

Characterization of plasmids associated with antibiotic resistant bacteria in the North-West Province

VS Visser
22973893

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Supervisor: Prof C Bezuidenhout
Co-supervisor: Dr C Mienie

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Abstract

Antibiotic release in natural environments select for antibiotic resistant bacteria. These antibiotic resistant bacteria are in many cases associated with plasmids, containing one or more resistant genes. Plasmids are mobile genetic elements and are involved in transmitting the antibiotic resistance from bacterial species off-spring or to other bacterial species. These plasmids may also transfer virulence genes from one bacterial strain to another, rendering non-pathogenic strains with virulence capabilities. This is a significant public health concern. The present study aimed to characterize plasmids associated with antibiotic resistant bacteria in selected surface water systems in the North West province, South Africa. Several multiple antibiotic resistant bacteria previously isolated from these water sources were screened for the presence of plasmids. A total of 20 parental plasmids were successfully isolated from 20 multiple-antibiotic resistant (MAR) bacterial species using the traditional alkaline lysis method. Plasmid DNA were transformed into 10- β *E. coli* host strain. Transformants was selected using one of the antibiotics in the resistance profile of the original MAR parental strain for selection. Furthermore, antibiotic resistance profiles of the transformants were determined and compared to those of parental strains. Finally, polymerase chain reaction (PCR) amplification and sequencing analyses were used to determine whether genes responsible for antibiotic resistance could be amplified. In addition, PCR was also used to amplify the incompatibility (Inc) group markers and classify these accordingly. Selected bacterial strains were resistant to two or more antibiotics of different classes. Among all the isolates, resistance patterns were in this specific order ampicillin (17/20), tetracycline (12/20), erythromycin (11/20), kanamycin (9/20), streptomycin (5/20), neomycin (4/20), and the least resistance to chloramphenicol (1/20). Results indicate that the Schoonspruit river had a generally diverse resistance to various antibiotic groups, compared to the Harts river and Barbers Pan. All selected plasmids belonged to the IncP group. It is known that this group of plasmids are responsible for conferring resistance to a broad spectrum of antibiotics due to the accessory modules or antibiotic resistance genes they may contain. Amplification of antibiotic resistance genes, only detected those encoding β -lactamases (*ampC*) and efflux pumps (*tetA*), but no genes encoding aminoglycoside resistance (e.g. *nptII* genes). Furthermore, susceptibility profiles of parental strains differed from the transformed *E. coli* plasmids. Suggesting that the specific phenotype was not entirely encoded by genetic elements on the plasmid. Other mechanisms may thus be responsible for the resistance phenotype. In this work, the IncP plasmid harbouring these antibiotic resistance genes were mainly isolated from *Enterobacteriaceae* family. Plasmids harbouring the *ampC* genes were the only ones that were able to transform through electroporation with sufficient transformation efficiencies that ranged between 3.1 X

10^8 and 7.1×10^8 transformants per microgram DNA. Both pHR2 and PSR8 were somewhat smaller than pHR5 and had higher transformation efficiencies. However, further optimization is advised using a wider range of bacterial host strains as this may also influence the uptake of plasmid DNA. Furthermore, this study also demonstrated that these plasmids can be transferred among bacteria through the bacterial transformation process. Therefore, plasmids belonging to the IncP group may be responsible for the rapid dispersal of these antibiotic genes in the aquatic environment in the North West province. This may possibly be a hazard for human and animal health as these types of plasmids may confer resistance to broad spectrum antibiotics.

Keywords: plasmids; IncP; antibiotic resistance genes; *ampC*; *tetA*; transformation efficiency.

Preface

The research discussed in this dissertation for the M.Sc. degree in Microbiology was conducted in the Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, South Africa. This work was conducted over a two-year period, under the supervision of Prof. Carlos Bezuidenhout and Dr. Charlotte Mienie.

The research done and presented in this dissertation represents original work undertaken by the author and has not been previously submitted for degree purposes to any other university. The use of work of other researchers, is duly acknowledged in the text. References were done according to the specifications provided by the NWU Harvard Referencing Guide.

The general microbiological data formed part of a WRC funded research project (K5/2347//3). The candidate was part of the research team that were responsible for collecting some of the data. It was agreed that all participants would use data from the set and it is thus unavoidable that overlaps of the actual data in this dissertation, some M.Sc. dissertations and the WRC final report (K5/2347//3) will exist.

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“In this world you will have trouble, but take heart! I have overcome the world.”

John 16:33

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List of Abbreviations

10-β <i>E. coli</i>	10-beta Competent <i>Escherichia coli</i> cells/ transformation host strain
<i>ampC</i>	Antibiotic resistant ampicillin coding gene
BLAST	Basic Local Alignment Search Tool
BLASTn	Blast Local Alignment Search Tool for nucleotides
bp	Base pairs
BSA	Bovine serum albumin
CaCl ₂	Calcium chloride
cds	Codon deoxyribonucleic acid sequence
CLSI	Clinical and Laboratory Standards Institute
CLUSTAL W	Compare a widely used method of multiple sequence alignment
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic
dNTPs	Deoxynucleoside triphosphate
EDTA	Ethylene Diamine Triacetic Acid
ESBL	Extended spectrum beta-lactamase producers
E-value	Expected value
F primer	Forward primer
g	Relative centrifugal force
HGT	Horizontal gene transfer
<i>HindIII</i>	A restriction endonuclease
Inc	Incompatibility
LB-agar	Luria-Bertani agar
LB-Broth	Luria-Bertani Broth
MAR-isolates	Multiple antibiotic resistant isolates
MEGA	Molecular Evolutionary Genetics Analysis
MFS	Major facilitator superfamily
MgCl ₂	Magnesium chloride
MGE	Mobile genetic elements
MIC	Minimum inhibitory concentration
NaCl ₂	Sodium chloride
NCBI	National Centre for Biotechnology Information
NEB	New England Biolabs
<i>npt (II and III)</i>	Neomycin phosphotransferase genes
<i>oriT</i>	Origin of transfer

<i>oriV</i>	Origin of replication
PCR	Polymerase chain reaction
pH	The co-logarithm of the activity of dissolve hydrogen ions (H ⁺)
pUC19	Plasmid cloning vector in <i>Escherichia coli</i>
R primer	Reverse primer
R-factor	Resistance-plasmid
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SA	South Africa
<i>sul</i> (<i>I</i> and <i>II</i>)	Sulfonamide resistance genes
TAE	Tris-acetate-EDTA
<i>tetA</i>	Tetracycline resistance gene
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
UK	United Kingdom
US	United States
USA	United States of America
WWTP	Wastewater treatment plant
β	Beta

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Chapter 1

Introduction

1.1 General introduction and problem statement

Release of antibiotic into natural environments selects for resistant bacteria (Martínez, 2009). Antibiotic use in human and veterinary medicine is critical to treat infectious diseases. However, overuse and misuse of antibiotics in humans, medicine and in animal food production are some of the factors impacting on the increased antibiotic resistant bacteria in the environment. (Gilchrist *et al.*, 2007; CDDEP, 2015). Use of antibiotics as growth promoting agents in livestock, leads to a drastic increase of antibiotic resistant bacteria. Such bacteria could thus directly reach workers which are in contact with animals (Marshall and Levy, 2011). Unnecessarily prescribed antibiotics by physicians for non-bacterial infections and inappropriate use of antibiotics are some of the other factors that also lead to the challenge of increased antibiotic resistance genes in the environment (Noornissabegum *et al.*, 2014; Essak *et al.*, 2016).

In 2010 the global usage of antibiotics in livestock were estimated to be 63,151 tons and is most probably twice as high for human consumption. These figures are most likely to increase by 67% by 2030 (Van Boeckel *et al.*, 2015). According to the U.S. Centres for Disease Control and Prevention (CDC, 2013) antibiotic resistant bacteria accounts for an estimated two million bacterial infections and 23,000 annual deaths in the United States. This leads to economic losses of \$20 billion as well as decreased production costs of up to \$35 billion. However, in developing countries limited data exists on economic losses (Laxminarayan, 2014). The implications of this is globally felt in cases where antibiotics are no longer effective in combatting illnesses, showing resistance to first line antibiotics and putting more stress on last resort antibiotics (CDDEP, 2015). Over the past thirty years, little advances had been made with regard to the development of new antibiotics (Spellberg *et al.*, 2004; Högberg *et al.*, 2010).

Many concerns have been raised with regard to the overuse of antibiotics that adds to the acquired resistance as well as mutations in bacteria (Gilchrist *et al.*, 2007; Centner, 2016; Holmes *et al.*, 2016). The presence of antibiotics in water systems may serve as a selection pressure and reservoir for resistance genes (Aarestrup *et al.*, 2003; Akinbowale *et al.*, 2007;). Antibiotic resistance may be plasmid encoded (Manjusha and Sarita, 2013). Plasmids are mobile genetic elements and have the ability to carry and transfer genes between different bacteria as well as parental strains and off-spring (Actis *et al.*, 1999; Bennett, 2008; Martínez, 2009). The genetic elements (plasmids) have been associated with the rapid spread of

antibiotic resistance in various niches. They could also carry virulence factors (determinants) that present bacteria with pathogenicity features. Thus if antibiotics in an environment select for plasmids that carry both antibiotic and virulence determinants, these could be transferred to non-pathogenic bacteria (Hayes, 2003; Schlüter *et al.*, 2007a; Fondi *et al.*, 2010), affecting human health and microbial evolution of environmental strains (Martínez, 2009). Clinically relevant antibiotic resistance genes have been found in several non-pathogenic bacteria in soil and aquatic environments (Heuer *et al.*, 2002; Baquero *et al.*, 2008; Noornissabegum *et al.*, 2014). This could be due to selection pressures of antibiotic residues in such environments.

Genes that plasmids carry could be responsible for conferring resistance to most clinically important classes of antibiotics including aminoglycosides, tetracyclines, macrolides and β -lactams amongst others (Bennett, 2008; Martínez, 2009; Manjusha and Sarita, 2013). In these cases, the resistance genes could code for efflux pumps, enzymes that modify or inactivate specific antibiotics and molecules that interfere with membrane permeability (Mazel and Davies, 1999; Alekshun and Levy, 2007).

