons containing trimethoprim and aminoglycoside resistance determinants as part of R-plasmids in *A. salmonicida*. The R-plasmids were transferred to a new host, which displayed the resistance phenotype encoded by the genes contained within the integrons. In the present study, the transformed *Aeromonas* spp. plasmids that transferred cotrimoxazole resistance to *E. coli* DH5α did not co-transfer resistance to the tested aminoglycosides. Transferred resistance to cotrimoxazole, which consists of trimethoprim and a sulphonamide compound, could furthermore be an indication of the presence of integron structures on the transferred *Aeromonas* spp. plasmids. The conserved 3'-region of integrons encode for sulphonamide resistance (*sulI*) as described by Collin and Hall (1995). Schmidt *et al.* (2001b) also found a strong correlation between the presence of class 1 integrons and combined trimethoprim/sulphonamide resistance among aeromonads isolated from rainbow trout in Danish aquaculture systems. The distribution of class 1 integrons among aquatic organisms vary in regard to the bacterial species, geographical location and antimicrobial therapy, ranging from 3.6% to 45% (Rosser & Young, 1999; Schmidt *et al.*, 2001b). In this study, none of the plasmids isolated from the wild-type *Aeromonas* spp., nor the transformed plasmids showed the presence of a possible integron structure, which is unexpected since Jacobs and Chenia (2007) identified the presence of class 1, 2 and 3 integrons in 51.4%, 27% and 10.8% of the 37 *Aeromonas* spp. isolates, respectively, using genomic DNA as template for PCR amplifications. It is possible that the integrons identified by Jacobs and Chenia (2007) may be present in the plasmid-free *Aeromonas* spp. isolates that were not screened during this survey.

The prevalence of plasmid-mediated quinolone resistance is increasingly being observed among clinically important pathogens such as *E. coli* and *Klebsiella pneumoniae* (Jacoby *et al.*, 2003; Jacoby *et al.*, 2006; Rodriguez-Martinez *et al.*, 2003; Wang *et al.*, 2006; Wang *et al.*, 2003). Different quinolone resistance genes have been identified, including *qnrA, qnrB* and *qnrS* (Bonemann *et al.*, 2006; Jacoby *et al.*, 2006). The prevalence of the plasmid-encoded *qnr* genes is not limited to the clinical setting and consistency they have also been identified in the environmental setting. Bonemann *et al.* (2006) characterized the *qnrS2* gene as part of an IncQ-like plasmid isolated from a

wastewater treatment plant. When investigating the prevalence of the *qnrA* resistance determinant among Gram-negative aquatic bacteria, it is speculated that the sub-inhibitory levels of quinolones found in water systems provides the selective pressure which enhances the transfer of this gene to other bacterial species (Poirel *et al.*, 2005). The high incidence of transferable nalidixic acid reported in the present study could be seen as an indication of the possibility that plasmid-mediated quinolone resistance exists in other environmental compartments. This correlates with the speculation that aquatic environments may serve as a reservoir of resistance genes such as *qnr* (Bonemann *et al.*, 2006; Szczepanowski *et al.*, 2004; Tennstedt *et al.*, 2003) Although only nalidixic acid resistance was transferred to *E. coli*, decreased susceptibility to ciprofloxacin and/or ofloxacin was also observed (decreasing zone diameters). This is indicative that different quinolone resistance gene could be present on the studied *Aeromonas* plasmids. The co-transfer of quinolone and β-lactam resistance has been reported in *K. pneumoniae* strains obtained from a clinical setting (Chen *et al.*, 2006; Rodriguez-Martinez *et al.*, 2003). This could explain the high prevalence (47.8%) of nalidixic acid resistance expressed by the transformants which also exhibited resistance to β-lactam antibiotics (Table 3.5).

MAR index values displayed by the transformants were much higher than expected, since many of the *Aeromonas* spp. from which the majority plasmids were isolated had been obtained from a tilapia system which had no prior exposure to antimicrobial agents. At least 50% of the wild-type *Aeromonas* spp. isolates had a MAR index value of > 0.3 (Jacobs & Chenia, 2007), indicating a high level of exposure to antimicrobial agents, possibly natural occurring antibiotics produced by molds, yeasts and actinomycetes. Fifty-seven percent of the transformants displayed MAR indices of > 0.5, indicating that a significant percentage of resistance genes in the *Aeromonas* spp. population examined were plasmid-borne. Comparison of the wild-type *Aeromonas* spp. isolates and the *E. coli* DH5α transformants revealed a number of differences including resistance to a greater variety of antimicrobial agents in the *E. coli* DH5α host to which the wild-type *Aeromonas* spp. appeared susceptible. This might be attributed to differences in the expression levels of transferred genes in the new host, differences in copy number of the plasmids in different hosts, the orientation/shuffling of resistance determinants in integron structures if present, differences in the integron promoter

activity which leads to differences in expression of inserted gene cassettes, and intrinsic differences between *E. coli* and *Aeromonas* cells, i.e., the presence of other cell components such as outer-membrane proteins responsible for the efflux of antibiotics out of the bacterial cell (Collis & Hall, 1995; Hall & Collis, 1998; Recchia & Hall, 1995; Taylor *et al.*, 2004). Cells harbouring plasmids encoding resistance determinants are able to regulate their adaptation properties to the immediate environment by changing the plasmid copy number and hence the level of expression of the present resistance genes (Toranzo *et al.*, 1984). Enne *et al.* (2006) reported the lack of expression of antibiotic resistance genes present on R-plasmids although the genes were intact, indicating that the expression of gene systems can be switched off and silenced. This gene silencing might be due to single-site mutations or other low-frequency genetic changes which occur within the population, rather than loss of the resistance genes themselves and might explain differences in antibiotic resistance phenotypes (Enne *et al.*, 2006).

MDR is frequently observed among members of the *Aeromonadaceae* family (Chang & Bolton, 1987). However, the transfer of these MDR phenotypes is of concern. Differences were observed in the present study with respect to resistance displayed by wild-type *Aeromonas* spp. isolates and the phenotypes displayed when their resident plasmids were transferred to *E. coli*. Since the carriage of antibiotic resistance genes is generally assumed on the basis of phenotype, investigation was only focused on resistant isolates. This did not allow the discrimination between the presence of resistance genes which are expressed at low levels resulting in a susceptible phenotype and silenced genes. In future, isolates expressing susceptible phenotypes should not be discarded as this could be an indication of the presence of genes conferring only low levels of resistance and providing insight into the true state of antibiotic resistance. No guidelines currently exist for the use of antimicrobial agents in South African aquaculture making it important to evaluate the status of antibiotic resistance among potential fish pathogens isolated from this environmental compartment. The widespread nature of genes encoding resistance to an extensive spectrum of different, often clinically important antibiotics within this group of bacteria could provide a genetic pool for the exchange of antibiotic resistance genes among other aquatic organisms. These plasmid-mediated antibiotic resistance genes may contribute to the spread of antibiotic resistance within aquacultural settings.

Having identified the presence of several plasmid-mediated antibiotic resistance and accessory genes in the *Aeromonas* spp. isolates, the investigation of different backbone DNA structures of selected plasmids will be the focus of the next section. Southern hybridization experiments with different replication and mobilization genes will be undertaken to identify the prevalence of different replicon-types. Furthermore, selected plasmids will be characterized by endonuclease restriction and mobilization frequencies.

Chapter 4

Characterization and mapping of selected *Aeromonas* plasmids

4.1. Introduction

Plasmids are extra-chromosomal circular or linear fragments of DNA that replicate autonomously in the host cell. All plasmids, irrespective of size, contain an essential origin of replication region which contain the genes and loci involved in replication and its control (Couturier *et al.*, 1988; del Solar *et al.*, 1998). This basic replicon consists of a *cis*-acting region which supports autonomous replication, genes involved in the control of replication initiation and *rep* gene(s) encoding proteins involved in replication control (Couturier *et al.*, 1988; del Solar *et al.*, 1998). Many plasmids, however, harbour an array of different accessory genes providing resistance mechanisms, encoding proteins involved in virulence and enzymes required for the degradation and utilization of complex chemical compounds, all of which provide a competitive advantage to the host (Dennis, 2005; Majumdar *et al.*, 2006; Taylor *et al.*, 2004). Clinical microbiologists are mainly interested in plasmids from the perspective of the presence of accessory genes and the impact they have on the pathogenicity and treatment of disease-causing bacteria. Plasmid biologists, on the other hand, are interested in the backbone genes and mechanisms involved in plasmid replication, mobilization and maintenance.

Classification of bacterial plasmids became important during the 1950's with the identification and characterization of R-plasmids and their distribution in the environment (Couturier *et al.*, 1988). Plasmid identification has traditionally been performed by incompatibility testing, donor-specific phage propagation and molecular sizing, but a recent shift to a molecular approach has been observed (Smalla *et al.*, 2000). DNA hybridizations with specific replication genes, *oriT* and *oriV* regions and PCR-based detection has proved to be a more accurate approach to study the prevalence of different plasmid families in various environments (Gotz *et al.*, 1996).

Although a number of studies (Akinbowale *et al.*, 2006; Boyd *et al.*, 2003; Brown *et al.*, 1997; Chang & Bolton, 1987; L'Abee-Lund & Sørum, 2000; Palu *et al.*, 2006; Radu *et al.*, 2003; Schmidt *et al.*, 2001a; Son *et al.*, 1997) have documented the presence or absence of plasmids and their association with antibiotic resistance in *Aeromonas* spp. isolates from both clinical and natural environments, only a few studies have examined these plasmids with respect to their replication and mobilization genes (Casas *et al.*, 2005; L'Abee-Lund & Sørum, 2002; Rhodes *et al.*, 2000; Rhodes *et al.*, 2004; Sørum *et al.*, 2003). The transfer of conjugative R-plasmids isolated from *Aeromonas* spp. ranged between 1.7×10^{-7} to 1.9×10^{-3}, depending on plasmid size, conjugation systems, transfer (*tra*) genes encoding mating-pair formations and the type of recipient used during matings (Bruun *et al.*, 2003; Snyder & Champness, 2003). In natural environments, transfer frequencies are much higher than under laboratory conditions and can occur at frequencies up to 3.4×10^{-1} (Sandaa & Enger, 1994). Furthermore, plasmids capable of replicating in a wide variety of hosts, the so-called broad-host-range plasmids, demand investigation with respect to the potential role they play in interspecies genetic exchange (Gotz *et al.*, 1996). Many *Aeromonas* spp. R-plasmids have been shown to be stably maintained in various hosts including *E. coli* and *Y. ruckeri* (Bruun *et al.*, 2003). The reverse scenario holds true as different R-plasmids from other organisms such as *Vibrio cholerae*, *E. coli*, and *Pseudomonas aeruginosa* can reside within environmental *Aeromonas* spp. strains (Kruse & Sørum, 1994; Olsen & Wright, 1976).

The increase in antibiotic resistance among *Aeromonas* spp. from both clinical and environmental settings has led to the investigation of plasmid-types present in environmentally important *Aeromonas* spp. R-plasmids belonging to incompatibility groups IncQ, IncC, IncU, as well as cryptic plasmids, ColE1- and ColE2-type have been isolated from this genus (Boyd *et al.*, 2003; Casas *et al.*, 2005; Chang & Bolton, 1987; L'Abee-Lund & Sørum, 2000; L'Abee-Lund & Sørum, 2002; Sørum *et al.*, 2003). Plasmids harbouring similar replicon structures have been isolated from different environmental settings and geographical areas suggesting that micro-organisms form the link between different settings and that plasmids play an important role in the dissemination of accessory genes (Rhodes *et al.*, 2000; Smalla *et al.*, 2000). Incompatibility group U and Q plasmids are frequently isolated from tetracycline-

resistant *Aeromonas* spp. strains and have been the focus of R-plasmid research (L'Abee-Lund & Sϕrum, 2002; Rhodes *et al.*, 2000; Rhodes *et al.*, 2004). IncQ plasmids pRAS3.1 and pRAS3.2 have been characterized with regards to replication mechanisms and mobilization, while the backbone genes responsible for the replication, transfer and maintenance of IncU plasmids remain poorly understood (L'Abee-Lund & Sϕrum, 2002; Rhodes *et al.*, 2004). The IncU plasmids are, however, able to mobilize the IncQ plasmids and in *A. salmonicida* subsp. *salmonicida*, IncQ and IncU plasmids, pRAS3 and pRAS1, respectively, co-existed in the same host (L'Abee-Lund & Sϕrum, 2002).

Apart from examining the role of plasmids in conferring antibiotic resistance phenotypes to *Aeromonas* spp. isolates from an aquaculture environment, preliminary work was undertaken to identify plasmid backbone structures targeting IncU, IncQ and ColE1-type plasmid families. Selected plasmids were also characterized by endonuclease restrictions and partial maps were constructed. Furthermore, mating assays were undertaken to assess the potential of these selected plasmids to be mobilized and to determine the rate of transfer.

4.2. Materials and Methods

4.2.1. Replication in a (*polA*$^-$) mutant *E. coli* strain

Plasmids were transformed using the heat shock method (Ausubel *et al.*, 1998) into chemically competent DNA polymerase 1 (*polA*$^-$) mutant *E.coli* GW125a cells (Table 4.1). For selection purposes, the same antibiotics used in the initial selection of the plasmids in *E. coli* DH5α were used. Selection was as follows; plasmid p3A5 was selected with 100 µl/ml ampicillin; plasmids p4C1, p8C5, p9C1, p13C1, p15C1, p16C1, p24C1, p33C1 and p34C2 were selected with 50 µl/ml chloramphenicol; and plasmids p2T1, p2T2, p4T1, p11T1, p11T14, p12T1, p13T1, p25T1, p31T1, p31T2, p31T5, p36T2 were and p37T1 selected with 30 µl/ml tetracycline. Positive transformants were screened by plasmid isolations as described previously in Chapter 2, section 2.2.2.

Table 4.1. Bacterial strains and vectors used during general transformation, cloning and mating experiments.