Plasmids are categorized into various incompatibility groups (Couturier *et al.*, 1988; Wang *et al.*, 2009) of which the IncN, IncW, IncQ and IncP groups have been detected in environmental microorganisms (Götz *et al.*, 1996). These plasmids have the ability to transfer and replicate in broad host range bacteria including pathogens (Schlüter *et al.*, 2007a).

Limited data on plasmids associated with antibiotic resistant bacteria in surface water systems are available for the North West province. The characterization of plasmids from these water sources may shed more light on the presence and dynamics of antibiotic resistant bacteria in aquatic environments and its potential for dispersal between different bacterial strains.

1.2 Aim

To isolate and characterize plasmids associated with antibiotic resistant bacteria from selected surface water sources in the North West province, South Africa.

1.3 Objectives

Specific objectives of this study were to:

- Screen multiple antibiotic resistant bacteria from water sources for plasmids that could potentially be responsible for the observed antibiotic resistance
- Isolate and purify plasmids

- Transform isolated plasmids into a 10-β *E. coli* strain and select transformants using one of the antibiotics in the resistance profile for selection
- Determine the antibiotic resistance profile of the transformed 10-β *E. coli* strain and compare it to the parental strain
- Determine the presence of selected genes responsible for the antibiotic resistance and the incompatibility group that the plasmids belong to, using PCR and sequencing data.

Chapter 2

Literature Review

2.1 Impacts of antibiotics in the environment

Antibiotics have been in use in human and veterinary medicine for several decades and major resources have been committed towards understanding the impacts of these uses on resistance development. However, limited attention has been given to their impacts in environmental settings (Davies and Davies, 2010; Pruden *et al.*, 2013). The significant therapeutic role that these agents have played, aided in ways of preventing and treating infections, as well as limiting the transfer of particular diseases in human and animals (Cohen, 1992; Allen *et al.*, 2010). In animal husbandry, antibiotics are also used as growth promoters in livestock (Kümmerer, 2003; 2004; Pruden *et al.*, 2013).

The metabolism of most antibiotics in humans and animals are not fully understood. It is however, known that they are not completely metabolized in the human body and are excreted by the kidneys and liver and end up in the sewage (Kümmerer, 2003; 2009). Wastewater treatment plants were not designed to remove these substances and although some reduction occurs, these antibiotics may end up in the treated sewage effluent and eventually the environment, primarily in the aquatic systems. Moreover, these substances might also find their way in soil, sediment or groundwater sources as a result of manure application in agricultural fields (Kümmerer, 2003; 2004; 2009). This might have adverse effects on organisms in water and terrestrial environments and finally reach humans through drinking water (Kümmerer, 2009).

Studies have reported on the presence of antibiotics in low quantities (ng to µg/L) in different environments ranging from surface water, hospital effluent, sewage treatment plants, wastewater to groundwater systems (Golet *et al.*, 2001; Sacher *et al.*, 2001). This was true for tetracycline that was detected in soil (several 100 µg/kg) after the application of manure (Hamscher *et al.*, 2002; De Liguroro *et al.*, 2003). Moreover, the detection of several other antibiotics such as ciprofloxacin (249-405 ng/L) and norfloxacin (45-120 ng/L), have been reported in the final effluent of wastewater treatment plants (WWTP's), assuming that human activities were more responsible (Golet *et al.*, 2001). For this reason, risk assessment is thus advised in such areas to analyse the impact of antibiotic pollution on natural ecosystems (Martínez, 2009).

Microbes play an important role in some geochemical and nutrient cycles and is vital for fertile soil and breaking down organic material (Kümmerer, 2004). The dynamics of bacterial inhabitants in soil are regulated by antibiotics naturally produced by fungi and bacteria (Kümmerer, 2004). However, antibiotics used currently are more stable and artificially produced, making them difficult to degrade and this prolong their existence in the environment (Kümmerer, 2004; Roose-Amsaleg and Laverman, 2015). Kümmerer *et al.* (2000) focussed on the biodegradability of some clinically important antibiotics such as ciprofloxacin, ofloxacin, and metronidazole, using specific test systems. They found that these industrially produced antibiotics were much more resistant to biodegradation compared to natural antibiotics. Biodegradation of organic material is the principle on which sewage treatment plants work and if the system cannot degrade the organic material such as antibiotics then these are toxic and could negatively affect the system (Kümmerer *et al.*, 2000).

Antibiotics do not only alter the population dynamics of microorganisms but also selects for resistance (Martínez, 2008; 2009). If antibiotics in the environment, select for resistant pathogens then it may negatively affect the human and animal health. Direct contact by humans could result in infection by a pathogen that would not respond to treatment. Bacteria could also be transferred to fish or other food sources in water bodies and land in the human food web (Sørum, 1998; Cabello, 2006; Tamminen *et al.*, 2011; Cabello *et al.*, 2013). This could be affecting food security in rural communities but may also affect the economic output of aquaculture and in the process intensify the spread of these resistance determinants (Rhodes *et al.*, 2000; Cabello, 2006).

2.2 Levels of antibiotics in aquatic systems

Antibiotics are seen as environmental pollutants (Valavanidis *et al.*, 2014). The levels of antibiotics (ng/L) in water systems and their relating health issues are of great concern (Fatta *et al.*, 2007). Several classes of antibiotics (aminoglycosides, β -lactams, fluoroquinolones, macrolides, sulphonamides and tetracyclines), generally used in animal husbandry and in humans to combat illnesses have been detected in environmental waters (Table 2.1; Kümmerer, 2004; Le-Minh *et al.*, 2010; Huang *et al.*, 2011; Sim *et al.*, 2011). The detection of these pollutants in aquatic environments (Table 2.1) may pose a threat to its surrounding communities (Huang *et al.*, 2011).

Most of these antibiotics are broad spectrum antibiotics and their concentrations range from a level below detection (macrolides) in surface water to elevated levels (β -lactams) in the final effluent of wastewaters. Large amounts of commonly used antimicrobials are received by municipal WWTP's on a daily basis (Manaia *et al.*, 2011). The β -lactams are the most

extensively used antibiotics in human therapy (Schlüter *et al.*, 2007a). In the animal production of swine, poultry and beef a considerable high amount of antibiotics are used including macrolides, sulphonamides, tetracyclines, β -lactams and aminoglycosides (Huang *et al.*, 2011). At sub-therapeutic levels these antibiotics are also used to prevent illnesses and to promote growth in livestock (Le-Minh *et al.*, 2010; Huang *et al.*, 2011).

The overuse of antibiotics may be the result of their occurrence in environmental surface water as they sometimes get passed by sewage treatment plants (Hamscher *et al.*, 2002), which might have originated from hospitals and agricultural lands (Baquerro *et al.*, 2008). These possible contaminants may complicate water reuse and -resource planning in the industry (Huang *et al.*, 2011). Thus, measures need to be put in place in reducing the load of antimicrobial agents and resistant microbes present in wastewater, such as the management of manure and wastewater and optimized disinfectant protocols (Baquerro *et al.*, 2008).

Table 2.1: Levels of antibiotics detected in environmental sources relevant to the study.

Antibiotic substance	Class	Concentration detected (ng/L)	Source	Reference
amoxicillin	β -lactams	27 000	Untreated wastewater	Huang <i>et al.</i> , 2011
chloramphenicol	Phenicol	15	Wastewater	Kowalski <i>et al.</i> , 2011
tetracycline	Tetracyclines	5.4 - 8.1	Surface and groundwater	Javid <i>et al.</i> , 2016
trimethoprim	Sulfonamides	<10 & 70	River and wastewater	Ashton <i>et al.</i> , 2004
erythromycin	Macrolides	<4	Surface water	Thomas and Hilton, 2004

2.3 Antibiotics usage patterns globally and in South Africa

The prompt development of antibiotic resistance and the possibility of a post-antibiotic era is alarming (McKenna, 2013). Concerns have been raised about the overuse of antibiotics in veterinary practices and its contribution to mutations and acquired resistance in bacteria (Gilchrist *et al.*, 2007; Holmes *et al.*, 2016; Centner, 2016).

Data in Table 2.2 shows that usage of antibiotics in South Africa and the United Kingdom (UK) and United States of America (USA) is generally increasing if the period 2000 to 2014 is considered (CDDEP, 2015). The broad spectrum penicillins are reported as the most extensively used antibiotics globally and the data in Table 2.2 is supporting this report (Mölstad *et al.*, 2002). This may contribute to increased resistance to this antibiotic class (Gilchrist *et al.*, 2007; GARP, 2011; Holmes *et al.*, 2016). In South Africa the use of aminoglycosides has

increased considerably over this period. However, usage of other antibiotics has stayed relatively constant. What is of concern is that these are the only antibiotics for which records exist in South Africa. Some of the drugs are also used in agriculture and these are not accounted for in this table.

The effects of antibiotics misuse are mostly felt in poverty stricken communities where bacterial infections may be common and treatment may be unconventional (Mendelson, 2015). However, antibiotic resistance is a phenomenon that affects all communities and surveillance is important. South Africa is on par with regard to antibiotic resistance surveillance compared to other African countries (GARP, 2011; Essak *et al.*, 2016). Measures are taken in improving the correct diagnoses and prescription of antibiotics, reducing antibiotic usage for veterinary, aquaculture and agriculture purposes and introduction into the environment, developing innovative antimicrobials, guaranteed access to qualitative health care and augmented surveillance systems (Holmes *et al.*, 2016). This is, however, still human and veterinary medicine centred and surveillance in the environment is lacking. The latter surveillance is an important aspect that is not recognised in the antibiotic stewardship programme.

Table 2.2: Common use of antibiotics from different classes in two global countries and in South Africa from 2000–2014 (CDDEP, 2015).

Country:	America				United Kingdom				South Africa			
Year:	2000	2005	2010	2014	2000	2005	2010	2014	2000	2005	2010	2014
Antibiotics (Standard unit per 1000/ population):												
Broad spectrum penicillins	1158	1309	1155	1167	6874	7224	7458	7242	6005	6974	10526	9242
Aminoglycosides	31	33	25	27	141	176	110	137	70	79	79	114
Cephalosporins	328	355	388	393	1747	1850	1243	777	1153	980	1098	1056
Fluoroquinolones	229	268	352	360	614	783	570	494	404	847	1270	1173
Macrolides	344	253	273	292	3419	3275	3180	2959	2209	1818	3322	1535
Tetracyclines	708	800	700	555	3359	3398	3400	3372	2591	1785	2377	1738

2.4 Classes of antibiotics, mode of action and resistance

In Table 2.3 classes of antibiotics that are relevant to the present study, their modes of action as well as the primary effect of these substances are shown (Peach *et al.*, 2013; Hoerr *et al.*, 2016). These agents can either be bacteriostatic or bactericidal (Pankey and Sabath, 2004). The relevant antibiotics listed in Table 2.3 have four key mechanisms which include the inhibition of bacterial DNA, cell wall, protein and folate synthesis (Davies, 1994; Alekshun and Levy, 2007). Resistance to antibiotics may be encoded on plasmids or in the chromosome (Manjusha and Sarita, 2013) and the genes responsible for resistance mechanism to the antibiotics in Table 2.3 could be on both (Boissinot *et al.*, 1987; Livermore, 1995; Henriques *et al.*, 2006). Thus if a selection pressure in the form of antibiotics occur in the aquatic environment then this may serve to maintain the reservoir for resistance genes in that environment (Aarestrup *et al.*, 2003; Akinbowale *et al.*, 2007).

Table 2.3: Different classes of antibiotics as well as their mode of action (Peach *et al.*, 2013; Hoerr *et al.*, 2016).

Inhibitors of protein synthesis:				Resistance	
Class	Antibiotic	Mode of action	Effect	Chromosomal	Plasmid
Aminoglycosides	kanamycin	Inhibition of 30s subunit	bactericidal	<i>nptII</i> , <i>nptIII</i> (Woegerbauer <i>et al.</i> , 2014)	<i>nptII</i> (Fall <i>et al.</i> , 2007)
Tetracyclines	tetracycline	Inhibition of 30s subunit	bacteriostatic	<i>tetA</i> (Balassiano <i>et al.</i> , 2007)	<i>tetA</i> (Rychlik <i>et al.</i> , 2006; Christabel <i>et al.</i> , 2012)
Inhibitors of cell wall synthesis:					
β -lactams	ampicillin	Inhibition of penicillin binding	bactericidal	<i>ampC</i> (Schwartz <i>et al.</i> , 2003)	<i>ampC</i> (Jacoby, 2009; Peter-Getzlaff <i>et al.</i> , 2011)
Inhibitors of DNA and protein synthesis:					
Sulfonamides	sulfamethoxazole	inhibitor for DHPS involved in folate synthesis	bacteriostatic	<i>sulI</i> (Rychlik <i>et al.</i> , 2006)	<i>sulI</i> (Christabel <i>et al.</i> , 2012) <i>sulIII</i> (Rychlik <i>et al.</i> , 2006)

2.5 Plasmids

2.5.1 Structure of plasmids

Plasmids are double stranded DNA molecules that can replicate independent of the chromosome of their host (Actis *et al.*, 1999; Thomas and Nielsen, 2005; Carattoli, 2009). They are found in Gram-positive and Gram-negative bacteria and in some fungi and yeast species (Actis *et al.*, 1999). These genetic elements are compact and carry genes required for bacterial growth under stressed conditions (Thomas and Nielsen, 2005). They may have genetic elements allowing their hosts to have advantages over other microbes sharing the same environmental niche. An example is plasmids encoding enzymes that could inactivate antibiotics (Actis *et al.*, 1999).

Besides these genes that are involved in the survival of the host, plasmid systems also have genes responsible for their replication and the mechanisms for the control of plasmid copy number and inheritance (Hayes, 2003). These mechanisms also enable lateral transfer of plasmids via conjugation, between microorganisms from different genera and kingdoms. Many plasmids encode for systems that are based on toxin-antitoxin factors, which help in eliminating daughter cells that were unable to inherit the plasmid during cell division (Hayes, 2003; Carattoli, 2009). Plasmids may also carry genes responsible for pathogenicity (virulence determinants) in specific organisms. In this manner, these virulence determinants carried by plasmids may be mobilized and transferred between different bacteria in the environment (Heuer *et al.*, 2002; 2012; Binh *et al.*, 2008).

The R (resistance) plasmids (Novick *et al.*, 1976) harbour multiple genes that display resistance to a broad range of antibiotics, heavy metals (Foster, 1983) and including disinfectant agents such as formaldehyde (Kummerle *et al.*, 1996). In addition, the investigation of plasmids on molecular and genetic level gave insight to the structure and role of integrons and transposons in the transfer of resistance genes (Hall and Collis, 1995; Actis *et al.*, 1999). The presence of integrons and transposons in transmissible plasmids may be disadvantageous as they may possibly cause the inter- or intracellular transfer of resistance genes (Couturier *et al.*, 1988; Actis *et al.*, 1999). Sizes of these extra chromosomal elements may vary from a few to several hundred kilo base pairs (Couturier *et al.*, 1988; Zielenkiewicz and Ceglowski, 2001; Dib *et al.*, 2015). Various different types of plasmids may co-exist in a single bacterium (Manjusha and Sarita, 2013).

2.5.2 Classification of plasmids

Plasmids are classified into incompatibility groups based on their origin of replication (Couturier *et al.*, 1988; Actis *et al.*, 1999; Wang *et al.*, 2009). This classification scheme was

established in the 1970's (Datta and Hedges, 1972; Couturier *et al.*, 1988). Plasmid incompatibility can be described as the inability of two plasmids to co-exist in the same cell without any selective pressure (incompatibility (Inc) groups; Novick *et al.*, 1976; 1987; Velappan *et al.*, 2007). This phenomenon provides insight into the relatedness between plasmids. Most species of *Pseudomonas* and the *Enterobacteriaceae* families carry plasmids that belong to over 30 incompatibility groups (Popowska and Krawczyk-Balska, 2013).

Specific genes are required that control the initiation of replication of plasmids. Primers are available for amplification of these origins of replication and origin of transfer regions (Smalla and Sobecky, 2002). Even though plasmids need specific molecules to start the replication process, they still depend on their host-encoded factors. Initiation of plasmid replication starts in the pre-determined *cis-site* (*ori*) and continues with a theta replication or rolling circle mechanisms (Adamczyk and Jagura-Burdzy, 2003). The antisense RNA molecules as well as repeated sequences of DNA are closely located to the *ori* site and it determines the copy number and incompatibility group that these plasmids belong to (Actis *et al.*, 1999).

Insight into the relationship of plasmid characteristics and the host taxonomy is needed to fully comprehend the spread of plasmids between bacteria (Smalla *et al.*, 2015). In order for newly isolated plasmids to be identified, it is necessary to first classify these into known plasmid categories. Thus further, categorizing these plasmids into specific hosts will be more informative when studying or using them as genetic tools in microbial engineering or industrial processes (Shintani *et al.*, 2015).

Several studies used polymerase chain reaction (PCR) based methods (Götz *et al.*, 1996; Turner *et al.*, 1996) or hybridization with specific probes (Couturier *et al.*, 1988; Smit *et al.*, 1998) for the characterization of plasmids. In addition, these techniques were also used for the detection of replicon-specific sequences in total DNA, directly isolated from sediments (Sobecky *et al.*, 1997; Cook *et al.*, 2001), soils (Götz *et al.*, 1996; van Elsas *et al.*, 1998) or other environments.

2.5.3 Broad-host-range plasmids

Plasmids that belong to the incompatibility groups of IncN, IncP, IncQ and IncW have been identified in environmental bacteria by PCR based methods (Götz *et al.*, 1996). These plasmid types have the ability to replicate independently in a large range of phylogenetically distinct hosts including pathogens. Most of the broad host range plasmids such as the IncP (Adamczyk and Jagura-Burdzy, 2003) and IncQ (Rawlings and Tietze, 2001), isolated from Gram-negative bacteria (*Pseudomonas spp.* and *Escherichia coli*), are the best studied

groups. Plasmids that belong to the IncQ group, have been reported as promiscuous genetic elements and so far its backbone consist of a plasmid maintenance and mobilization region. Their widespread nature in broad host range bacteria is, however not known (Rawlings and Tietze, 2001).

The first IncP group of plasmids were originally isolated in clinical isolates from Birmingham hospital in the United Kingdom and was found to harbour genes that enabled their host to survive in the presence of antibiotics (Adamczyk and Jagura-Burdzy, 2003). Furthermore, it was discovered that their genomes contain a rudimentary backbone that is disrupted by mobile genetic elements (MGE's) with diverse phenotypical characteristics (Adamczyk and Jagura-Burdzy, 2003). These elements are responsible for the transfer of resistance determinants to almost all of the clinically important antibiotics from different classes. In addition, most of the accessory genes present on plasmids are mainly composed of MGE's that harbour determinants which confer resistance to various macrolide, β -lactam, tetracycline, aminoglycoside, sulphonamide and trimethoprim antibiotics as well as mercury ions and disinfectants (Schlüter *et al.*, 2007a). They are also highly promiscuous and can transfer between and replicate in particularly pathogenic and non-pathogenic species (Schlüter *et al.*, 2007a), This may negatively affect human health (Popowska and Krawczyk-Balska, 2013) as these types of plasmids confer resistance to antibiotics used for treating infections (Dröge *et al.*, 2000).

Several studies reported that these wide host range plasmids were present in soils (Götz *et al.*, 1996; Sen *et al.*, 2011; Heuer *et al.*, 2012), freshwaters (Haines *et al.*, 2006), fertilizers as well as wastewater (Götz *et al.*, 1996; Haines *et al.*, 2006; Binh *et al.*, 2008; Sen *et al.*, 2011; Heuer *et al.*, 2012).

2.5.4 Plasmids associated with antibiotic resistance genes

In a recent study by Laroche-Ajzenberg *et al.* (2014) the link between plasmids and the multiple-resistance phenotype displayed in microbial species was demonstrated. A number of plasmids, that could be linked to multiple drug resistance, were isolated from environmental water sources (groundwater, surface water) that had anthropogenic impacts. A number of conjugative plasmids were identified, including one of 60 kb that belonged to the IncP group. Several other plasmids from a range of incompatibility groups were also isolated, illustrating plasmid diversity in the environment.

Conjugative plasmids may contribute to the spread of antibiotic resistance in the microbial populations. Plasmids are associated with the dissemination of antibiotic resistance and this

is cause for concern for human health as it limits the effective treatment of severe illnesses (Levy and Marshall, 2004; Strahilevitz *et al.*, 2009).