Bacterial strains/Plasmids	Description	Reference/Source
Strains		
E. coli GW125a	*recA, polA* mutant of AB1157	(Dorrington & Rawlings, 1989)
E. coli ACSH50I^{q}	*rspL* △(*lac-pro*) (F' *traD36 proAB lacI^{q}* △M15) Cm^{r}	(Smith & Rawlings, 1998)
E. coli S17-1	*RecA pro hsdR* (RP4-2 Tc::Mu Km::Tn7)	(Simon *et al.*, 1983)
E. coli HB101	F^{-} △(*mcrC-mrr*) *hsdS20 recA13 ara-14 proA2 lacY1 λ^{-} galK2 rpsL20*(Smr) *Xyl-5 mt1-1supE44*	(Boyer & Roulland-Dussoix, 1969)
Plasmids and constructs		
RSF1010	IncQ plasmid isolated from *E. coli*	(Guerry *et al.*, 1974)
pTC-F14	Natural 14.4 kb plasmid from *Acidithiobacillus caldus* strain "f"	(Gardner *et al.*, 2001)
pGEMRSFREP1	Apr, pGEM-T® vector with PCR amplified RSF1010 *mobA* to *cac* fragment (bp 3161-5926)	(Gardner & Rawlings, 2004)
pMmob	Amp^{r} , 5554 bp *Bam*HI-*Xba*I fragment of pTC-F14 containing all mobilization genes and the *repB* primase, cloned in to pUC19	(van Zyl *et al.*, 2003)
pTCM1	Conjugative plasmid isolated from the bio-mining organism *At. caldus* MNG	(L.J. van Zyl, unpublished data)
pBluescriptKS (+/-)	Cloning vector, Amp^{r}, β-Gal	Stratagene

4.2.2. Identification, characterization and mapping of IncQ-like plasmids from *Aeromonas* spp.

4.2.2.1. Analysis of plasmid replication and mobilization genes by Southern hybridization

All DNA fragments used as probes were separated in 1% agarose gels together with O'GeneRuler™ 100 bp DNA Ladder Plus for sizing purposes (Fermentas, Canada) and visualized by UV transillumination. Constructed probes were purified to remove any residual dNTP's and/or primer using a GFX™PCR DNA and gel band purification kit (Amersham Biosciences). All probes constructed during this study were labeled using the DIG system (Roche, Germany) and Southern hybridization experiments were conducted as described previously in Chapter 3, section 3.2.3.

4.2.2.1.1. repC gene

The 1149 bp *repC* gene fragment (Fig. 4.1) was amplified from *A. salmonicida* plasmid pRAS3.1 using the RepC-F (5'-TAA TGA TAT CGC AGA ACT ACG-3') and RepC-R (5'-ATA TCC TAG GTC TTG AAC AGG-3') primer set (W. Loftie-Eaton, personal communication).

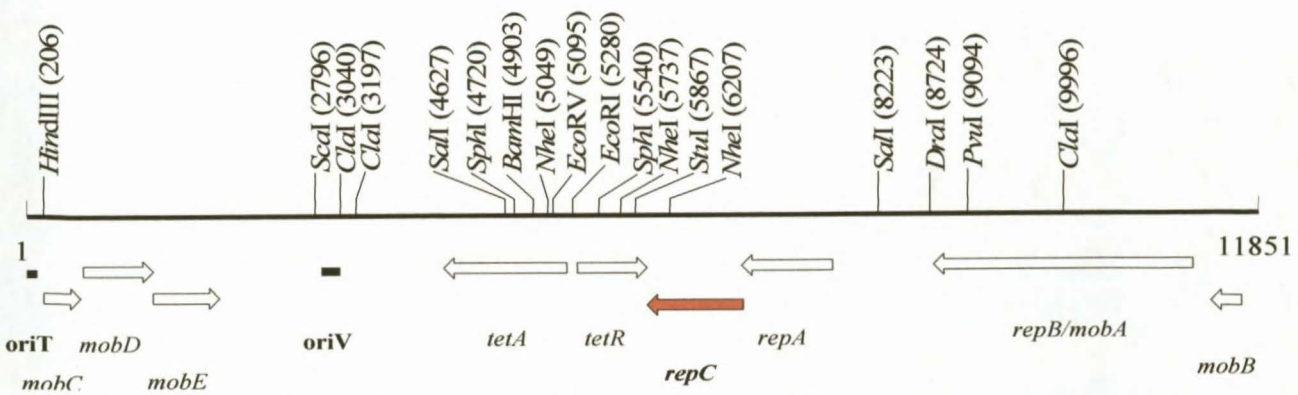

Figure 4.1. Restriction map of plasmid pRAS3.1 indicating the *repC* gene amplified by PCR (W. Loftie-Eaton, personal communication)

Twenty-five µl reaction volume mixtures contained 100 ng of template DNA, 200 µM of each of the dNTP's (Roche, Germany), 50 pmol of the forward and reverse primers, 1.5 mM $MgCl_2$ and 1 U Super-Therm *Taq* DNA polymerase (JMR Holdings, Kent, UK) together with 1 × reaction buffer. PCR cycling parameters included 30 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min in a PCRSprint thermal cycler (Hybaid). An initial denaturing step at 94 °C for 2 min and a final elongation step at 72 °C for 10 min were included.

4.2.2.1.2. repB gene

Replication gene, *repB*, encoded by the IncQ plasmid RSF1010, was used as probe during DNA-DNA hybridizations (Derbyshire & Willets, 1987) (Fig. 4.2). The *repB* gene fragment was obtained by double digestion of plasmid RSF1010 with *Eco*RV and *Sca*I at 37 °C for 3 h, which yielded the 940 bp *repB'* gene.

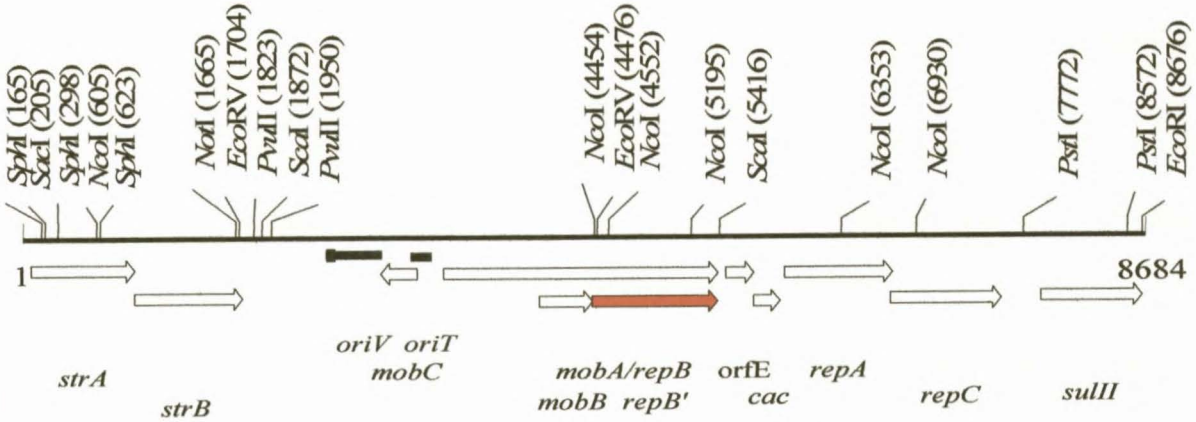

Figure 4.2. Partial restriction map of plasmid RSF1010 showing the different mobilization and replication genes, i.e., *mobC*, *mobB/repB'* and *mobA/repB* (Derbyshire & Willets, 1987).

4.2.2.1.3. Three-mob system

Construct pGEMRSFREP1 (Table 4.1) which consisted of the cloning vector pGEM-T® with the mobilization genes (*mobABC*) of RSF1010 cloned into the multiple

cloning site was used to obtain the mobilization genes for the use as a probe (Gardner & Rawlings, 2004) (Fig. 4.3).

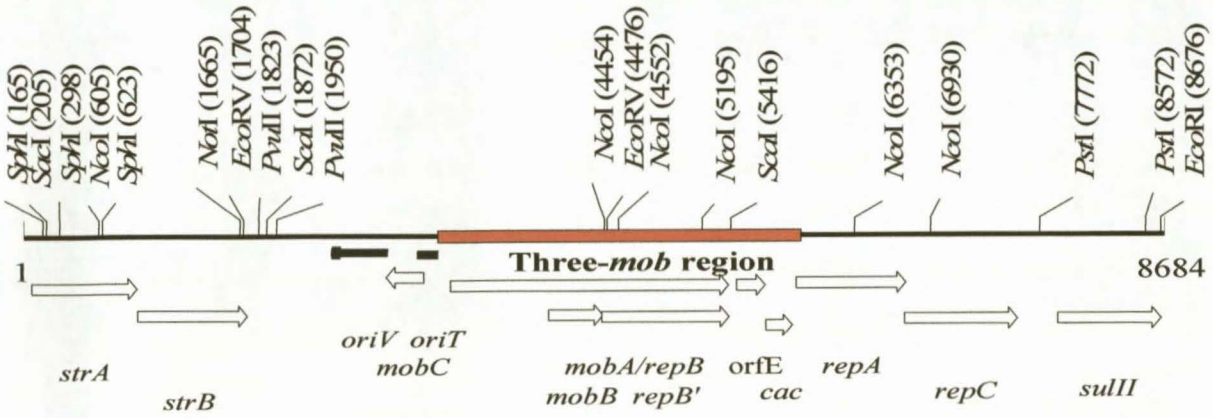

Figure 4.3. The three-*mob* region of the IncQ plasmid RSF1010 was used to establish the prevalence of IncQ-like plasmids transformed into *E. coli* DH5α (Derbyshire & Willets, 1987).

A 2765 bp fragment containing the *mobB* and *mobA/repB* genes was excised through endonuclease digestion with *Apa*I and *Pst*I for 1 h at 37 °C. A second digestion with *Sca*II for 1 h at 37 °C removed the *orfE* and *cac* genes to yield the 2347 bp mobilization region of plasmid RSF1010 (Fig. 4.3).

4.2.2.1.4. Five-mob system

Construct pMmob (Table 4.1) was digested with *Bam*HI and *Xba*I for 1 h at 37 °C to yield the 5554 bp fragment containing the mobilization genes as well as the *repB* gene encoding the primase of plasmid pTC-F14 (van Zyl *et al.*, 2003) (Fig. 4.4).

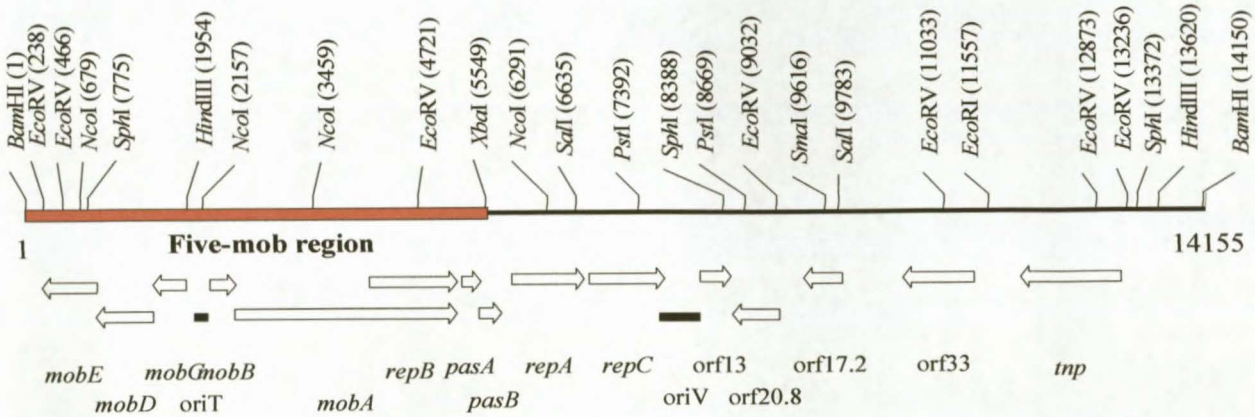

Figure 4.4. Bio-mining plasmid pTC-F14 containing a five-*mob* system, which was used as a probe during Southern hybridization experiments (Gardner *et al.*, 2001).

4.2.2.2. Restriction mapping

Restriction endonucleases were used to map two possible IncQ-like plasmids, p31T1 and p36T2. Frequently- and infrequently-cutting enzymes, which were used for single digests, included *Xba*I, *Hin*dIII, *Pst*I, *Kpn*I, *Eco*RI, *Eco*RV, *Sal*I and *Bam*HI (Fermentas, Canada; Roche, Germany). Restriction digests contained 10-20 µg plasmid DNA, 10 × complementary restriction buffer, respective enzymes and double distilled water to constitute a 20 µl restriction mixture. Plasmid restriction mixtures were incubated at 37 °C for 1 h and subjected to electrophoresis in 1% agarose gels. For accurate sizing, the O'GeneRuler™ 1 kb Plus DNA ladder Plus (Fermentas, Canada) was included and band sizes were calculated using UviPhotoMW software (UVItec, Cambridge, UK). Once the number of bands produced was calculated, double digests were performed and plasmid maps constructed in DNAMAN (Version 4.13, Lynnon BioSoft, Canada).

4.2.2.3. Mating assays

For mating purposes, plasmids were transformed into *E. coli* S17-1 and HB101 (Table 4.1) (Simon *et al.*, 1983; van Zyl *et al.*, 2003). Plasmid-containing *E. coli* S17-1 and HB101, which served as the donor, and recipient (ACSH50I^{q}) cells (Table 4.1) were

cultured separately overnight at 37 °C under constant agitation with 10 µg/ml tetracycline and 20 µg/ml chloramphenicol, respectively, for selection purposes. Cells were harvested by centrifugation at 8000 rpm for 3 min and washed three times in a saline solution (0.8% wt/vol NaCl). Donor and recipient were mixed in a 1:10 ratio and 100 µl spotted on LA plates and incubated overnight at 37 °C. Bacterial growth was collected from the plate and suspended in 5 ml saline solution. Cells were washed, pelleted by centrifugation and resuspended in 1 ml of saline. Serial dilutions (10^{-1} to 10^{-6}) were then plated onto LA media containing 10 µg/ml tetracycline and 20 µg/ml chloramphenicol together with 10 µg/ml tetracycline which was used for selecting donor and transconjugant cells, respectively. Mating experiments were done in triplicate and the transfer frequency was calculated as the number of transconjugants per donor during the overnight mating period. During the mating experiments, plasmid pRAS3.1 isolated from *A. salmonicida*, was included to establish if the transfer frequency of study plasmids were comparable to that of an IncQ-like plasmid obtained from the aquaculture environment.