In most instances the resistance genes are carried directly on the plasmid, while in some cases these are found in integrons within variable gene cassettes. Both these scenarios make it possible for the relocation of the antibiotic resistance genes in the genome or between microorganisms (Garcillán-Barcia *et al.*, 2011). For example, the distribution of β -lactamase genes are found directly within bacterial plasmids (Adamczuk *et al.*, 2015). It has been suggested that in the pre-antibiotic era these genes might not have been harboured on plasmids. This suggests that the presence of antibiotic resistance genes and their spread between pathogenic microorganisms is the result of antibiotic therapy due to selective pressure by the antibiotic (Datta and Hughes, 1983; Hughes and Datta, 1983; Allen *et al.*, 2010). Additionally, epidemiological studies reported that the emergence of antibiotic resistance plasmids started to occur within five years after the antibiotic have been used for treatment (Cohen, 1992).

Antibiotic resistance genes can be transferred via horizontal gene transfer (HGT; Götz *et al.*, 1996; Popowska and Krawczyk-Balska, 2013). This process (HGT) can be described as a process by which foreign DNA is acquired through conjugation, transduction or transformation (Davison, 1999) from the same or different species. The acquisition of resistance genes through HGT greatly influences the development and dissemination of these genes in pathogenic bacteria (Davies, 1994; Alonso *et al.*, 2001). Recently, plasmids belonging to the IncL/M group were associated with the spread of β -lactam resistance genes between the *Enterobacteriaceae* (Adamczuk *et al.*, 2015).

According to Ledda and Ferretti (2014) plasmids are under a great amount of strain due to essential genes they may contain. Moreover, these genes may execute important functions in bacteria. These authors imply that increased antibiotic stress, from human impact, may result in greater length and quantity of antibiotic resistance genes coded for by plasmids.

2.6 Methodology to isolate and study plasmids

2.6.1 Isolation of plasmid DNA

2.6.1.1 Alkaline lysis method

Many procedures such as polymerase chain reaction (PCR), cloning and DNA sequencing require good quality plasmid DNA for successful analyses (Kotchoni *et al.*, 2003). The alkaline lysis method has a long history in the analysis of plasmids and was described by Birnboim and Doly (1979). This is a straight forward and reliable technique, but should be performed

very cautiously (Prazeres *et al.*, 1999). Even though plasmid DNA extraction protocols may employ several approaches, most of them share three common integral steps (i) complete lysis of bacterial cells and isolation of intracellular nucleic acid, (ii) removal of cellular debris and (iii) ensuring pure and good quality nucleic acid is recovered (Lever *et al.*, 2015).

The amplification of plasmid DNA provide a way of increasing this yield. When optimal cell density is reached, the addition of chloramphenicol (a protein synthesis inhibitor) prior to extraction and a further incubation step aid in plasmid replication of up to several thousands. Plasmid amplification is a crucial step for increased yield of low copy number plasmids (Brown, 1986).

The technique of plasmid separation and manipulation have been widely applied in molecular biology (Sayers *et al.*, 1996). Several approaches have been developed for the rapid extraction of plasmid DNA from bacteria (Kotchoni *et al.*, 2003; Ojo and Oso, 2009) as well as commercial kits (Yang and Yang, 2012; Becker *et al.*, 2016). Numerous studies have employed the alkaline lysis method (Christabel *et al.*, 2012; Manjusha and Sarita, 2013; Çimen *et al.*, 2015), mostly for small scale plasmid analysis. However, this method could also be considered for the preparation of large quantity plasmid DNA (Sambrook *et al.*, 1989).

2.6.1.2 Commercial Kits

Commercially available kits such as the Qiagen plasmid extraction (Qiagen, Hilden, Germany) and NucleoSpin plasmid extraction (Macherey-Nagel, Düren, Germany) kits may be employed for the rapid extraction of plasmid DNA. However, the alkaline lysis method appears to be the most widely used and forms the basis of these kits. Numerous authors prefer traditional plasmid isolation methods in which minor modifications had been made (Akkurt, 2012; Yang and Yang, 2012; Kušec *et al.*, 2015). This may be attributed to the costliness, individual handling and specificity of standard kits. Moreover, the properties of environmental samples may differ which may lead to discrepancies in the design of generally optimized methods (Lever *et al.*, 2015). Modifications of extraction methods may aid in such instances, although it can be difficult to adjust commercial kits since recipes of reagents are generally exclusive (Lever *et al.*, 2015).

Nevertheless, these commercially available kits are advantageous in many ways including efficient extraction protocols, ready to use reagents and its time efficiency (Lever *et al.*, 2015). It has been proven to deliver sufficient results under specified analysis conditions (Smith *et al.*, 2003; Schill, 2007; Lusk *et al.*, 2012; Pérez-Losada *et al.*, 2016).

2.6.2 Bacterial transformation

The introduction of foreign DNA into bacterial species is very important in molecular biology (Dower *et al.*, 1988). It is necessary to develop transformation systems, mediated by plasmids, which contribute to the genetic manipulation of microorganisms. This involves introduction of foreign DNA into the bacterial strain, where the genes present on the exogenous DNA are then expressed. The DNA is caused to be stably maintained and replicated in the host, which results in the expression of the desired phenotypical traits (Ruiz-Díez, 2002).

2.6.2.1 Electroporation

Several methodologies can be used to introduce foreign DNA into prokaryotes and eukaryotes. However, transformation through electroporation is a frequently used method (Ahmad *et al.*, 2014). Electroporation is a method of exposing living cells with a sudden and intense electric pulse. This causes pores in cell membranes (Calvin and Hanawalt, 1988; Wani *et al.*, 2013) so that foreign molecules or plasmid DNA can move into the host cell (Prasanna and Panda, 1997; Wani *et al.*, 2013).

According to Prasanna and Panda (1997) electroporation is more efficient than other conventional methods such as the CaCl_2 method. They found that it delivers greater transformation efficiencies (10^8 – 10^9) for bacterial isolates such as *Escherichia coli*. The method is also technically easy to use, can be done in a short period of time, and it is highly reproducible. Reports indicated that bacteria could be transformed with large DNA molecules (Eynard *et al.*, 1992). The method is versatile and various studies have also reported on the successful transformation of animal, plant, fungi as well as yeast cells through electroporation (Chu *et al.*, 1987; Ruiz-Díez, 2002; Kawai *et al.*, 2010; Wani *et al.*, 2013).

2.6.2.2 Calcium Chloride (CaCl_2) method

Traditional methods, based on chemical and physical approaches are available for the transformation of *Escherichia coli* strains with plasmid DNA (Roychoudhury *et al.*, 2009). The calcium chloride-heat shock method was first described in the 1970's by Cohen and co-workers (1972). They successfully transformed the R-factor and recombinant plasmids into *Escherichia coli* strains. This convenient method has been widely applied ever since.

The calcium chloride-heat shock method works on the principle of treating *E. coli* cells with ice cold calcium chloride, followed by treatment with heat shock, which enables the uptake of plasmid DNA into the cell (Mandel and Higa, 1970; Cohen *et al.*, 1972). A large amount of research has been conducted using this method by introduction of plasmid DNA into bacterial host strains (Tu *et al.*, 2005; Li *et al.*, 2010). However, it has been shown that optimization of

the calcium chloride method of transformation is necessary for efficient transformation efficiency and frequency. This can be achieved by increasing the concentration of CaCl_2 and modifying the heat shock method. Furthermore, they successfully isolated plasmid DNA after transformation, confirming that the competent *E. coli* cells were able to acquire the desired plasmid.

2.6.3 DNA sequencing

Sequencing analysis can be employed to confirm the presence of specific genes such as antibiotic resistance genes on plasmids, which are comprised of many accessory genetic elements including integrons, transposons, insertion elements (IS) and other genetic modules. These accessory elements play a key role in the survival of the bacterial host, providing them with adaptive traits such as antibiotic or metal resistance and the ability to degrade pollutants (Tennstedt *et al.*, 2005; Schlüter *et al.*, 2007a).

Antibiotic resistance genes encoding tetracycline, kanamycin and β -lactam resistance have been reported in several studies focussing on plasmid sequencing. In Tauch *et al.* (2003) it was revealed that one of the conjugative antibiotic resistance plasmids (pB4), contained specific regions that code for multidrug resistance (MDR) efflux genes and β -lactam resistance genes. Several plasmids (pB8, pB10 and pTB11) encoding for β -lactamases have been detected (Tennstedt *et al.*, 2003). The gene cassettes present on these plasmids are all similar to each other (Schlüter *et al.*, 2003, 2005; Tennstedt *et al.*, 2005). In a similar study Tennstedt *et al.* (2005), identified the presence of *tetA* and *tetR* genes encoding resistance to tetracyclines on plasmid pTB11. This resistance module seemed rather common in all of their sequenced IncP plasmids, as they detected the presence of the *tetA* gene in all of their analysed plasmids. In addition, kanamycin resistance genes were also detected.

Fully analysed plasmids were compared to known sequences of the Birmingham plasmids at DNA sequence level for more insightful knowledge on the evolutionary history of these plasmids as well as their accessory genetic elements (Tennstedt *et al.*, 2005). Their findings revealed that the composition of the backbone modules of plasmid pTB11 matched that of the Birmingham or IncP plasmids (RP1, RP4, RK2, R18 and R68; Pansegrau *et al.*, 1994). The origin of replication (*oriV*) is identical (99.9%) to that of the references' *oriV*. Their origin of transfer replication (*oriT*) is 100% identical to the *oriT* of the Birmingham plasmids (Tennstedt *et al.*, 2005).

Detailed phylogenetic analysis of the antibiotic resistance and degradative plasmids, belonging to the IncP group, illustrated that they share a common ancestor (Dröge *et al.*, 2000;

Schlüter *et al.*, 2003; Trefault *et al.*, 2004; Heuer *et al.*, 2004). This is revealed especially in the multi-resistance plasmids pB10 and pJP4, sharing the same genomic composition. These plasmids code for degradative functions of chlorinated compounds (Trefault *et al.*, 2004). The core/ backbone genes share a 99.9-100% similarity at nucleotide sequence level.

Thus it is evident that using more advanced technologies such as next generation sequencing aids in the rapid analysis of microbial structure and diversity (Acosta-Martínez *et al.*, 2008; Barriuso *et al.*, 2011; Akinsanya *et al.*, 2015). Sanger sequencing is employed for plasmid sequence analysis and with cost becoming less prohibitive, the next generation sequencing of whole plasmids will become more popular (Schlüter *et al.*, 2007a; Buzoianu *et al.*, 2012; Kozich *et al.*, 2013; Akinsanya *et al.*, 2015). However, this is a very expensive approach, requires fine detail, delivers large amounts of data and it is time constraint with a lot of technical and bioinformatics challenges (Acosta-Martínez *et al.*, 2008; Kozich *et al.*, 2013). Thus, antibiotic resistance genes detected in plasmids were identified using Sanger sequencing for analysis purposes.

2.7 Chapter Summary

In this literature review it was highlighted that antibiotics play an essential role in our everyday life. Their widespread use and misuse have steered the development of antibiotic resistance bacteria and antibiotic resistance genes. These genes have been associated with the presence of plasmids in antibiotic resistant bacteria isolated from water environments. They encode for enzymes, mediated through different mechanisms which might explain the ineffectiveness of antibiotic therapy in humans and animals. Bacteria harbouring plasmids have showed resistance to a wide range of antibiotics.

Molecular techniques such as PCR have been applied in the classification of incompatibility groups based on their origin of replication. Plasmids belonging to the IncQ, IncW and IncP have previously been isolated from bacteria in soil, manure and water environments. These plasmids have been detected in a wide range of bacteria, including pathogens that were isolated from environmental water systems, especially those that belong to the IncP group. The development and spread of these genetic elements is a significant public health concern.

Molecular cloning has been used to further analyse plasmids on a genetic level. The most frequently used method for introducing foreign DNA into a bacterial host strain is the electroporation technique, which was reported to deliver greater transformation efficiency than the calcium chloride-heat shock method.

It has been documented that it is more advantageous to use advanced techniques such as the next generation sequencing to fully analyse plasmids. This may aid in exploring the evolutionary history and antibiotic resistance genes they may contain. However, such approaches are not always feasible and conventional old techniques (Sanger sequencing) are more accessible and affordable.

Chapter 3

Materials and Methods

3.1 Bacterial strains used for plasmid analysis

Available multiple-antibiotic resistance bacteria were obtained from parallel studies on antibiotics resistant bacteria in aquatic systems. Samples were from the Harts river and Barbers Pan (2014) and the Schoonspruit river (2016) from various sites (listed in Appendix A). The specific isolates were chosen as they were resistant to more than two antibiotic classes representing aminoglycosides, β -lactams and tetracyclines.

3.2 Plasmid DNA extractions

Ten millilitres of Luria Bertani-Broth (LB Broth; Merck, Germany) containing the appropriate concentration (10-100 $\mu\text{g/ml}$) of selected antibiotics (ampicillin, tetracycline and kanamycin) were prepared. A purified, single colony of the bacterial strain was inoculated in LB-Broth, containing one of the selected antibiotics, and grown overnight at 37°C with constant shaking at 150 rpm. An hour and a half before extraction chloramphenicol (170 $\mu\text{g/ml}$) was added to stop bacterial growth but continue plasmid replication (Sambrook *et al.*, 1989).

Plasmid DNA extractions were performed according to Birnboim and Doly (1979) with modifications. Overnight cultures that were treated with chloramphenicol were centrifuged (13 400 g for 1 minute), the supernatant removed and pellets resuspended in 0.6 ml solution I (50 mM Tris-HCl pH8; 10 mM EDTA; 50 mM glucose). A vortex and pipetting step ensured that the cells were completely resuspended. Two microliter of RNase A (Sigma, US) (10 mg/ml) was added followed by half an hour incubation step at 42°C. To this mixture 0.6 ml solution II (200 mM NaOH; 1% SDS) was added and the cells were mixed by gently inverting the tube 6-8 times. Solution III (0.6 ml; 3M Potassium Acetate, pH5.5) was then added and the tube was gently inverted 6-8 times.

This resulted in the formation of a clear/white precipitate. The Eppendorf tube mixture were centrifuged for 10 minutes at 13 400 g. The clear supernatant was then transferred to a fresh/sterile microfuge tube. To the mixture 900 μl of 100% ethanol was added and mixture was gently inverted. A twenty-minute centrifugation step at 13 400 g at 4°C followed. The supernatant was removed and in the final wash step 100 μl of 75% ice cold ethanol was added and centrifuged at 13 400 g for thirty seconds. All excess ethanol was removed using a pipette and the Eppendorf tube was left open to ensure that pellet was completely dry. Finally, the pellet was resuspended in 50 μl of distilled deionized water and stored at -20°C.

3.3 Spectrophotometric and agarose gel electrophoresis

Plasmid DNA quantity and quality was determined using the Nanodrop, ND-1000 spectrophotometer (Nanodrop Technologies, US) immediately after collection of DNA. Successful plasmid DNA isolation was also confirmed using 1.5% (w/v) gel electrophoresis. Five microliters of plasmid DNA was mixed with 2 μ l of loading dye (6X Orange Loading Dye, ThermoScientific, US), containing GelRed (1000X) (Biotium, US) and was loaded onto the 1.5% agarose gel. The electrophoresis buffer used was 1X TAE (20 mM Acetic acid, 100 mM EDTA, 40 mM Tris at pH 8.0). A 1 kb molecular weight marker (1 kb, O'GeneRuler, Fermentas, US) was used to estimate the molecular weight of plasmid DNA. Conditions for gel electrophoresis were set at 80 Volts for 45 minutes in a Mini Sub-cell GT and Power-Pac (Bio-Rad, US). Images were captured using a ChemiDoc system (Bio-Rad, US).

3.4 Determining the DNA fragment sizes

The *HindIII* enzyme (Promega Corporation, USA) was used in this study to digest the plasmids. A final volume of 10 μ l restriction enzyme reaction consisted of 1 μ l 10X Buffer E (Promega Corporation, USA), 0.2 μ l BSA (Promega Corporation, USA), 0.2 μ l restriction enzyme, 1 μ l of DNA template and nuclease-free water (ThermoFischer, US) to make up the final volume. The reaction mixture was incubated at 37°C for 4 hours. Ten microliters of the reaction and 2 μ l of 6X Orange loading dye (ThermoFischer, US) containing GelRed (Biotium, US) was loaded onto a 1.5% agarose gel (see section 3.3 for electrophoresis details).

3.5 Bacterial transformation

The electroporation protocol was performed according to the manufacturer's (Bio-Rad, US) with minor modifications. Fifty microliter (μ l) of 10- β competent *E. coli* (New England Biolabs: NEB, US) cells were thawed at room temperature and immediately placed on ice. Both 0.1 cm cuvettes (Bio-Rad, US) and plasmid DNA were separately placed on ice. Two microliters (μ l) of plasmid DNA was mixed with the 50 μ l cell suspension and kept on ice for 30-60 seconds. Mixtures were then transferred to 0.1 cm cuvettes (kept on ice). The cuvette was placed in the safety chamber (also kept on ice) and covered with the slide. The pulser (Bio-Rad, US) was set at 1.8 kilo Volts (kV) by simultaneously depressing the raise and lower buttons. A pulse was applied to the cuvette (cells) by simultaneously pressing both the pulse buttons. After removal of the cuvettes, 1 ml of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) medium (New England Biolabs, US) was immediately added to the cells to obtain maximal transformation efficiency of *E. coli*. This step was very crucial in ensuring recovery of transformants. The cell mixture was transferred to a sterile 1.5 ml Eppendorf tube and incubated for one hour at 37°C, with vigorous shaking

at 225 rpm. Pulse parameters like the actual voltage (in kV) and time constant (in milliseconds) was recorded. Spread plates were prepared on LB-agar plates containing selected antibiotics (ampicillin, tetracycline and kanamycin) at various concentrations ranging from the lowest (10 µg/ml) to the highest (100 µg/ml). Plates were incubated for 16 hours at 37°C. To determine the transformation efficiency, the ratio of the number of transformants to the concentration of DNA (per µg) used, was calculated.

3.6 Susceptibility profiles determination

Antibiotics used to determine the susceptibility profiles of the transformants included: ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), kanamycin (30 µg), neomycin (30 µg), streptomycin (10 µg) and tetracycline (30 µg) (Oxoid, UK). Mueller-Hinton agar was used. Hundred microliters (µl) of each culture was spread plated onto the media. The plates were left to dry before applying the discs onto the surfaces. Inhibition diameter zones were measured (in mm) after an incubation step of 16 to 18 hours at 37°C. Measurements were compared to CLSI (2014) standards.

3.7 Minimum inhibitory concentration (MIC) of original and transformed strains to selected antibiotics

Most of the MAR-isolates obtained from colleagues were resistant to ampicillin, tetracycline and kanamycin. Therefore, minimum inhibitory concentration (MIC) of these antibiotics was determined for selected bacterial strains, using the E-test strips for ampicillin (Oxoid, UK), tetracycline (Oxoid, UK) and kanamycin (Davies Diagnostics, Italy). This was done according to the instructions of the manufacturer.

3.8 PCR amplification of Incompatibility (Inc) groups

The IncP, IncQ and IncW primers were specifically chosen as all the isolates included in this study were obtained from environmental water samples. In order to detect and identify plasmids with broad host ranges, PCR amplification was done according to Götz *et al.* (1996). All PCR protocols were performed using the Techne Prime elite Thermo Cycler (Bibby Scientific Limited; UK). A total volume of 25 µl mixture was made up of 2X PCR master mix (0.4 mM dNTPs, 4 mM MgCl₂ and 0.05 U/µl Taq DNA polymerase (ThermoFischer, US)), nuclease-free water (ThermoFischer, US), specific (10 pmole/µl) primers (Table 3.1; Inqaba Biotech, SA) and DNA template. The same protocol was used for the amplification of each Inc primer set (Götz *et al.*, 1996; Mahlatsi, 2013). The initial denaturation step was for 300 seconds at 94°C. Thirty-five (35) cycles consisting of denaturation for 60 seconds at 94°C, primer annealing step for 60 seconds irrespective of the annealing temperatures as listed in

Table 3.1, and a primer extension for 60 seconds at 72°C with a final extension of 600 seconds at 72°C.

3.9 PCR amplification of genes responsible for antibiotic resistance

3.9.1 β -lactam resistance

The specific primers for the *ampC* gene, encoding for β -lactamase activities (Schwartz *et al.*, 2003), was selected. PCR mixtures were prepared as described in section 3.8. The initial denaturation step was at 94°C for 30 seconds. Thirty-five cycles consisted of denaturation for 30 seconds at 94°C, primer extension for 60seconds at 72°C and a final extension of 420 seconds at 72°C. Both forward and reverse primers are listed in Table 3.1.