4.2.3. Identification of possible IncU plasmids

To identify the prevalence of IncU plasmids among the transformed *Aeromonas* plasmids, a putative replication protein, RepB' present on plasmid pTCM1 was used as a probe for Southern hybridization experiments (L.J. van Zyl - personal communication). The first open reading frame (ORF) containing the putative replication protein similar to that of *Rhodospeudomonas palustris*, together with ORF's 2, 3 and 4 were cloned into pUCBM21 vector (L.J. van Zyl, personal communication) (Fig. 4.5). The 2.4 kb fragment was restricted with *Stu*I for 1 h at 37 °C and separated in a 1% agarose gel by electrophoresis.

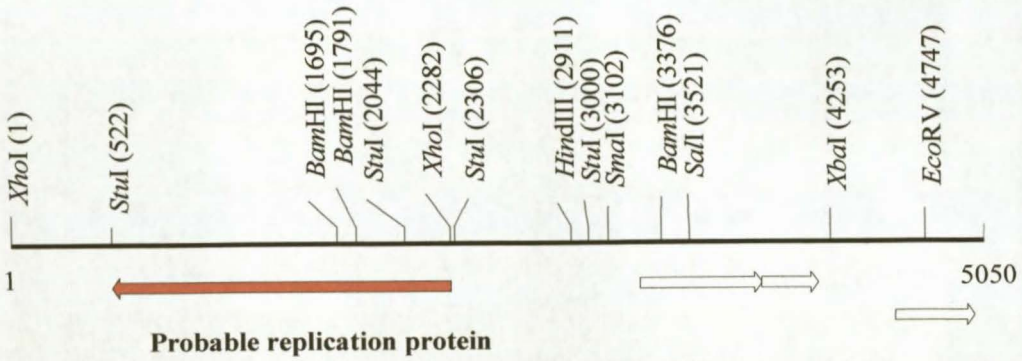

Figure 4.5. Open reading frames 1 to 4 of plasmid pTCM1 isolated from the bio-mining organism, *At. caldus* MNG. These ORF's encode a putative replication protein similar to *Rhodospeudomonas palustris* (L.J. van Zyl, personal communication).

4.2.4. Identification, characterization and mapping of the ColE1-type plasmid, p37T1, from *A. sobria*

4.2.4.1. Restriction mapping

The 26.6 kb plasmid, p37T1, was digested with an array of frequently-cutting and rare cutting-enzymes including *Eco*RV, *Sma*I, *Cfo*I, *Xba*I, *Not*I, *Taq*I, *Nhe*I, *Msp*I, *Alu*I, *Xho*I, *Sal*I, *Hinf*I, *Ase*I, *Rca*I, *Apa*I, *Hin*dIII, *Bam*HI, *Kpn*I, *Eco*RI and *Pst*I (Fermentas, Canada; Roche, Germany) (Sambrook *et al.*, 1989). Single digests with the above-mention restriction endonucleases were performed at 37 °C or 25 °C depending on the optimum temperature specified by the respective manufacturers. Double digests were performed to construct a partial map of p37T1 (Sambrook *et al.*, 1989). All restriction digests were performed according to the manufacturers' recommendations and specifications. Restriction fragments were subjected to electrophoresis in 1% agarose gels and fragment sizes were calculated using UviPhotoMW software (UVItec, Cambridge, UK) and linear plasmid maps were constructed in DNAMAN (Version 4.13, Lynnon BioSoft, Canada).

4.2.4.2. Replicon typing

*Apa*I, *Kpn*I and *Pst*I single digests of plasmid p37T1 yielded fragments that were self-ligated to form circular DNA segments (Fig. 4.6). Ligation mixtures contained 10-20 µg of respective fragment, 5 U T4 DNA ligase (Fermentas, Canada) and 10 × ligase buffer (Sambrook *et al.*, 1989). These constructs were transformed into *E. coli* DH5α and plated onto 30 µg/ml tetracycline containing LA plates, incubated overnight at 37 °C followed by the investigation of the replication potential of the constructs. As positive control, unrestricted plasmid was transformed into *E. coli* DH5α and the transformation mixture plated out onto LA plates containing 30 µg/ml tetracycline.

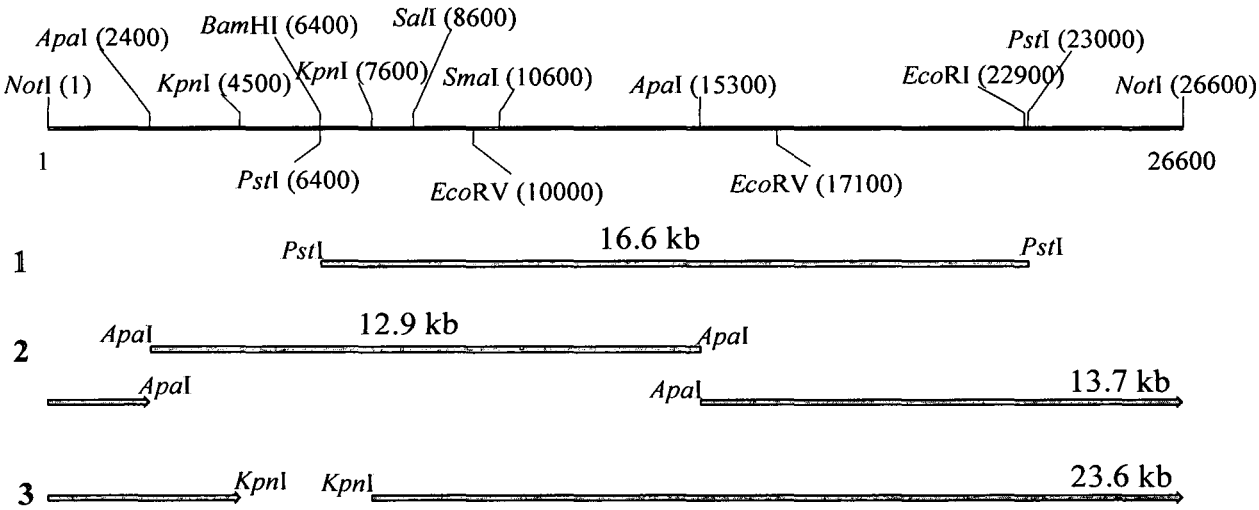

Figure 4.6. Linear restriction map of plasmid p37T1 indicting the *Apa*I, *Kpn*I and *Pst*I fragments used to identify the smallest replication unit.

4.2.4.3. Cloning and Sequencing of p37T1 DNA fragments

Plasmid p37T1 and the vector pBluescript(KS) were subjected to restriction digests for 3 h at 37 °C followed by the inactivation of the enzymes by heating the mixture at 75 °C for 10 min (Ausubel *et al.*, 1998) (Table 4.1). Plasmid bands were separated by electrophoresis in 0.8% LMP agarose gel. DNA fragments were visualized under UV light and bands corresponding to the predicted size were excised from the gel in the smallest volume possible (20 – 50 µl). Gel slices containing DNA were melted at

70 °C for at least 10 min. Ligation mixtures (200 µl) contained 35 µl of the melted gel containing the insert, 5 µl of the digested vector, 50 U T4 DNA ligase (Fermentas, Canada) and 10 × ligase buffer. Fragments were allowed to ligate overnight at room temperature. Sixty µl of the ligation mixture was used to transform 100 µl of chemically competent *E. coli* DH5α cells after which the cells were plated out on LA (X-Gal/IPTG) plates containing 100 µg/ml ampicillin. Positive constructs (Table 4.2) were selected based on the formation of white colonies of which 16 were screened for each fragment cloned. The 3.1, 3.5 and 4 kb fragments cloned into pBluescript(KS) were sequenced from both ends and compared to existing sequence data in the NCBI database.

Table 4.2. DNA fragments of plasmid p37T1 cloned into pBluescript(KS) for mapping and sequencing purposes.

Construct	Description	Source
A	1.8 kb *Apa*I-*Hin*dIII fragment of plasmid p37T1cloned into pBluescript(KS)	This study
B	1.9 kb *Kpn*I-*Bam*HI fragment of plasmid p37T1cloned into pBluescript(KS)	This study
C	2.2 kb *Hin*dIII-*Bam*HI fragment of plasmid p37T1cloned into pBluescript(KS)	This study
D	2.4 kb *Not*I-*Apa*I fragment of plasmid p37T1cloned into pBluescript(KS)	This study
E	3 kb *Pst*I-*Not*I fragment of plasmid p37T1cloned into pBluescript(KS)	This study
F	3.1 kb *Eco*RI-*Not*I fragment of plasmid p37T1cloned into pBluescript(KS)	This study
G	3 5 kb *Not*I-*Pst*I fragment of plasmid p37T1cloned into pBluescript(KS)	This study
H	4 kb *Apa*I-*Bam*HI fragment of plasmid p37T1cloned into pBluescript(KS)	This study
I	2.2 kb *Bam*HI-*Sal*I fragment of plasmid p37T1cloned into pBluescript(KS)	This study
J	4.4 kb *Hin*dIII-*Sal*I fragment of plasmid p37T1cloned into pBluescript(KS)	This study
K	7.3 kb *Eco*RI-*Hin*dIII fragment of plasmid p37T1cloned into pBluescript(KS)	This study
L	7.7 kb *Kpn*I-*Apa*I fragment of plasmid p37T1cloned into pBluescript(KS)	This study
M	7.7 kb *Apa*I-*Pst*I fragment of plasmid p37T1cloned into pBluescript(KS)	This study
N	10.9 kb *Apa*I-*Not*I fragment of plasmid p37T1cloned into pBluescript(KS)	This study
O	14.3 kb *Sal*I-*Eco*RI fragment of plasmid p37T1cloned into pBluescript(KS)	This study

4.2.4.4. Localization of accessory genes

Plasmid p37T1 was restricted with *Not*I, *Sma*I, *Ase*I and *Apa*I (both single and double digests) and blotted onto a nylon membrane as described previously in Chapter 3, section 3.2.3.1. The *tetA*, Tn*1721*, *intI* and *bla*-TEM gene probes created in Chapter 3, section 3.2.3.2. were used in Southern hybridization experiments to localize each of the respective genes on p37T1. All Southern hybridization experiments were conducted as described previously in Chapter 3, section 3.2.3. The Furushita *et al.* (2003) primer sets (Table 4.3) were used to identify the other presence of possible tetracycline resistance genes on plasmid p37T1.

Table 4.3. Sequences of primers used to identify the presence and type of tetracycline resistance determinants (Furushita *et al.*, 2003).

Gene	Primer set	Sequence	Predicted size (bp)
tetA	tetAC-F	5'-CGCYTATATYGCCGAYATCAC-3'	417
tetC	tetAC-R	5'-CCRAAWKCGGCWAGCGA-3'	
tetB	tetBDEFHJ-F	5'-GGDATTGGBCTTATYATGCC-3'	967 or 964
tetD	tetBD-R	5'-AMTACKCCCTGYAATGCA-3'	
tetE	tetBDEFHJ-F	5'-GGDATTGGBCTTATYATGCC-3'	650
tetH	tetEHJ-R	5'-AWDGTGGCDGGAATTTG-3'	
tetJ			
tetG	tetGY-F	3'-TATGCRTTKATGCAGGTC-5'	971 or 911
tetY	tetGY-R	5'-GACRAKCCAAACCCAACC-3'	

Twenty-five µl PCR reaction mixtures included 100 ng unrestricted plasmid DNA, 200 µM of each dNTP's (Roche, Germany), 50 pmol of each primer, 1.2 mM $MgCl_2$ and 1 U Super-Therm *Taq* DNA polymerase (JMR Holding, Kent, UK) together with 1 × reaction buffer. PCR cycling parameters consisted of 35 cycles of 94 °C for 30 s; 50 °C for 1 min and 72 °C for 1.5 min in a PCRSprint thermal cycler (Hybaid), although the annealing temperature for the tetAC primer set was 55 °C. Initial denaturing steps of 94 °C for 2 min and final elongation steps of 72 °C for 10 min were included to ensure sufficient denaturation and elongation. Five µl of the PCR reaction mixture was subjected to electrophoresis in a 1% agarose gel, stained in ethidium bromide and

visualized under UV light. The O'GeneRuler^{TM} 100 bp DNA Ladder (Fermentas, Canada) was included for accurate sizing of the amplicon.

E. coli DH5α-containing constructs (Table 4.2) were streaked out onto tetracycline-containing LA plates to confirm the presence of possible tetracycline resistance genes. The presence of bacterial growth after overnight incubation at 37 °C was considered as an indication of the presence of tetracycline resistance genes.

4.2.4.6. Mating experiments

ColE1-type plasmid p37T1 was transformed into *E. coli* HB101 for mating purposes. The mating experiments were performed as described previously in section 4.2.1.4.

4.2.5. Homology between p37T1 and other *Aeromonas* spp. plasmids

Total DNA preparations of 37 *Aeromonas* spp. isolates restricted with *Hin*dIII were separated in 1% gels and blotted onto nylon membranes as described previously in Chapter 3, section 3.2.3. A DNA probe of plasmid p37T1, which was restricted with *Sal*I, was created as described in Chapter 3, section 3.2.3.2. This probe was used to establish the prevalence of similar sequences among the *Aeromonas* spp. study population.

4.3. Results

4.3.1. Identification of plasmids replicating in the absence of DNA polymerase 1

Of the 23 plasmids capable of replicating in *E.coli* DH5α (Table 4.2), 21 (91%) were able to successfully replicate independently of host-encoded DNA polymerase 1. Two plasmids, i.e., p37T1 (26.6 kb) and p11T1 (23.5, 5.0 kb), were unable to replicate in the *polA*⁻ mutant *E. coli* GW125a strain and appear to be dependent on host-encoded factors to initiate replication. Plasmids p37T1 and p11T1 were isolated from *A. sobria* G2 and *A. ichtiosomia* (Chapter 2, section 2.3.1); respectively and possibly harbour a ColE1-type replicon.

4.3.2. Identification, characterization and mapping of IncQ-like plasmids

4.3.2.1. Identification of IncQ-like plasmids

To assess the prevalence of possible IncQ-like plasmids, Southern hybridizations with the *repB* gene isolated from plasmid RSF1010 were undertaken. Similarly, Southern hybridizations with the *repC* gene amplified from plasmid pRAS3.1 were carried out. Only 2 of the 23 transformed plasmids were identified as possible IncQ-like plasmids based on hybridization with the *repC* probe. Plasmids isolated from isolates *A. sobria* G1 (AE31) and *A. hydrophila* G1 (AE36), i.e., p31T1, p31T2, p31T5 and p36T2, gave positive hybridization signals (Fig.4.7B). This suggested that study plasmids (p31T1 and p36T2) harboured similar replication genes to that of pRAS3.1 and may be related to the IncQ-like plasmids. The three bands observed represent different plasmid conformations, i.e., supercoiled, circular and linear. No positive signals were observed for any of the *repC*-positive plasmids using the *repB* probe, although a strong hybridization signal was obtained for plasmid RSF1010 (Fig. 4.8B), suggesting that the identified IncQ-like plasmids contained different RepB proteins.

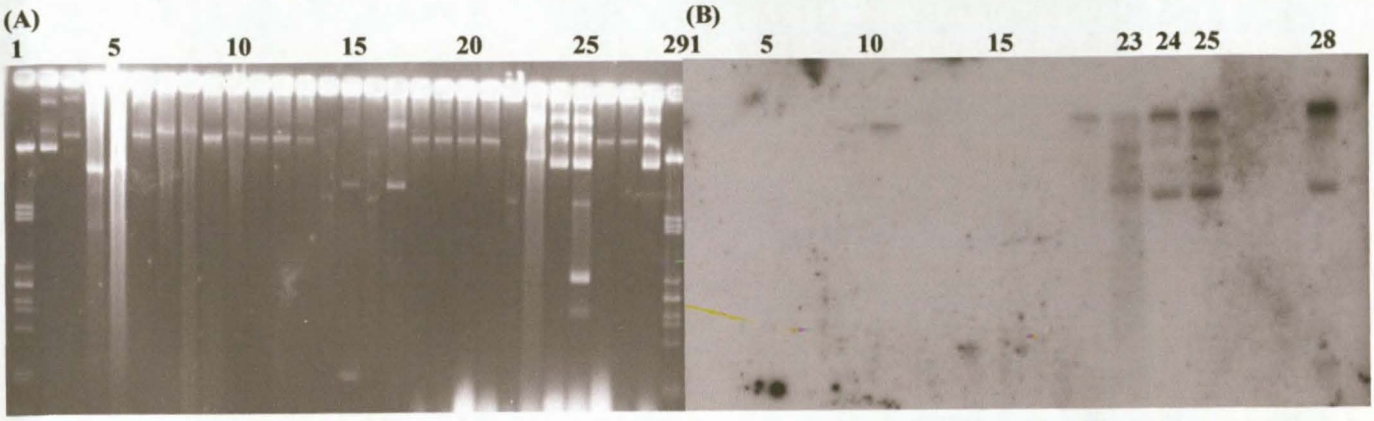

Figure 4.7. Identification of possible *repC* genes in the 23 *E. coli* DH5α transformants containing *Aeromonas* spp. plasmids. **(A)** Lanes 1 and 29: DNA molecular-weight marker (*Eco*RI and *Hin*dIII digest of λ DNA); lanes 2-28: pTC-F14, pTF-FC2, pRAS3.1, pRAS3.2, p37T1, p2T1, p2T2, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13T1, p13C1, p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2. **(B)** Southern blot hybridization with the *repC* gene probe from pRAS3.1.

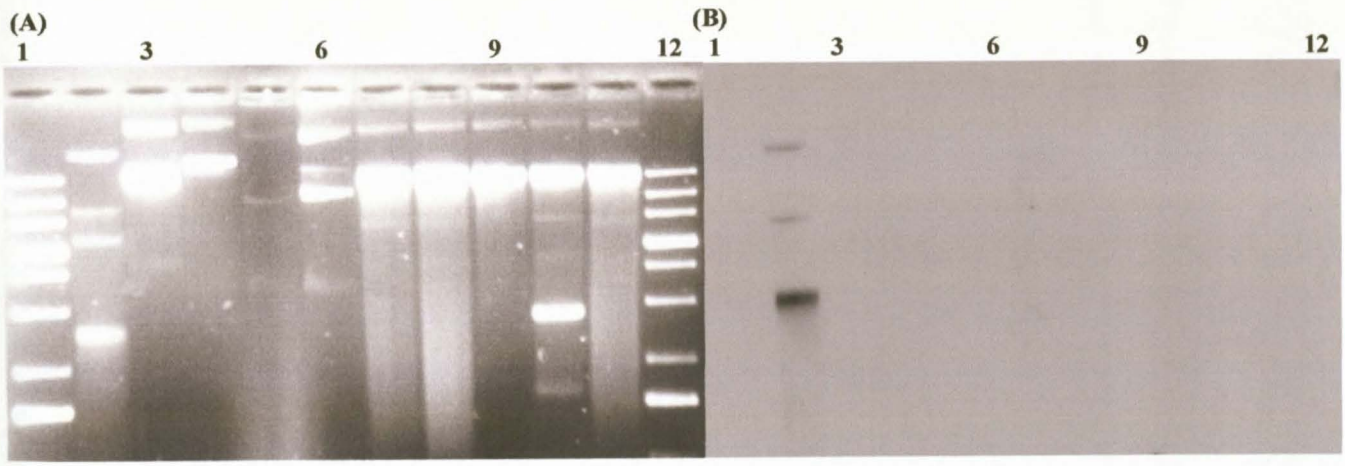

Figure 4.8. Plasmid DNA profiles of possible IncQ-like plasmids transformed into *E. coli* DH5α from *Aeromonas* spp. isolates AE31 and AE36. **(A)** Lanes 1 and 12: O'GeneRuler™ 1 kb DNA ladder Plus (Fermentas, Canada); lanes 2 – 11: plasmids RSF1010, pTC-FC2, pTF-F14, pRAS3.1, pRAS3.2, p31T1, p31T2, p31T5a, p31T5, p36T2. **(B)** Southern hybridizations with the *repB* gene of plasmid RSF1010.

To further investigate the possibility of IncQ-related plasmids within the *Aeromonas* spp. study population, the mobilization systems were compared to that of RSF1010 and pTC-F14 which are classified as IncQ and IncQ-like plasmids, respectively (Derbyshire & Willets, 1987; Gardner *et al.*, 2001). Using the three-*mob* (*mobABC*) system of the IncQ plasmid RSF1010 as a probe, a single positive hybridization signal

was observed for the smaller plasmids that co-transferred with p31T5 (Fig. 4.9B and Table 3.4).

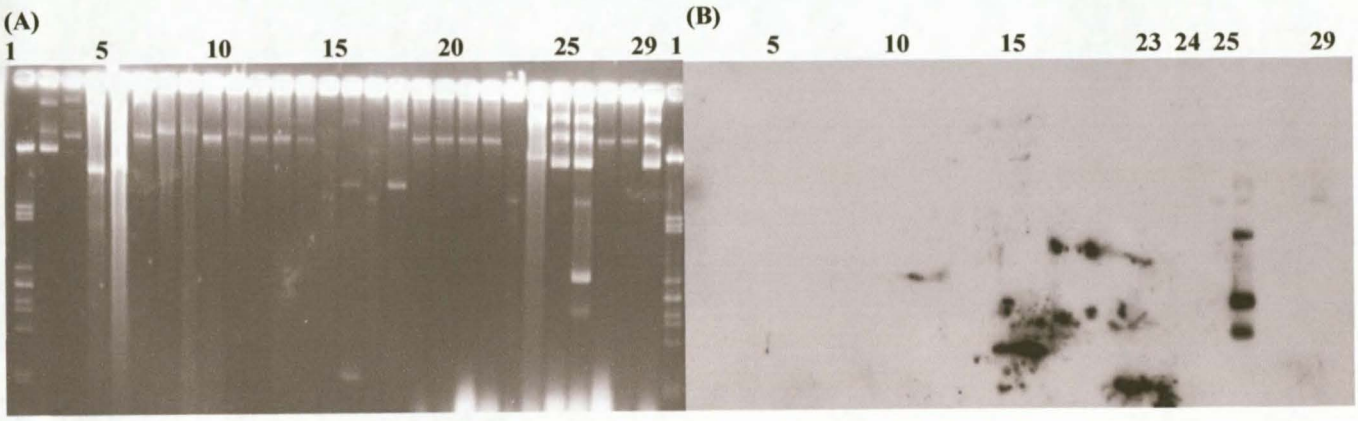

Figure 4.9. Identification of transformed *Aeromonas* spp. plasmids harbouring mobilization systems similar to the three-*mob* system of plasmid RSF1010. **(A)** Lanes 1 and 29: DNA molecular-weight marker (*Eco*RI and *Hin*dIII digest of λ DNA); lanes 2-28: pTC-F14, pTF-FC2, pRAS3.1, pRAS3.2, p37T1, p2T1, p2T2, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13T1, p13C1, p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2. **(B)** Southern blot hybridizations with the three-*mob* system of plasmid RSF1010.

The same result was obtained using the five-*mob* system (*mobABCDE*) of the IncQ-like plasmid, pTC-F14 (Fig.4.10B). No hybridization signals using the two mobilization regions were obtained for the plasmids to which the pRAS3.1 *repC* gene probe hybridized. However, positive hybridization signals were observed for the 1.9 and 4.8 kb plasmids that were co-transferred with plasmid p31T5, as obtained with the three-*mob* system. The lack of positive hybridization signals with the two mobilization gene systems suggests that the study plasmids contain different mobilization genes and could possibly belong to a new sub-group of IncQ-related plasmids. Overall, the results obtained in this section suggested that the study IncQ-like plasmids not only contained different mobilization genes, but different replication proteins as well.

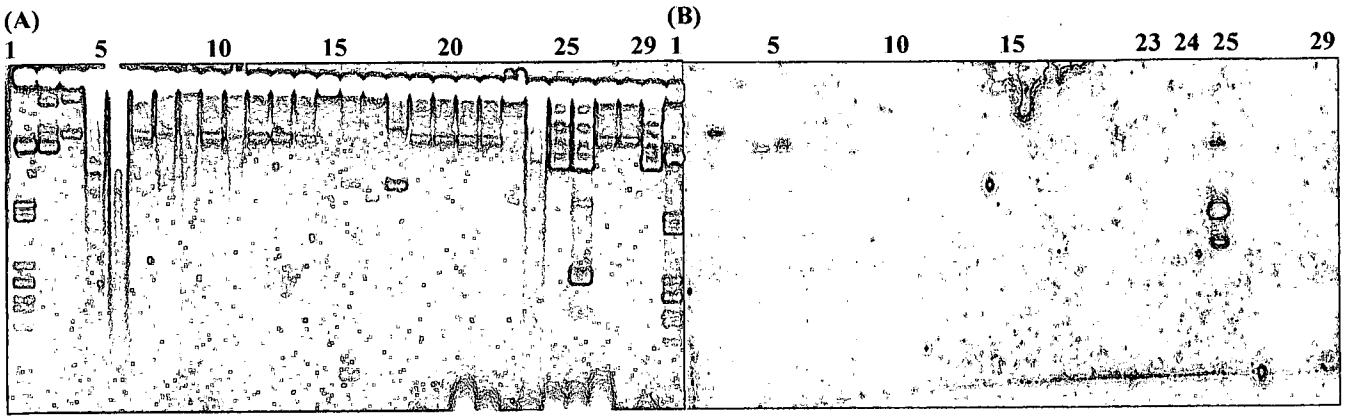

Figure 4.10. Identification of transformed *Aeromonas* spp. plasmids harbouring mobilization regions similar to the five-*mob* system of the bio-mining plasmid pTC-F17. **(A)** Lanes 1 and 29: DNA molecular-weight marker (*Eco*RI and *Hin*dIII digest of λ DNA); lanes 2-28: pTC-F14, pTF-FC2, pRAS3.1, pRAS3.2, plasmids p37T1, p2T1, p2T2, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13T1, p13C1, p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2. **(B)** Southern blot hybridizations with the five-*mob* system of plasmid pTC-F14.

4.3.1.3. Mapping of possible IncQ-like plasmids

Single endonuclease digestions of IncQ-like plasmid DNA were performed with *Eco*RI, *Bam*HI, *Kpn*I, *Xho*I, *Sal*I and *Eco*RV, respectively, and double digestions were also performed with combinations of the above-mentioned enzymes. Endonuclease digestions of plasmids p31T1 and p36T2 revealed that these plasmids, isolated from *A. sobria* G1 and *A. hydrophila* G1 isolates, respectively, were identical (Fig. 4.11). *Hin*dIII and *Xba*I digestions did not result in plasmid restriction, i.e., no corresponding restriction sites were present on either of these plasmids, while *Pst*I restriction digests resulted in seven fragments and *Kpn*I in 4 fragments. These restrictions could be used with further endonuclease restriction digests to construct a detailed map.