3.9.2 Tetracycline resistance

The *tetA* primers (Aarestrup *et al.*, 2003) as listed in Table 3.1 were used for detection of tetracycline resistance genes. The following were the final cycling conditions used. An initial denaturing step of 300 seconds at 94°C. Subsequently a 35 cycle step consisting of a denaturing step at 94°C (60 seconds), extension of 55°C (60 seconds) and a final extension at 72°C (90 seconds).

3.9.3 Aminoglycoside resistance

The specific primers used (Woegerbauer *et al.*, 2014) are listed in Table 3.1. PCR conditions were as follow: initial denaturing step was set at 95°C for 3 minutes. The 35 cycle step consisted of a denaturation step at 94°C for 1 minute, the annealing temperature of 56°C for 1 minute, an extension step of 72°C for 1 minute and the final extension of 72°C for 5 minutes.

Table 3.1: Specific primers for PCR amplification of three Incompatibility groups (IncP-9, IncQ and IncW) and antibiotic resistance genes. Both F (forward) and R (reverse) were used.

Specific primers	Oligonucleotide Sequences (5' - 3')	Anneal. temp. (°C)	Size (bp)	Reference
Incompatibility primers				
IncP-9				
<i>ori</i> 3Fd	5'- CCA CCG ACA CTG ATG GTC TG -3'	54	800	Krasowiak <i>et al.</i> , 2002
<i>rep</i> 3Rc	5'- ACC GTG ATG CGT ATT CGT G -3'			
IncQ				
<i>oriV</i> 1	5'- CTC CCG TAC TAA CTG TCA CG -3'	57	436	Götz <i>et al.</i> , 1996
<i>oriV</i> 2	5'- ATC GAC CGA GAC AGG CCC TGC -3'			
IncW				
<i>oriV</i> 1w	5'- GAC CCG GAA AAC CAA AAA TA -3'	58	1 140	Götz <i>et al.</i> , 1996
<i>oriV</i> 2w	5'- GTG AGG GTG AGG GTG CTA TC -3'			
Antibiotic Resistance Genes primers				
β-lactams				
<i>ampC</i> F	5'- TTC TAT CAA MAC TGG CAR CC -3'	49	550	Schwartz <i>et al.</i> , 2003
<i>ampC</i> R	5'- CCY TTT TAT GTA CCC AYG A -3'			
Tetracyclines				
<i>tetA</i> 1	5'- GTA ATT CTG AGC ACT GTC GC -3'	55	888	Aarestrup <i>et al.</i> , 2003; Christabel <i>et al.</i> , 2012
<i>tetA</i> 2	5'- CTG CCT GGA CAA CAT TGC TT -3'			
Aminoglycosides				
<i>nptII</i> F	5'- ATG ATT GAA CAA GAT GGA TTG C -3'	56	795	Woegerbauer <i>et al.</i> , 2014
<i>nptII</i> R	5'- TCA GAA CTC GTC AAG G -3'			

3.10 Agarose gel electrophoresis of PCR products

In this study agarose gel electrophoresis was used to confirm the success of DNA isolations, estimation of the size of plasmids using restriction enzymes as well as PCR amplifications of antibiotic resistance genes, including plasmid incompatibility groups (Section 3.3).

3.11 Sequencing data analysis

PCR products were purified using a NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Germany). DNA quality and quantity was determined using the NanoDrop, ND-1000 spectrophotometer (Nanodrop Technologies, US). The BigDye Cycle Sequencing Terminator Kit v3.1 (Applied Biosystems, UK) was used for sequencing PCR reactions. A final volume of 20 µl PCR reaction contained 1:10 diluted Ready Reaction Premix (2.5X), BigDye Sequencing Buffer (5X), 3.2 pmole of the specific primer (Inqaba Biotech, SA), Template DNA (20 ng) and nuclease free water (Fermentas Life Sciences, US). The C1000 Thermocycler (Bio-Rad, US) was used for thermal cycling. The following thermal cycling conditions was used; 96°C for 60 seconds, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and finally 60°C for 240 seconds. The second clean-up of PCR products was done using a ZR DNA-Sequencing Clean-up kit (Zymo Research, US) according to the manufacturer's instructions.

Sequencing of amplicons was done using an ABI 3130 Genetic Analyser (Applied Biosystems, UK). The chromatograms obtained were viewed in Geospiza Finch TV (version 1.4) software and BLAST (Basic Local Alignment Search Tool) searches (<http://www.ncbi.nlm.nih.gov/BLAST>) were performed for identification purposes. Subsequently to the sequencing analysis, a phylogenetic tree (neighbor-joining) was constructed to illustrate the association of plasmids and similar genes (*ampC*, *tetA* and *IncP*) detected in various bacterial strains. For this reference gene sequences were chosen for *IncP*, *ampC* and *tetA* from the NCBI database. MEGA 6 (version 6.06) software program (Tamura *et al.*, 2013) was used for phylogenetic analysis.

Chapter 4

Results

4.1 Introduction

This chapter gives an overview of the results obtained from the characterization of plasmids, isolated from several Gram negative, multiple antibiotic resistant *E. coli* strains from parallel studies. Characterization was done to determine plasmid size, transforming them into a 10- β *Escherichia coli* host strain and to determine whether antibiotic resistance phenotypes were introduced by the plasmid. Furthermore, plasmids were categorized into specific incompatibility groups, tested for the presence of antibiotic resistance genes using polymerase chain reaction (PCR). Moreover, sequencing was done to determine the identities of the amplified antibiotic resistance genes. These results were then used to construct phylogenetic trees to observe a possible close association between genes harboured by plasmids and relevant reference genes obtained from the NCBI database.

4.2 Susceptibility profiles of selected antibiotic resistant bacteria

Bacterial strains were selected based on their antibiotic susceptibility profiles (Table 4.1). Isolates from the Harts river- Barbers Pan and the Schoonspruit river in the North West province were all Gram negative bacilli. All of the 20 bacterial strains were resistant to at least 2 or more antibiotic groups/classes.

From Table 4.1 it is evident that isolates from the Harts river and Barbers Pan were generally resistant and had reduced susceptibility to ampicillin, tetracycline and erythromycin. Samples from the Harts river (HR3–HR8) showed a similar trend in resistance to ampicillin and erythromycin. Furthermore, HR7 and HR8 samples showed reduced susceptibility to kanamycin and tetracycline (Table 4.1). Barbers Pan samples showed resistance to ampicillin, tetracycline and erythromycin and reduced susceptibility to kanamycin and neomycin (Table 4.1). Thus, similar antibiotic resistance phenotypes were observed for (HR3–HR6 and HR7–HR8, respectively), and Br1–Br3 samples. Bacterial isolates with the exact same susceptibility patterns were assumed to be clones from the same bacterial strain.

Isolates from the Schoonspruit river showed multiple resistances to a broad spectrum of antibiotics, ranging from three to five different antibiotics (Table 4.1). Nine of the samples were resistant to tetracycline and kanamycin, while only eight were resistant to ampicillin. These multiple resistance profiles may be an indication of multiple genes present on plasmids or of multiple plasmids present in one bacterium (Nsofor and Iroegbu, 2013b; Manjusha and Sarita,

2013). Among all the isolates, resistance patterns were in this specific order ampicillin (17/20), tetracycline (12/20), erythromycin (11/20), kanamycin (9/20), streptomycin (5/20), neomycin (4/20), and the least resistance to chloramphenicol (1/20). Results indicate that the Schoonspruit river, site downstream from a WWTP, had a generally diverse resistance to various antibiotic groups, compared to the Harts river and Barbers Pan.

Table 4.1: Susceptibility profiles of original strains obtained from previous studies.

Water Source	Site	Sample name	Gram Stain (+/-)	Antibiotics						
				A (10 µg)	K (30 µg)	Ch (30 µg)	S (10 µg)	T (30 µg)	E (15 µg)	N (30 µg)
Harts river	Before WWTP at Lichtenburg	HR1	-	I	S	S	S	I	R	I
	Before WWTP at Lichtenburg	HR2	-	I	S	S	S	S	R	S
	Before WWTP at Lichtenburg	HR3	-	R	S	S	S	I	R	I
	Before WWTP at Lichtenburg	HR4	-	R	S	S	S	I	R	I
	Before WWTP at Lichtenburg	HR5	-	R	S	S	S	I	R	I
	Before WWTP at Lichtenburg	HR6	-	R	S	S	S	I	R	I
	Before WWTP at Lichtenburg	HR7	-	R	I	S	S	I	R	S
	Before WWTP at Lichtenburg	HR8	-	R	I	S	S	I	R	S
Barbers Pan	North of Goosepan	Br1	-	R	I	S	S	R	R	I
	North of Goosepan	Br2	-	R	I	S	S	R	R	I
	North of Goosepan	Br3	-	R	I	S	S	R	R	I
Schoonspruit river	Downstream from WWTP	SR1	-	R	R	S	I	R	I	R
	Downstream from WWTP	SR2	-	I	R	S	I	R	I	R

Downstream from WWTP	SR3	-	R	R	S	R	R	I	I
Downstream from WWTP	SR4	-	R	R	S	I	R	I	R
Downstream from WWTP	SR5	-	R	R	S	R	R	I	I
Downstream from WWTP	SR6	-	R	R	S	R	R	I	I
Downstream from WWTP	SR7	-	R	R	S	I	R	I	R
Downstream from WWTP	SR8	-	R	R	R	R	R	I	I
Downstream from WWTP	SR9	-	R	R	S	R	R	I	I

S – susceptible, I – intermediate resistant and R – resistant. Abbreviations for antibiotics include; A (ampicillin), K (kanamycin), C (chloramphenicol), S (streptomycin), T (tetracycline), E (erythromycin), N (neomycin).