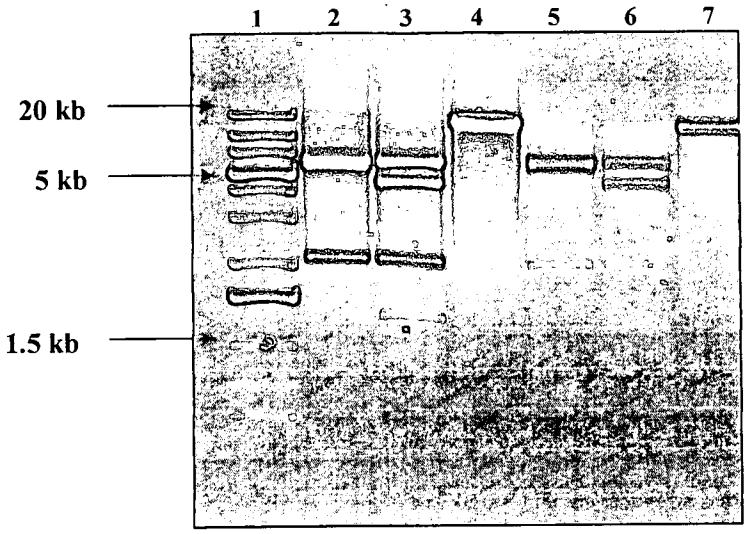

Figure 4.11. Endonuclease digestions of plasmids p31T1 and p36T2. Lane 1: O'GeneRuler™ 1 kb DNA ladder Plus (Fermentas, Canada); lanes 2-4: plasmid p31T1 restricted with *Sal*I, *Sal*I + *Eco*RI, and *Eco*RI, respectively; lanes 5-7: plasmid p36T2 restricted with *Sal*I, *Sal*I + *Eco*RI, and *Eco*RI, respectively.

A preliminary map was drawn up based on the restriction digestions and is depicted in Fig. 4.12.

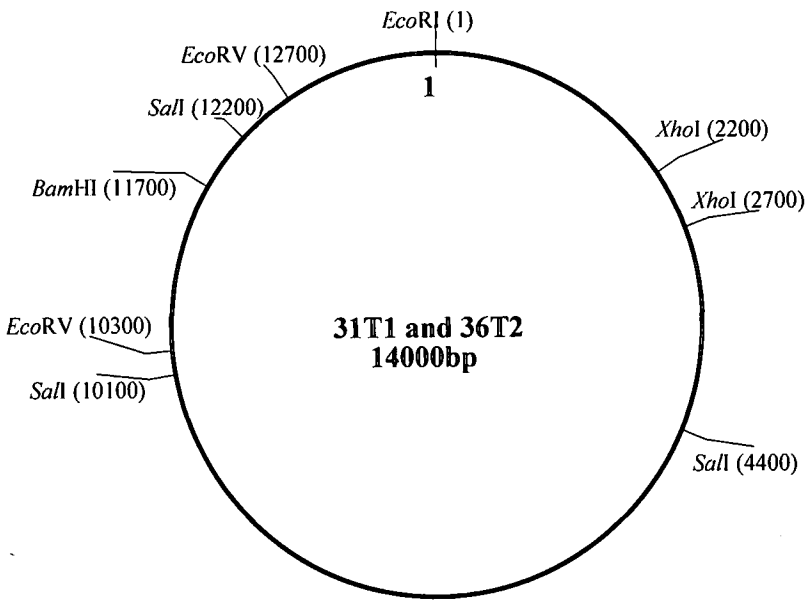

Figure 4.12. Preliminary restriction map of the IncQ-like plasmids p31T1 and p36T2 isolated from *A. sobria* G1 and *A. hydrophila* G1, respectively.

4.3.1.4. Mating frequencies of IncQ-like plasmids

The IncQ-like plasmids, p31T1 and p36T2, were not self-transmissible, since no conjugal transfer of the plasmids was observed during mating experiments using *E. coli* HB101 as donor. To assess if the two 14 kb IncQ-like plasmids, p36T2 and p31T1 plasmids were mobilizable and to determine their mating frequencies, they were transformed into an *E. coli* S17-1 strain which has an RP4 plasmid derivative integrated into the chromosome to provide the mating pair formation functions required for plasmid mobilization (Simon *et al.*, 1983). The mating frequency of the two IncQ-like plasmids and pRAS3.1 is shown in Table 4.4.

Table 4.4. Mating frequencies of IncQ-like plasmids and pRAS3.1 following overnight mating.

Strain	Plasmid transferred	Plasmid size (kb)	Transfer frequency
AE31	p31T1	14	2.25×10^{-5}
AE31	p31T5	14; 4.8; 1.9	1.12×10^{-3}
AE36	p36T2	14	3.59×10^{-2}
A. salmonicida	pRAS3.1	11,851	saturation*

* Mating period of 30 min (personal communication - W. Loftie-Eaton)

Plasmid preparation p31T5 contained the 14 kb IncQ-like plasmid (p31T1) along with two smaller plasmids of 4.8 and 1.9 kb, while p31T1 and p36T2 preparations contained the single 14 kb IncQ-like plasmids. The general transfer frequency of the screened plasmids was low and only detectable after a 16 h mating period. Plasmid p31T1 displayed the lowest transfer frequency, while p31T5 showed a 100-times higher frequency than that of p31T1 (Table 4.4). The presence of the 4.8 and 1.9 kb plasmids could have impacted the transfer rate, since a difference was observed between the transfer frequencies of p31T1 and p31T5. The highest transfer frequency was observed for p36T2 which was 2000- and 30-times greater than that of p31T1 and p31T5, respectively (Table 4.4). No similarity was observed between the transfer frequencies of the study plasmids and pRAS3.1, since the extended mating time of 16 h resulted in the saturation of the transconjugants of the pRAS3.1 mating. Plasmid pRAS3.1 achieved saturation after 30 min, meaning that the mating frequency was greater than 1, indicating a high transfer frequency (personal communication, W. Loftie-Eaton). Transformed plasmids were successfully re-isolated from the recipient after conjugation (Fig. 4.13).

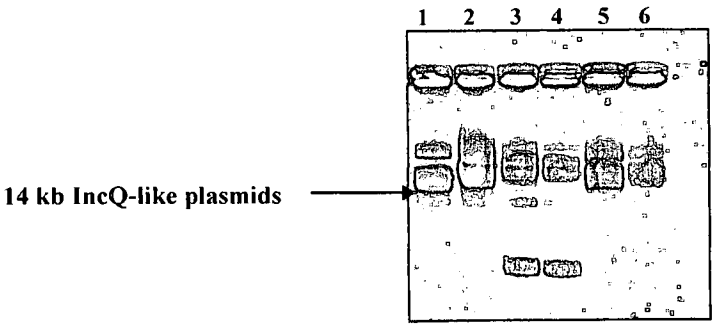

Figure 4.13. Plasmid profiles of transformants containing IncQ-like plasmids from *Aeromonas* spp. isolates AE31 and AE36 and transconjugant cells after mating of plasmids p31T1, p31T5 and p36T2 with recipient, *E. coli* ACSH50I^{q}. Lanes 1-6: p31T, pT31 transconjugant, p31T5, p31T5 transconjugant, p36T2, p36T2 transconjugant.

4.3.2. Identification of possible IncU plasmids

Plasmid profiles of *E. coli* DH5α transformed with *Aeromonas* spp. plasmids were screened for the presence of possible IncU-type replicons, with the putative replication protein identified on plasmid pTCM1. A single positive hybridization signal was obtained for the 4.8 kb plasmid, co-transferred with the 14 kb IncQ-like plasmid p31T5, using the putative replication protein of *Rhodospeudomonas palustris* which showed a high level of nucleotide similarity to a IncU RepB protein (Fig. 4.14B).

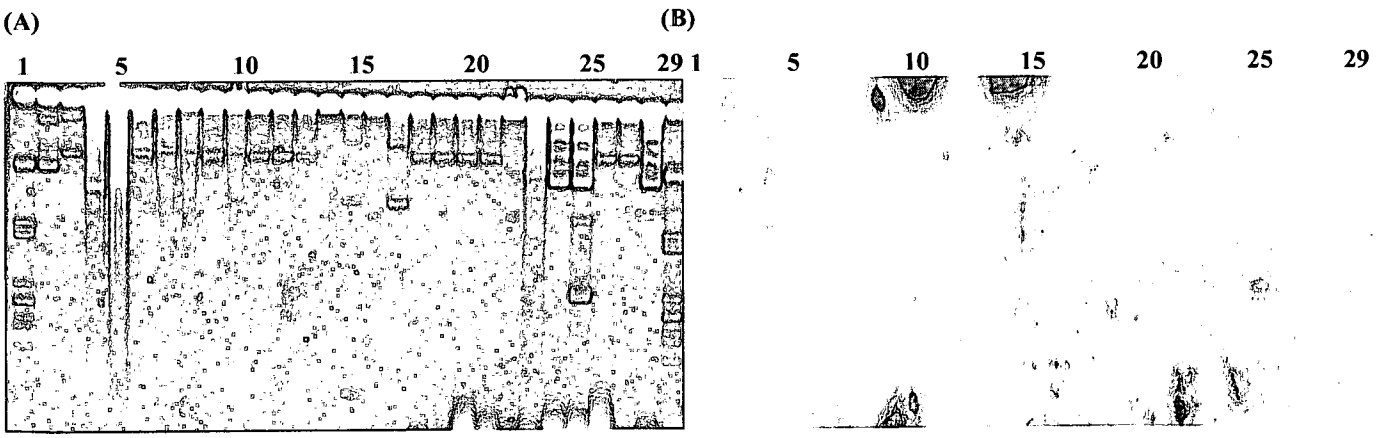

Figure 4.14. Plasmid profiles of 23 *Aeromonas* plasmids transformed into *E. coli* DH5α and identification of possible IncU replicons. **(A)** Lanes 1 and 29: DNA molecular-weight marker (*Eco*RI and *Hin*dIII digest of λ DNA); lanes 2-28: pTC-F14, pTF-FC2, pRAS3.1, pRAS3.2, p37T1, p2T1, p2T2, p3A5, p4T1, p4C1, p8C5, p9C1, p11T1, p11T14, p12T1, p13T1, p13C1, p15C1, p16C1, p24C1, p25T1, p31T1, p31T2, p31T5, p33C1, p34C2, p36T2. **(B)** Southern blot hybridization with the *repB* gene of plasmid pTCM1 resulted in a single positive hybridization signals for the smaller sizes plasmid that was co-transferred with plasmid p31T5.

Although the stringency of the hybridization assay was decreased (probe hybridization temperature lowered from 42 °C to 37 °C and lower stringency washes), no other signals were obtained.

4.3.3. Characterization of the ColE1-type plasmid, p37T1 isolated from *A. sobria*

4.3.3.1. Map construction of p37T1

Endonuclease restriction of plasmid p37T1 revealed the presence of unique *Not*I and *Ase*I sites (Fig. 4.15). The unique *Not*I site was used as a point of reference for mapping purposes.

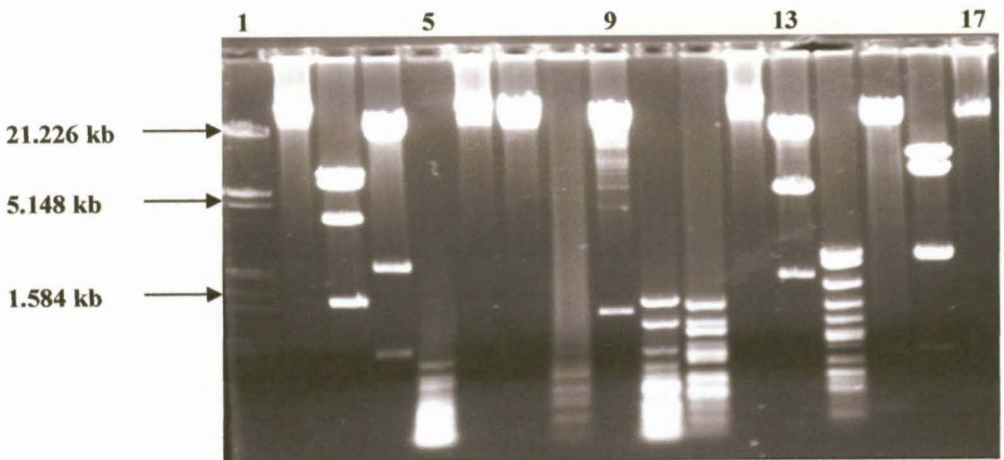

Figure 4.15. Endonuclease digests of plasmid p37T1. Lane 1: λ DNA restricted with *Eco*RI and *Hin*dIII; lanes 2–17: uncut plasmid DNA (p37T1) from isolate AE37, plasmid p37T1 restricted with *Eco*RV, *Sma*I, *Cfo*I, *Xba*I, *Not*I, *Taq*I, *Nhe*I, *Msp*I, *Alu*I, *Xho*I, *Sal*I, *Hin*fI, *Ase*I, *Rca*I and *Apa*I, respectively.

Following double digests, it was apparent that *Xba*I and *Xho*I did not have restriction sites on the plasmid, since double digests with *Apa*I and these two enzymes resulted in only two fragments, which were identical to that produced following *Apa*I restriction (Fig 4.16). Frequently-cutting enzymes such as *Cfo*I, *Taq*I, *Nhe*I, *Msp*I, *Alu*I and *Hin*fI were not considered for map construction due to the large number of fragments obtained following respective restrictions.

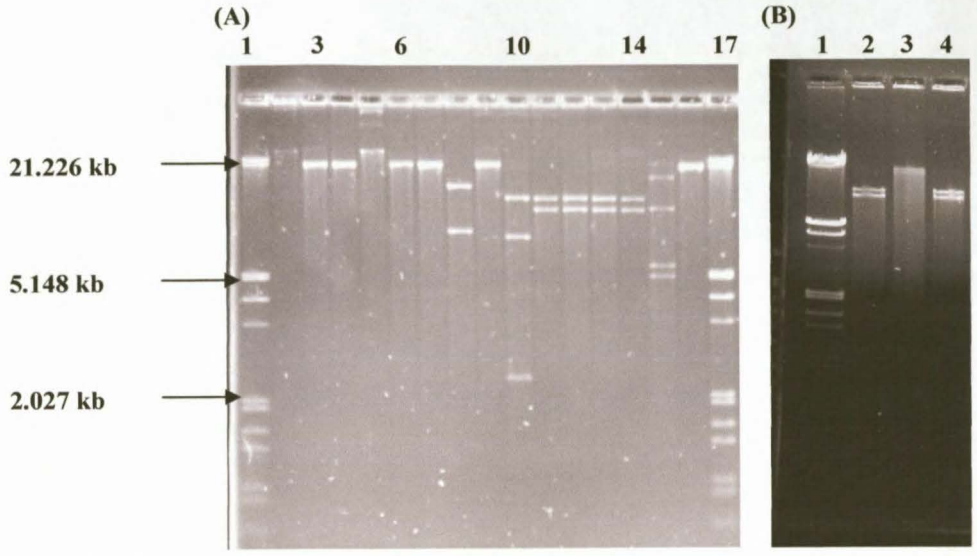

Figure 4.16. Double and single endonuclease restriction of plasmid p37T1. **(A)** Lanes 1 and 17: λ DNA restricted with *Eco*RI and *Hin*dIII; lanes 2–16: uncut plasmid DNA (p37T1) from isolate AE37, plasmid p37T1 restricted with *Not*I, *Not*I + *Xho*I, *Xho*I, *Xho*I + *Ase*I, *Ase*I, *Ase*I + *Not*I, *Not*I, NotI + *Apa*I, *Apa*I, *Apa*I + *Xho*I, *Apa*I, *Apa*I, *Apa*I + *Ase*I and *Ase*I. **(B)** Lane 1: λ DNA restricted with *Eco*RI and *Hin*dIII; lanes 2-4: p37T1 restricted with *Apa*I, *Xba*I and *Apa*I + *Xba*I.

A preliminary map was constructed based on the fragment profiles obtained following different double digests (Fig. 4.17).

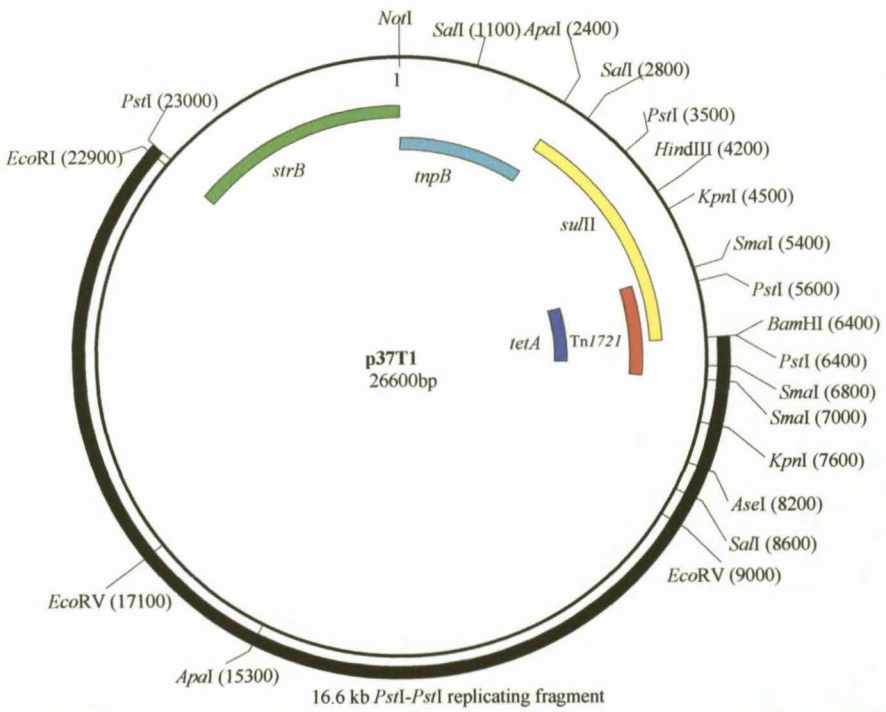

Figure 4.17. Restriction map of plasmid p37T1 isolated from *A. sobria* G2, constructed after single and double digests with restriction endonucleases.

4.3.3.2. Identification of the replicon

The 4 self-ligated (Fig.4.6) fragments (16.6 kb *Pst*I-*Pst*I, 12.9 and 13.7 kb *Apa*I-*Apa*I and 23.6 kb *Kpn*I-*Kpn*I fragments) were screened for their replicating capabilities in *E. coli* DH5α. Of the self-ligated fragments, the 16.6 kb *Pst*I fragment represented the smallest DNA segment capable of replicating in *E. coli* DH5α (Fig. 4.17, indicated by solid black line).

4.3.3.3. Sequencing of p37T1

Partial sequencing of plasmid p37T1 restriction clones revealed the presence of antibiotic resistance determinants and genes responsible for transposition of associated genes. Sequences obtained from cloned fragments were compared to other known sequences by the NCBI Blast program (Altschul *et al.*, 1997) and the *strB*, *tnpB* and *sulII* genes were identified and located. The 3.1 kb *Eco*RI-*Not*I fragment showed 99% nucleotide sequence identity to the 836 bp streptomycin resistance genes, *strB*, identified as part of plasmids obtained from several pathogenic organisms including pU302L isolated from *Salmonella typhimurium* and p9123 isolated from *E. coli* (Fig. 4.17, indicated by solid green line).

Sequencing data obtained from the 2.4 kb *Not*I-*Apa*I fragment revealed the presence of a transposase (*tnpB*) gene with 98% nucleotide sequence identity to the 539 bp truncated gene present on the *V. cholerae* plasmid, pKA1 (Fig. 4.17, indicated by solid turquoise line). Hundred percent nucleotide sequence identity between the sequence obtained and *tnpB* encoded by *orfA* of *E. coli* and the truncated form present in the *V. cholera* SXT genome was also observed. A high level of nucleotide sequence homology was obtained for the 4 kb *Apa*I-*Bam*HI fragment, which showed 100% nucleotide homology to the 815 bp sulphonamide resistance determinant, *sulII*, present on the IncQ plasmid RSF1010, p9123 and pNG828-5, all isolated from *E. coli*, pK245 isolated from *K. pneumoniae*, pSY7K isolated from *S. enteritidis* and plasmid pIE1115 from an uncultured bacterium (Fig. 4.17, indicated by solid yellow line).

4.3.3.4. Presence of the tetA gene and Tn1721 on p37T1

The 417 bp *tetAC* gene was successfully amplified from plasmid p37T1 (Fig. 4.18). No amplicons were obtained for the *tetBD, tetGY* or *tetEHJ* genes indicating that these tetracycline resistance genes were not present on plasmid p37T1. Restriction analysis revealed that the *tetA* gene was present on plasmid p37T1 (data not shown).

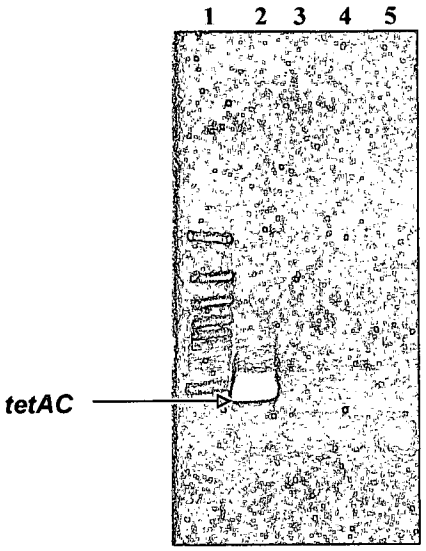

Figure 4.18. Agarose gel electrophoresis of tetracycline resistance gene amplified from plasmid p37T1 using primer sets described by Furushita *et al.* (2003). Lanes 1-5: O'GeneRuler™ 100 bp Ladder Plus (Fermentas, Canada), tetAC, tetBD, tetEHJ and tetGY amplicons.

To identify the location of the *tetA* resistance gene on p37T1, cloned fragments of the plasmid transformed into *E. coli* DH5α were plated onto 30 µl/ml tetracycline-containing plates. Vector containing the 2.2 kb *Bam*HI-*Sal*I fragment conferred tetracycline resistance to the transformed DH5α cells, which were capable of growth on LA plates containing 30 µg/µl of the antibiotic. The larger 4.4 kb *Hin*dIII-*Sal*I fragment which consists of regions flanking the 2.2 kb fragment, also conferred resistance to tetracycline.

Southern hybridizations with the 300 bp *tetA* gene probe (Schnabel & Jones, 1999), confirmed the presence of the tetracycline resistance gene on the 2.2 kb *Bam*HI-*Sal*I fragment. A positive hybridization signal was observed for the 1.4 kb *Sma*I-*Sma*I

fragment, which was used to locate the gene (Fig. 4.17, indicated by solid blue line and Fig. 4. 19). The 1.4 kb fragment forms part of the *Bam*HI-*Sal*I fragment (Fig. 4.17). Since *Not*I and *Ase*I only restricted plasmid p37T1 once, positive signals were also observed in lanes 1, 4 and 10 (Fig. 4.19).

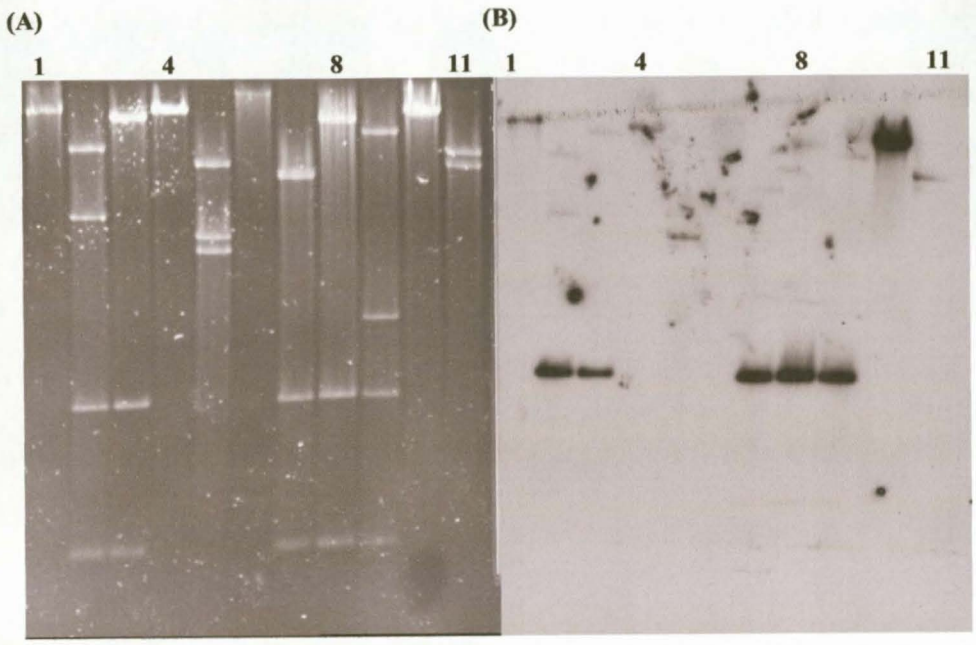

Figure 4.19. Restriction endonuclease profiles of plasmid p37T1 following single and double digests. **(A)** Lanes 1 -11: plasmid DNA restricted with *Not*I, *Not*I + *Sma*I, *Sma*I, *Ase*I, *Ase*I + *Apa*I, *Ase*I, *Sma*I + *Apa*I, *Sma*I, *Ase*I + *Sma*I, *Ase*I and *Apa*I. **(B)** Southern hybridization with the *tetA* probe (Schnabel & Jones, 1999) resulted in positive hybridization signals with the 1400 bp *Sma*I-*Sma*I fragment.

Similarly, when p37T1 restriction profiles were probed with the Tn*1721* probe, positive signals were used to identify the location of the transposon on p37T1 (Fig. 4.17, indicated by solid red line and Fig. 4.20). The 1400 bp *Sma*I-*Sma*I as well as the 200 bp *Sma*I-*Sma*I fragment produced strong hybridization signals with the Tn*1721* probe (Fig. 4.20).

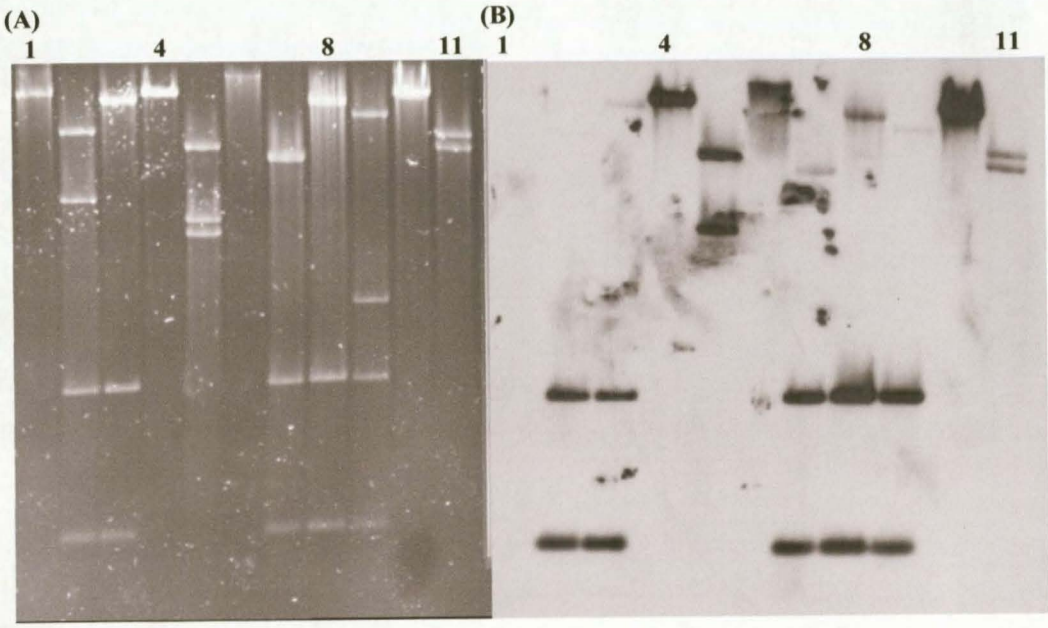

Figure 4.20. Restriction endonuclease profile generated of plasmid p37T1. **(A)** Lanes 1 -11: plasmid restricted with *Not*I, *Not*I + *Sma*I, *Sma*I, *Ase*I, *Ase*I + *Apa*I, *Ase*I, *Sma*I + *Apa*I, *Sma*I, *Ase*I + *Sma*I, *Ase*I and *Apa*I. **(B)** Southern hybridization with the Tn*1721* probe resulted in positive hybridization signals for the 1400 bp *Sma*I-*Sma*I fragment.