4.3 Plasmid DNA profiles

Plasmid DNA was isolated using the method described in Section 3.2. Concentrations of the plasmid DNA ranged between 17.1 and 104.0 ng/μl as determined by the NanoDrop, ND-1000 spectrophotometer (Nanodrop Technologies, US). An agarose gel image of the plasmid DNA is shown in Figure 4.1. Multiple bands were obtained (A to E). These bands are probably due to various forms of the plasmids or could be presenting different/multiple plasmids. Several of the bands exceeded 10 kilo base pairs (kb). Gel electrophoresis confirmed parental plasmids were present in all 20 antibiotic resistant strains the three water sources. In lanes marked pHR3, pHR5 and pHR6 similar patterns can be seen. Results also showed that band sizes from pHR7–pHR8 as well as pBr1–pBr3 could be identical. A similar band pattern was also observed in pHR1 and pBr1–pBr3. This trend overlaps with the susceptibility profiling in Table 4.1.

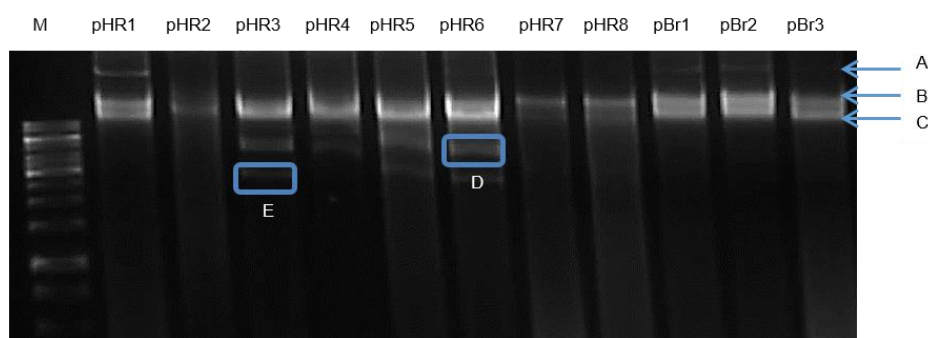


Figure 4.1: A 1.5% (w/v) gel red stained agarose gel image presenting parental plasmid DNA isolated from various strains from the Harts river (pHR1–pHR8) and Barbers Pan (pBr1–pBr3). In lane M is a 1 kb molecular weight marker. Banding positions marked A–E represents potentially different parental plasmids.

In the present study, the six base pair restriction endonucleases, *HindIII*, was used to digest the plasmids. The sizes of plasmids from these three water sources are listed in Table 4.2. The 1 kb ladder was used as the molecular weight marker. Gel electrophoresis illustrated that *HindIII* enzyme cleaved the plasmid DNA at one or multiple sites (Figure 4.2). Majority of the plasmid DNA only had one recognition site, while several were cleaved at three recognition sites resulting in the multiple bands observed. Fragment sizes ranged from 1,500 to 13,995 bp (Table 4.2). Results of the *HindIII* digestion of all 20 parental plasmids indicate that eleven contained single parental plasmids, seven harboured potentially three parental plasmids and two had double bands. The presence of multiple bands might be an indication of more than one plasmid in a single bacterium (Manjusha and Sarita, 2013).

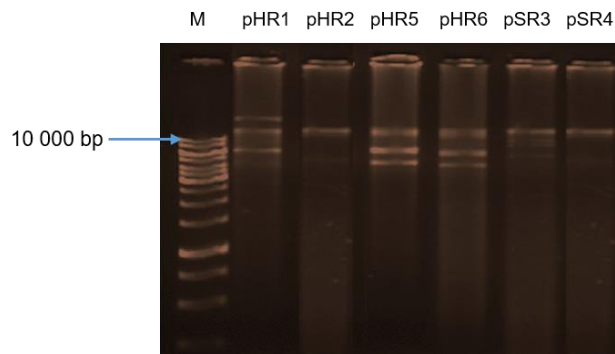


Figure 4.2: A 1.5% (w/v) gel red stained agarose gel electrophoresis image presenting digestion of parental plasmid DNA using *HindIII* enzyme. A 1 kb molecular weight marker is indicated in lane M. Lanes 2-6 were parental plasmids representative from both the Harts and Schoonspruit rivers.

Table 4.2: Estimated band sizes of parental plasmids using *HindIII* endonucleases.

Source	Site	Parental Plasmid	Estimated size in bp (using restriction enzyme <i>HindIII</i>)		
Harts river	Before WWTP at Lichtenburg	pHR1	13,995	10,000	5,998
		pHR2	10,000		
		pHR3	10,000	5,998	3,999
		pHR4	10,000	5,998	4,498
		pHR5	10,000	5,998	3,999
		pHR6	10,000	5,998	3,999
		pHR7	13,996		
		pHR8	13,996		
Barbers Pan	North of Goosepan	pBr1	10,000	3,499	2,999
		pBr2	10,000	5,000	
		pBr3	10,000	5,000	
Schoonspruit river	Downstream from WWTP	pSR1	10,000		
		pSR2	10,000	2,500	1,500
		pSR3	10,000		
		pSR4	10,000		
		pSR5	10,000		
		pSR6	10,000		
		pSR7	10,000		
		pSR8	10,000		
		pSR9	10,000		

4.4 Bacterial transformation and susceptibility profiles

For bacterial transformation three different antibiotics (ampicillin, kanamycin and tetracycline) were employed as selective agents. Various concentrations were used ranging from 10-100 µg/ml for all antibiotics. The electroporation technique with 10-β competent *E. coli* host strain was used which yielded a large number of colonies formed on plates that contained ampicillin (Figure 4.3). These results were observed for positive and negative controls and the three parental plasmids (pHR2, pHR5, pSR8). Furthermore, parental plasmid enumeration did not occur on media containing tetracycline and kanamycin, respectively.

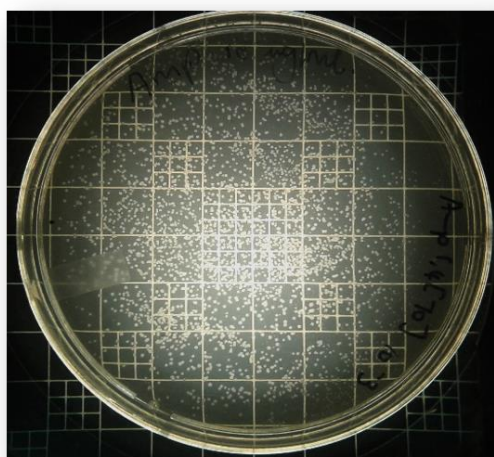


Figure 4.3: Transformants plated on ampicillin containing media after successful transformation using the electroporation technique.

Differences were also observed in the transformation efficiency ranging from $3,1 \times 10^8$ to $7,1 \times 10^8$ transformants per microgram DNA. Both pHR2 and pSR8 were 10,000 bp (Table 4.2) and had higher transformation efficiencies than pHR5, which were larger in size ranging from 3,999 to 10,000 bp. The antibiotic resistance profiles of the parental strain were not the same as the transformants (Table 4.3). Parental strains pHR2 and pHR5 showed resistance to erythromycin, while parental strains pHR5 and pSR8 were resistant to ampicillin. Furthermore, parental plasmid strain pSR8 was also resistant to kanamycin, chloramphenicol, streptomycin and tetracycline. It is rather clear from the transformation analysis, that the resistance markers encoded on plasmids were β-lactamase (ampicillin) and streptomycin.

Table 4.3: Comparison of the antibiotic resistance profiles of parental strains and transformants as well as the transformation efficiencies.

Parental Plasmid	Resistance profile of parental strain	Resistance profile of transformed <i>E. coli</i>	Plasmid encoded resistance	Transformation efficiency (per μg DNA)
pHR2	E	S	S (1)	$7,1 \times 10^8$
pHR5	E	S	S (1)	$3,1 \times 10^8$
pSR8	A, K, C, S, T	A	A (1)	$6,4 \times 10^8$

Abbreviations include; A (ampicillin), E (erythromycin), K (kanamycin), C (chloramphenicol), S (streptomycin) and T (tetracycline) indicate all the antibiotics that the strains were screened against. The numbers in brackets illustrates the amount of resistances encoded for on the plasmids.

4.5 Minimum inhibitory concentration (MIC) of selected antibiotics

Several bacterial strains were selected for the determination of minimum inhibitory concentrations for both parental strains (Table 4.4) and transformants (Table 4.5). The selected antibiotics included ampicillin, kanamycin and tetracycline. These antibiotics were also used as selective pressure during plasmid DNA transformation. Specific strains were selected in instances where two or more strains showed the same susceptibility profiles (Table 4.1). Four bacterial strains from the Harts river, one from Barbers Pan and eight from Schoonspruit river were tested.

Bacterial strains from the Schoonspruit river, showed high MIC values for tetracycline ($\geq 256 \mu\text{g/ml}$) and kanamycin ($\geq 256 \mu\text{g/ml}$) (Table 4.4). However, for ampicillin only one isolate (SR9) had a MIC value higher than $256 \mu\text{g/ml}$, while the rest were lower than $32 \mu\text{g/ml}$. In general, lower MIC values for the three antibiotics were detected from the Harts river and Barbers Pan. These results demonstrated that MIC values of parental strains as well as transformed strains did show differences in the MIC values (Tables 4.4 and 4.5). A similar trend was found between tetracycline and kanamycin resistance phenotypes where both expressed at high levels of MIC $\geq 256 \mu\text{g/ml}$.

Table 4.4: MIC of antibiotics for parental strains from Barbers Pan and both the Harts and Schoonspruit rivers using E-test strips (256 µg/ml).

Water source	Site	MIC (µg/ml)			
		Parental strain	Ampicillin (256 µg/ml)	Tetracycline (256 µg/ml)	Kanamycin (256 µg/ml)
Harts river	Before WWTP at Lichtenburg	HR1	≥8	≥4	≥2
		HR2	≥8	≥4	≥1
		HR5	≥256	≥4	≥4
		HR7	≥4	≥2	≥2
Barbers Pan	North of Goosepan	Br1	≥32	≥256	≥4
Schoonspruit river	Downstream from WWTP	SR1	≥16	≥256	≥256
		SR2	≥16	≥256	≥256
		SR3	≥16	≥256	≥256
		SR4	≥16	≥256	≥256
		SR5	≥16	≥256	≥256
		SR7	≥16	≥256	≥256
		SR8	≥32	≥256	≥256
		SR9	≥256	≥256	≥256

Table 4.5: MIC for transformants from both the Harts and Schoonspruit rivers using E-test strips (256 µg/ml).