To identify the location of the β-lactamase resistance determinant and class 1 integron, the *bla*-TEM and *intI* probes were used. No positive hybridization signals were observed for both probes which indicated that another type of β-lactamase resistance marker was present and confirmed the absence of a class 1 integron.

4.3.3.5. Mating experiments

The 27 kb ColE1-type plasmid, p37T1 was not transferred to the recipient during mating experiments and was therefore, not self-transmissible. No further experiments to mobilize the plasmid were conducted due to difficulties in selecting an appropriated antibiotic marker as the plasmid possessed gene conferring resistance to 14 different antibiotics.

2.4.5. Prevalence of plasmid p37T1

Screening the total DNA content of the *Aeromonas* spp. isolates with the *Sal*I digest of plasmid p37T1, which generated 3 fragments (Fig. 4.17) allowed the identification of 15 isolates, i.e., AE2, AE9, AE11, AE13, AE14, AE15, AE19, AE23, AE25, AE28, AE29, AE30, AE32, AE33 and AE34 which contained similar DNA sequences that resulted in positive hybridization signals. Fifty-three percent of these isolates, i.e., AE2, AE3, AE4, AE9, AE11, AE15, AE24, AE25, AE33 and AE34 contained plasmids as reported in Chapter 2, section 2.3.1. The distinct hybridization signals observed may be the result of sequence similarity of plasmids cleaved with *Hin*dIII or genomic DNA fragments generated by *Hin*dIII restriction.

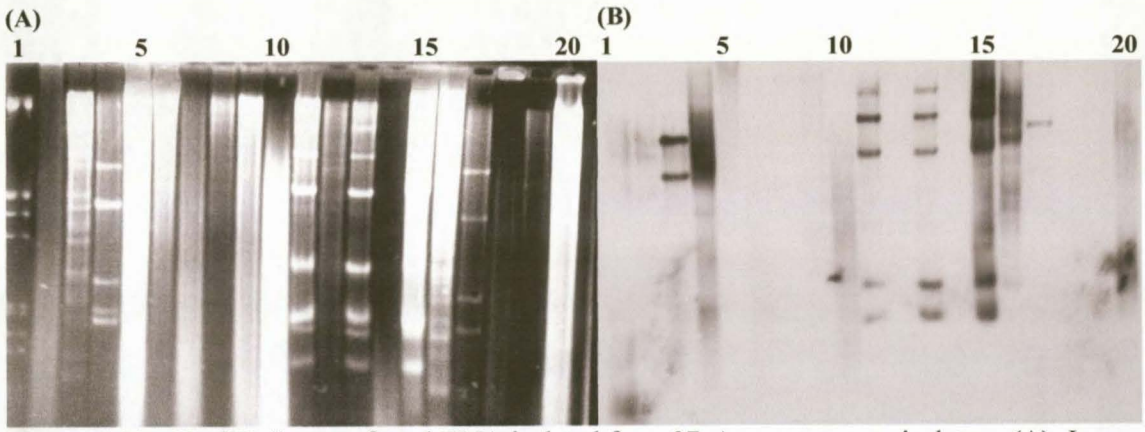

Figure 4.21. *Hin*dIII digests of total DNA isolated from 37 *Aeromonas* spp. isolates. **(A)** Lane 1: DNA molecular-weight marker (phage λ DNA digested with *Eco*RI and *Hin*dIII); lanes 2-20: plasmid p37T1, AE1, AE2, AE3, AE4, AE5, AE6, AE7, AE8, AE9, AE10, AE11, AE12, AE13, AE14, AE15, AE16, AE17, AE18. **(B)** Southern hybridization with the p37T1 (*Sal*I) probe to establish similarity between this plasmid and other possible *Aeromonas* spp. DNA

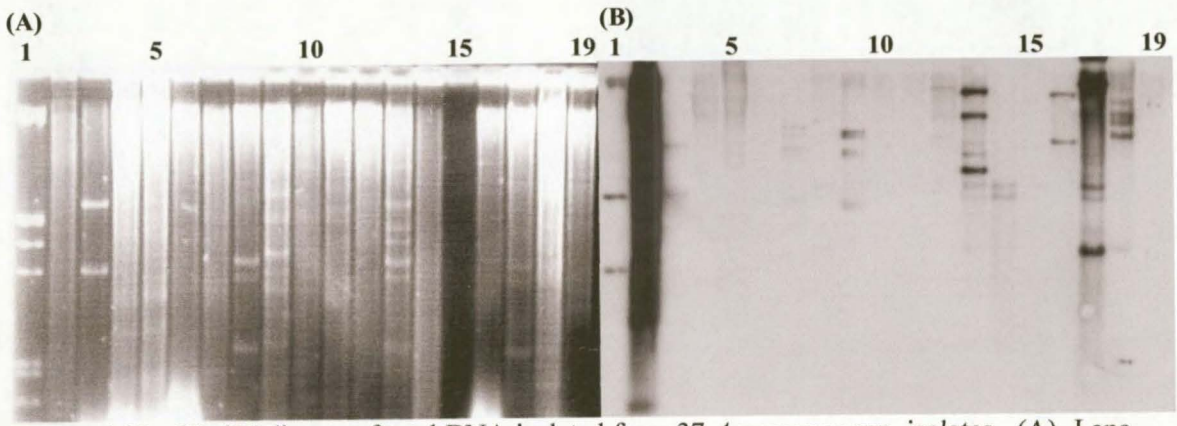

Figure 4.22. *Hin*dIII digests of total DNA isolated from 37 *Aeromonas* spp. isolates. (A) Lane 1: DNA molecular-weight marker (phage λ DNA digested with *Eco*RI and *Hin*dIII); lanes 2-19: plasmid p37T1, AE19, AE20, AE21, AE22, AE23, AE24, AE25, AE26, AE27, AE28, AE29, AE30, AE31, AE32, AE33, AE34, AE35. **(B)** Southern hybridization with the p37T1 (*Sal*I) probe to establish similarity between this plasmid and other possible *Aeromonas* spp. DNA

4.4. Discussion

The classification of R-plasmids isolated from environmental *Aeromonas* spp. is an important step in understanding the role that these plasmids play in the dissemination of antibiotic resistance determinants. The replication of plasmids independently of host-encoded factors allows plasmids to travel quickly between different hosts allowing the transfer of plasmids and the genes they carry. Furthermore, the classification of plasmids from different environments could provide insight into the movement of plasmids and the various resistance genes they encode between different environmental compartments (Rhodes *et al.*, 2000). This is an important factor regarding human health since contact between humans and diseased fish could give rise to infections not responsive to antibiotic treatment caused by opportunistic pathogens such as *A. hydrophila*, which acquired antibiotic resistance through horizontal gene transfer.

IncQ replicons contain three essential replication genes namely *repA, repB* and *repC*. These replication proteins are used to establish the relatedness of members of the IncQ plasmid family (Rawlings & Tietze, 2001). The isolation of possible IncQ-like plasmids from an aquatic environment is not unusual as plasmid pRAS3 was originally

isolated from the aquatic bacterium *A. salmonicida* (L'Abee-Lund & Sϕrum, 2002). Furthermore, plasmids harbouring IncQ-like replicons have been isolated from various other environmental niches, numerous hosts and geographical areas indicating their broad-host-range and promiscuous nature (Bonemann *et al.*, 2006; Dorrington & Rawlings, 1989; Rhodes *et al.*, 2004; Smalla *et al.*, 2000). The similarity in the gene organization of the replication and mobilization genes of pRAS3 and pTF-FC2 and pTC-F14 ranges between 89.5% and 98.2%, showing that these genes are conserved between plasmids (L'Abee-Lund & Sϕrum, 2002). The plasmids positive for the *repC* gene showed no homology to the described three-*mob* and five-*mob* regions of IncQ and IncQ-like plasmids. The smaller plasmids (4.8 kb and 1.9 kb) that co-transferred with p31T1 gave positive signals with both three- and five-*mob* systems. It is possible that these plasmids harbour a different replicon type that allows the different plasmids to be maintained in the same host cell. No homology was found between the *Aeromonas* spp. plasmids and the *repB* gene isolated from plasmid RSF1010. This does not correlate with the work of Smalla *et al.* (2000), where they used the *repB* gene, and *oriV* and *oriT* regions of RSF10101 to identify possible IncQ-related R-plasmids. Smalla *et al.* (2000) have noted a high degree of variability in the backbone DNA. When screening plasmids obtained by biparental matings with *Pseudomonas putida*, numerous positive hybridization signals with the IncQ *oriV* probe were obtained while hybridization with the IncQ *oriT* probe revealed the absence of *oriT*-specific sequences in several plasmids that gave positive and weaker signals with the *oriV* and *repB* probes, respectively (Smalla *et al.*, 2000). It is likely that plasmids p31T1 and p36T2, originally isolated from *A. hydrophila* and *A. sobria* strains, respectively, may possibly constitute a new group of IncQ-like plasmids. This is the first report of IncQ-related *Aeromonas* spp. plasmids isolated from a South African aquaculture system. Southern hybridization experiments did not allow discrimination between the different replication and mobilization genes, but were used primarily to establish the level of similarity and to identify plasmids potentially related to the IncQ plasmid family.

Plasmids p31T1 and p36T2 differ from pRAS3 in several respects. Firstly, p31T1 and p36T2 are approximately 14 kb compared to the smaller 11.851 kb and 11.823 kb pRAS3.1 and pRAS3.2, respectively (L'Abee-Lund & Sϕrum, 2002). Differences with

respect to tetracycline resistance genes of pRAS3 and p31T1 and p36T2 were observed with pRAS3 harbouring a *tetC* gene, while *tetA* gene was detected on p31T1 and p36T2, respectively. Futhermore, the tetracycline-associated transposon Tn*1721* was identified on p31T1 and p36T2 which was also absent on pRAS3. Plasmids p31T1 and p36T2 encoded resistance to erythromycin and the first generation quinolone, nalidixic acid, characteristics absent in pRAS3 (L'Abee-Lund & Sϕrum, 2002). This resistance phenotype is similar to that displayed by the 88469 bp IncQ-like plasmid pGNB2 which also carried remnants of Tn*1721* and the nalidixic acid resistance gene *qnrS2* (Bonemann *et al.*, 2006). Restriction analysis of plasmids p31T1 and p36T2 indicated differences with respect to unique sites and the number of restriction site when compared to the pRAS3 plasmids. The additional resistance determinants present on p31T1 and p36T2 and the presence of the tetracycline-associated transposon, Tn*1721* would account for the size difference.

The failure to transfer p31T1 and p36T2 to the recipient, *E. coli* ASCH50I^{q} through conjugation is in correlation with the conjugation experiments by L'Abeé-Lund and Sørum (2002). Plasmid pRAS3 was not transferred from donor to recipient, but was mobilized by the presence of other conjugative plasmids such as pRAS1 and pRAS2 (L'Abee-Lund & Sϕrum, 2002). No other plasmids were, however, used to establish the mobilization efficiency of this plasmid. The mobilization of p31T1 and p36T2 by RP4 (IncPα) is supported by studies showing that members of the IncQ plasmid family are readily mobilized by IncP plasmids (Derbyshire & Willets, 1987). Although both plasmids showed a positive hybridization signal with the *repC* probe, differences in the transfer frequencies were noted. Van Zyl *et al.* (2003) compared the transfer frequencies of the mobilization regions of two related IncQ-like plasmids, pTC-F14 and pTF-FC2 and concluded that pTC-F14 was transferred/mobilized at a 3000-fold lower frequency compared to that of pTF-FC2, although similar replicon types were present. It is clear that different plasmid families have an impact on the mobilization efficiencies of certain plasmids (van Zyl *et al.*, 2003). No comparison between the transfer frequencies of the plasmids used in this study and that of pRAS3 could be made due to the extended mating period allowed for p31T1 and p36T2. Plasmid pRAS3.1 mated with ASCH50I^{q} to saturation in less than an hour while no transconjugants were detected for p31T1 and

p36T2 in this same period. This indicated that plasmids p31T1 and p36T2 have a much lower mating frequency than that of pRAS3.1, which taken together with the Southern hybridization results suggests a novel mobilization system. The transfer of several *Aeromonas* spp. R-plasmids has been investigated and variations in the transfer frequencies have been indicated (Bruun *et al.*, 2003). However, the mobilization of non-transferable R-plasmids and their impact on horizontal gene transfer remains poorly understood. Furthermore, the transfer of R-plasmids has been shown to occur at a lower temperature of 15°C, suggesting the transfer of possible antibiotic resistance determinants to the broader bacterial population including psychrophilic and mesophilic organisms (Casas *et al.*, 2005).

Plasmid p37T1, a ColE-type plasmid isolated from *A. sobria*, has a narrow-host-range due to the dependence on host-encoded factors to initiate plasmid replication. The inability of plasmid p37T1 to be transferred by conjugation further emphasizes its narrow-host-range. Plasmids harbouring a ColE1-type replicon have been isolated from *A. salmonicida* from an aquatic environment (Boyd *et al.*, 2003). These plasmids characterized by Boyd *et al.* (2003) were cryptic in nature, 5.2 – 5.6 kb in size and did not contain any antibiotic resistance determinants. On the other hand, plasmid p37T1 was 26.6 kb in size and displayed resistance to 14 different antibiotics.

The presence of the *tetA* resistance determinant on plasmid p37T1 concurs with previous studies that indicate the frequent identification of *tetA* gene among aquatic bacteria and fish pathogens, and as part as R-plasmids (Adams *et al.*, 1998; Miranda *et al.*, 2003; Rhodes *et al.*, 2000). The association of *tetA* and the transposon, Tn*1721*, is also in correlation with the findings of Rhodes *et al.* (2000) who identified the presence of TetA and its association with Tn*1721* on ten R-plasmids isolated from both clinical and aquaculture settings.

The detection of the streptomycin resistance genes, *strA-strB*, present on plasmid p37T1 may be an indication of possible movement of genes between environmental niches. This gene pair was previously identified as part of pRAS2, isolated from the fish pathogen *A. salmonicida* subsp. *salmonicida*, and other R-plasmids isolated from manure

slurries and clinical environments (Chen *et al.*, 2006; L'Abee-Lund & Sϕrum, 2000; Smalla *et al.*, 2000). The high level of homology between the pRAS2 streptomycin resistance genes and those isolated from the plant-pathogenic bacterium, *Erwinia amylovora,* suggests the movement of resistance determinants between the environmental and clinical settings. The partial *strB* gene identified in this study, showed high levels of nucleotide sequence identity to the plasmid-mediated *strB* genes identified other aquatic organisms suggesting the movement of genetic material within this environmental compartment. The presence of the sulphonamide resistance gene, *sulII,* was unexpected since this plasmid does not confer clinically significant resistance to this compound (Chapter 3, section 3.3.2). Again, the homology between the *sulII* identified in this study and the *sulII* plasmid-encoded genes, suggests evidence of horizontal gene transfer. The presence of the *sulII* gene and its lack of expression may be the result of gene silencing (Collis & Hall, 1995; Enne *et al.*, 2006; Hall & Collis, 1998). The identification of a possible transposase (*tnpB*) may provide insight into mechanisms facilitating gene transfer and possibly the path of acquisition of associated genes. The location of the accessory genes and the location of the 16.6 kb *Pst*I-*Pst*I fragment capable of replication, suggests that the functionality of the replicon is ensured (Fig. 4.17). The 16.6 kb *Pst*I-*Pst*I replicating region contained only a few restriction sites which could contribute to the replicon being maintained without the loss of any essential replication and maintenance genes, following acquisition of antibiotic resistance genes and/or associated mobile genetic elements, e.g., transposons and integrons. However, the 12 kb variable region (Fig. 4.17) contained a higher number of restriction sites and it is possible that if genes were acquired/lost, these processes would potentially occur within this region. A similar organization of restriction sites have been observed for other R-plasmids such as plasmid pRAS1 (Sørum *et al.,* (2003).

Similarities identified in the present study between the ColE-type plasmid, p37T1 and the total DNA of other *Aeromonas* spp. isolates, were interesting. Although p37T1 has a narrow-host-range, plasmid p37T1 sequences appeared to be common to the studied *Aeromonas* population. During this experiment, no discrimination between backbone DNA and accessory gene were made, since the entire plasmid was used as a probe for screening purposes. This complicates interpretation of results, but remains an indication

that certain DNA structures/fragments proliferate within a specific environmental niche and/or bacterial population.

IncU plasmids have been identified in tetracycline-resistant *Aeromonas* spp. isolated from aquaculture settings (Casas *et al.*, 2005; L'Abee-Lund & Sørum, 2002; Rhodes *et al.*, 2000; Rhodes *et al.*, 2004; Sørum *et al.*, 2003). These large conjugative plasmids include the extensively studied pRAS1 plasmid. The 4.8 kb plasmid which displayed the only positive signal is not at all comparable to the previously isolated IncU plasmids due to its small size. Plasmid pFBAOT6 characterized by Rhodes *et al.* (2004) has a 31 kb region containing genes encoding replication, transfer and maintenance proteins. Current data and available DNA sequences is limited, but plasmids harbouring IncU replicons are frequently isolated from aquatic bacteria such as *A. salmonicida* (Casas *et al.*, 2005; Rhodes *et al.*, 2000).

This study represents the first of characterization of different plasmids isolated from various *Aeromonas* spp. obtained from South African aquaculture systems. The presence of broad-host-range conjugative/mobilizable plasmids in conjunction with the many antibiotic resistance markers raises concern with respect to the spread of these resistance determinants not only in the aquaculture environment, but also to and in clinical settings.

Conclusions

Plasmid diversity of members of the *Aeromonadaceae* family is well documented with plasmids ranging from 1.2 to 140 kb being isolated from both clinically and environmentally important species such as *A. salmonicida* and *A. hydrophila*, respectively (Akinbowale *et al.*, 2006; Brown *et al.*, 1997; Casas *et al.*, 2005; Chang & Bolton, 1987; Majumdar *et al.*, 2006; Palu *et al.*, 2006; Radu *et al.*, 2003; Son *et al.*, 1997; Toranzo *et al.*, 1984). Son *et al.* (1997) and Brown *et al.* (1997) also reported diversity with respect with to the number of plasmids present. In the present study, *Aeromonas* spp. isolated from a variety of different South African aquaculture fish species were screened for the presence of plasmids ranging from 1 to 200 kb in size. *Aeromonas* spp. isolates showed a high prevalence of plasmids with 54.1% of the isolates containing plasmids ranging in size from 1.2 to 200 kb which correlated with current reports (Akinbowale *et al.*, 2006; Brown *et al.*, 1997; Chang & Bolton, 1987; Majumdar *et al.*, 2006; Palu *et al.*, 2006; Radu *et al.*, 2003; Son *et al.*, 1997). The use of standard plasmid isolation protocols together with the method described by Barton *et al.* (1995) for the isolation of large plasmids allowed the investigation of the true plasmid content of the *Aeromonas* spp. isolates. Variation with respect to size and number of resident plasmids was observed and the presence of plasmids did not appear to be species-specific or limited to a specific fish host as has been observed by other researchers (Akinbowale *et al.*, 2006; Brown *et al.*, 1997; Son *et al.*, 1997). The sampled tilapia were not exposed to any antibiotics, while the koi and trout had been previously exposed, however, no correlation between exposure to antibiotics and presence of plasmids could be made.

Antibiotic susceptibility testing of both environmental and clinical *Aeromonas* spp. isolates revealed the emergence of MDR resistance phenotypes within this genus. Jacobs and Chenia (2007) reported high levels of antibiotic resistance among the studied *Aeromonas* spp. isolates, which is in correlation with previous antibiotic susceptibility testing done on environmental *Aeromonas* isolates (Adams *et al.*, 1998; Hatha *et al.*, 2005; Miranda & Castillo, 1998; Miranda *et al.*, 2003; Palu *et al.*, 2006; Vila *et al.*, 2002). The resistance phenotype has been linked to the presence of plasmids and resistance determinants successfully transferred to a various host cells (Bruun *et al.*,

2003; Casas *et al.*, 2005; L'Abee-Lund & Sørum, 2000; L'Abee-Lund & Sørum, 2002; Majumdar *et al.*, 2006; Sørum *et al.*, 2003; Son *et al.*, 1997). Plasmid-mediated antibiotic resistance phenotypes were investigated following the transformation of *Aeromonas* spp. plasmids into susceptible *E. coli* DH5α (Table 3.4). Transformed plasmids were selected with ampicillin, tetracycline and chloramphenicol, respectively, based on the susceptibility profiles reported by Jacobs and Chenia (2007). Transformants displayed differences in transferred antimicrobial resistance phenotypes (Table 3.5). The dominant transferred phenotype was β-lactam resistance with the co-transfer of unrelated antibiotics also being observed. Resistance phenotypes of the transformants suggested diversity of different resistance genes being encoded by the various plasmids. Differences between the resistance phenotypes of the wild-type *Aeromonas* spp. isolates and the transformants were also observed. Many (82%) of the wild-type *Aeromonas* spp. isolates contained multiple plasmids, while 60% of the transformants only contained single plasmids transferred to *E. coli* DH5α. The variation in antibiotic susceptibility between the wild-type *Aeromonas* spp. isolates and transformants may be primarily due to variations in plasmid content of transformants in comparison to wild-type *Aeromonas* spp. isolates. Other reasons for the antibiotic susceptibility testing differences might include increased/decreased expression in the specific host or the presence of integron structures with difference is the gene arrangement within the inserted gene cassettes.

The presence of selected plasmid-mediated antibiotic resistance determinants and integron structures was further investigated among the transformants and the 17 plasmid-containing *Aeromonas* spp. isolates. Current reports have indicated the presence of various different plasmid-mediated genes encoding resistance to aminoglycosides, tetracycline, β-lactam antibiotics, chloramphenicol and trimethoprim as part of integrons (Schmidt *et al.*, 2001a; Schmidt *et al.*, 2001b; Sørum *et al.*, 2003; Walsh, 2006). Southern hybridization experiments were undertaken with tetracycline (*tetA*), β-lactam (*bla*-TEM) and streptomycin (*strA-strB*) resistance gene probes, and the class 1 integrase (*intI*) probe to assess the presence of plasmid-encoded copies of these genes. Southern hybridization experiments revealed a strong correlation between the presence of the TetA resistance determinant and the tetracycline-associated transposon Tn*1721* as previously reported by Pezzella *et al.* (2004) and Rhodes *et al.* (2000). The use of tetracycline in

aquaculture systems world-wide has led to the increased occurrence of tetracycline resistance determinants and the association of tetracycline resistance determinants with transposons may lead to the further spread of this resistance genes (Chopra & Roberts, 2001; DePaola *et al.*, 1995; Rhodes *et al.*, 2000). The lack of identification of plasmid-encoded class 1 integrons and *bla*-TEM genes by DNA-DNA hybridizations and PCR amplification suggested the presence of other classes of integrons and/or different β-lactamase genes. Although plasmid-mediated antibiotic resistance was characterized among the transformable *Aeromonas* spp. plasmids, this was only a partial assessment of the total plasmid-encoded resistance markers since many of the detectable plasmids were not transformed into *E. coli* DH5α (Table 2.2 and Table 3.3). The overall lower level of prevalence that was observed compared to the results reported by Jacobs and Chenia (2007), suggested that these genes were chromosomally-encoded or present on the untransformed plasmids.

The 23 transformed *Aeromonas* spp. plasmids were characterized with respect to their ability to replicate independently of host-encoded factors and replicon-type. Approximately 21.7% of the detected plasmids were successfully transformed into *E. coli* with 91% being potentially identified as broad-host-range plasmids due to their ability to replicate independently of host-encoded factors. Improved transformation frequencies could be obtained by the use of other selective pressures and host cells. Cryptic plasmids have been previously identified in *Aeromonas* spp. (L'Abee-Lund & Sørum, 2002), however, the characterization of cryptic plasmids remains difficult. The isolation of cryptic plasmids in the present study was only due the co-transfer of these plasmids with other R-plasmids which provided a basis for selection. Transformants obtained from plasmids isolated from isolates AE11, AE12. AE13 and AE31 displayed different plasmid profiles, however, antibiotic susceptibility profiles were identical. This suggested that the additional plasmid/s that was co-transferred does not contribute to the antibiotic resistance profiles of the transformants. It is possible that the co-transferred plasmid/s confer other characteristics not assayed for in the present study. Of the transformed plasmids, only four could be identified as possible IncQ, IncU and ColE-type plasmids. Remaining transformed plasmids could only be grouped as broad- and narrow-host-range plasmids based on replication in the *polA*$^-$ mutant.

Selected plasmids (p31T1, p36T2 and p37T1) were characterized by restriction endonuclease digests, sequence analysis and mobilization. The most significant discovery was the isolation of two possible IncQ-like plasmids, p31T1 and p36T2. The isolation of these plasmids from different *Aeromonas* species suggests the movement of plasmids within the aquaculture environment. The promiscuous nature of IncQ plasmids is well documented (Bonemann *et al.*, 2006; Gardner & Rawlings, 2004; Rawlings & Tietze, 2001; Smalla *et al.*, 2000), but this is the first report of IncQ-like plasmids from a South African aquaculture setting. Results also indicated the possibility that these plasmids harboured a unique mobilization system since no hybridization signals with the mobilization systems of known IncQ and IncQ-like plasmids were obtained. The mobilization of plasmids p31T1 and p36T2 by RP4 occurred at a much lower frequency when compared to the transfer rate of IncQ-like plasmid pRAS3.1 (W. Loftie-Eaton, personal communication), while the endonuclease restriction profiles of these plasmids revealed no similarity to that of pRAS3.1 (L'Abee-Lund & Sϕrum, 2002). This suggests that these plasmids may be the first representatives of a new subgroup of IncQ plasmids. Sequencing of plasmid p36T2 is currently underway which will provide insight into the replication, mobilization, transfer and maintenance of the plasmid and the level of relatedness to other known IncQ and IncQ-like plasmids.

Restriction endonuclease mapping of plasmid p37T1 from *A. sobria*, revealed two distinct regions. The region harbouring the replicon contained fewer restriction sites, while the region harbouring the accessory genes contained the majority of the mapped restriction sites (Fig. 4.17). This suggested the conservation of the replicon and that the acquisition of additional accessory genes would occur within the variable region without effecting the survival of the plasmid (Fig. 4.17). Sequence analysis of the ColE-type plasmid, p37T1, revealed the presence of a partial *strB* gene adjacent to a transposase (*tnpB*) and the presence of the sulphonamide resistance determinant (*sulII*). These resistance genes have been identified as part of R-plasmids isolated from both *Aeromonas* spp. and other bacteria (Guerry *et al.*, 1974; L'Abee-Lund & Sϕrum, 2000). The organization of the genes revealed similarities to those identified on plasmid pRAS2 (L'Abee-Lund & Sϕrum, 2000). Although plasmid p37T1 was shown to be a narrow-

host-range plasmid, the presence of multiple resistance genes suggested genetic exchange which led to the acquisition of antibiotic resistance genes.

Future work will entail the characterization of the different plasmid-mediated β-lactam resistance determinants and the identification of other replicon-types within the *Aeromonas* spp. population. Additionally, the expression of the MDR in other host cells, e.g., susceptible plasmid-free *Aeromonas* spp., as well as other micro-organisms commonly found in aquaculture systems will be necessary to evaluate the impact plasmids have on the spread of antibiotic resistance genes, not only in the aquacultural setting, but in other environmental compartments.

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