Water source	Site	MIC µg/ml			
		Transformant	Ampicillin (256 µg/ml)	Tetracycline (256 µg/ml)	Kanamycin (256 µg/ml)
Harts river	Before WWTP at Lichtenburg	<i>E. coli</i> pHR2	≥0.5	≥1	≥0.75
		<i>E. coli</i> pHR5	≥1	≥0.06	≥0.064
Schoonspruit river	Downstream from WWTP	<i>E. coli</i> pSR8	≥0.25	≥0.06	≥0.25

4.6 PCR amplification of Incompatibility (IncP) group

All of the parental plasmids from the Harts, Schoonspruit rivers and Barbers Pan were tested for IncQ, IncW and IncP groups, but were only positive for IncP. Transformants also belonged to the IncP group of plasmids. Amplification products of the IncP group is shown in Figure 4.4.

Results from the IncP group showed variation in band sizes ranging from 600 to 900 bp. Parental plasmids from the Harts river (pHR1–pHR3) were more or less the same size (900 bp) compared to parental plasmids pHR4 and pHR5 which were smaller (Figure 4.4). Furthermore, parental plasmid pHR6 appeared to be much smaller in size compared to the rest of the parental plasmids. Results also showed that parental plasmids pHR7 and pHR8 were similar in band size (800 bp). The amplicons from Barbers Pan were of the same size. A similar trend where band sizes were more or less the same were also observed in plasmids pHR4-pHR5, pHR7-pHR8 and pBr1-pBr3 (Table 4.2).

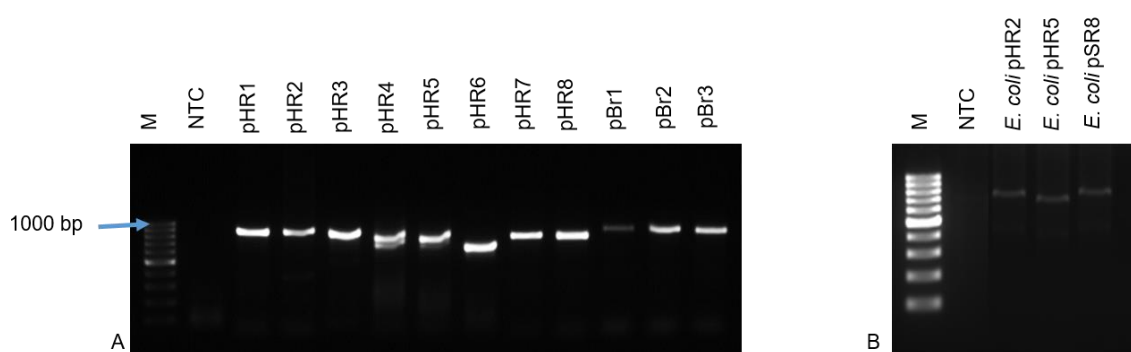


Figure 4.4: A 1.5% (w/v) gel red stained agarose gel image presenting parental plasmids of IncP from Barbers Pan (pBr1–pBr3), pSR8 from the Schoonspruit river and parental plasmids pHR1–pHR8 from the Harts river (Figure A). Transformed *E. coli* plasmids (pHR2, pHR5 and pSR8) also tested positive for the IncP group (Figure B). Lane M is the 100 bp ladder used and lane marked NTC represent the no template control.

4.7 PCR amplification of antibiotic resistance genes

4.7.1 β -lactam resistance

Successful amplification of the *ampC* gene was achieved (Figure 4.5). This is expected since several isolates from the Harts river and Barbers Pan showed resistance and reduced susceptibility to ampicillin (section 4.2; Table 4.1). Furthermore, results also showed that parental plasmids from the Harts River (pHR2 and pHR5) tested positive for the *ampC* gene. Only one of the parental plasmids from the Schoonspruit river (pSR8) were positive for the *ampC* gene although greater resistance was observed in most of the isolates (Figure 4.5). The expected band sizes were approximately 550 base pairs, however a variation in gene size were observed. Results also showed that the three isolated plasmids exceeded the expected band size of the genes.

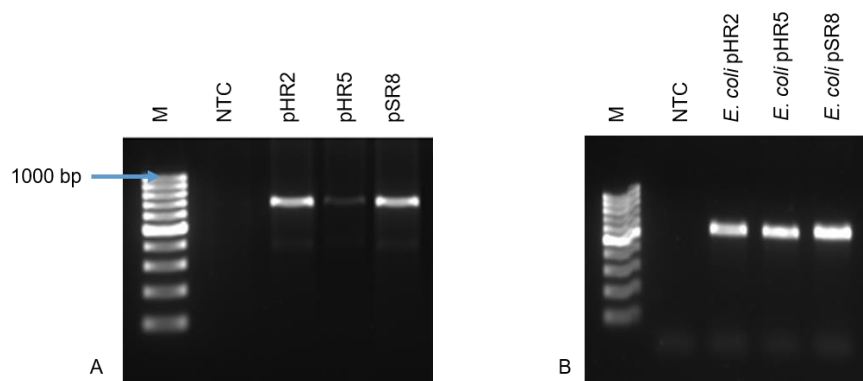


Figure 4.5: A 1.5% (w/v) agarose gel electrophoresis image for the detection of the β -lactamases activity. Amplification of the *ampC* gene were positive in three parental plasmids from the Schoonspruit (pSR8) and Harts river pHR2 and pHR5 (Figure A). After transformation these transformed *E. coli* plasmid also tested positive for the *ampC* gene (Figure B). Lane M represent the 100 bp ladder. Size of the amplicons were more or less in the range of 550 bp (Scwartz *et al.*, 2003).

4.7.2 Tetracyclines resistance

All three isolates from Barbers Pan were resistant to tetracycline (see Table 4.1). Thus, the *tetA* gene was successfully amplified in parental plasmids pBr1-pBr3. A similar trend was found in the Schoonspruit river isolates which also showed resistance to tetracycline. The *tetA* gene was only amplified in 5 of the parental plasmids from the Schoonspruit river. Expected amplicon sizes were more or less in the range of 888 bp (Christabel *et al.*, 2012). Variation in gene sizes may indicate the evolutionary history of the antibiotic resistance phenomenon.

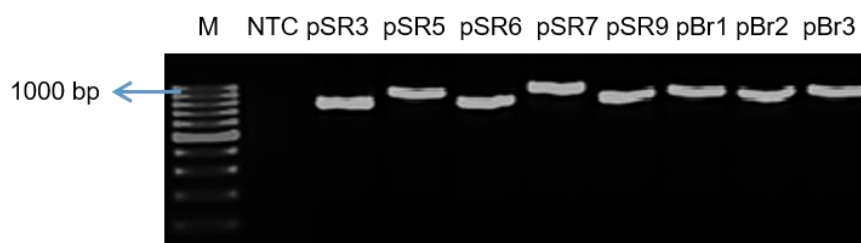


Figure 4.6: A 1.5% gel electrophoresis images illustrating amplification of the *tetA* gene. Lane marked M is the 100 bp ladder that was used, while NTC represents the no template control. Lanes 2-6 were parental plasmids from the Schoonspruit river (pSR3, pSR5-pSR7 and pSR 9) and in lanes 7-9 isolates from Barbers Pan (pBr1-pBr3).

4.7.3 Aminoglycoside resistance

Results in Figure 4.7 demonstrated that the positive control yielded a band of the expected size (795 bp). Furthermore, resistance and reduced susceptibility to kanamycin were detected in isolates from the three water sources (Table 4.1), none tested positive for the *nptII* gene.

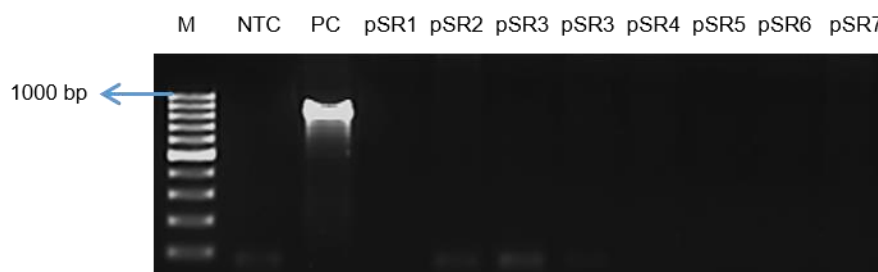


Figure 4.7: Agarose gel electrophoresis showing negative amplification of the *nptII* gene in parental plasmids from the Schoonspruit river. Lane M represent the 100 bp ladder, lane 1 the no template control (NTC) and the positive control (PC). The expected size of amplicons was 795 bp (Woegerbauer *et al.*, 2014).

4.8 Sequencing data analysis

The identities of the amplified PCR products were confirmed through sequencing using the protocols in Section 3.11. Sequencing was done in both directions (forward primers: *ampC* F, *tetA* 1 and *ori* 3Fd; reverse primers *ampC* R, *tetA* 2 and *rep* 3Rc).

A total of 12 amplicons from plasmids originating from the parental species were sequenced and included three *ampC*, four *tetA* and five IncP genes. These were successfully identified by BLASTn searches and compared to known reference genes from GenBank (see Table 4.6). High sequence (99%) similarities in most of the identified sequences was obtained, where in some instances several sequences was an exact match (100%). The expected (E) values were 0 and below.

Identification of the IncP origin of replication gene matched corresponding nucleotide sequences originally from *Escherichia coli* strains. For the *ampC* gene, nucleotide sequences were a 99% match to the corresponding reference genes encoding β -lactamase resistance. The *tetA* gene identities were an exact match (100%) to known *tetA* genes from Genbank that code for efflux pumps. All identified sequences matched with *Escherichia coli*, or other Gram-negative bacteria that belonged to the Proteobacteria. Phylogenetic trees for each of the genes were constructed and these demonstrated close associations.

Figure 4.8 illustrate the phylogenetic relationship of IncP genes. The tree is divided into two clusters, A and B which is further divided into subgroups. One of the identified plasmids (pHR2) from the Harts river grouped together with the IncP plasmid from an uncultured bacterium with a bootstrap value below 50%. The remaining plasmid isolates from the Harts river were grouped under subgroup B. Phylogenetic affiliation for this group is supported with high bootstrap values of 100%. This may suggest that these plasmids may be different from other IncP plasmids, as they were not closely grouped with other relevant plasmids.

Phylogenetic relationship of identified *ampC* plasmid isolates from both the Harts and Schoonspruit rivers are shown in Figure 4.9. The tree is also divided into two groups A and B, where A is further divided into subgroups. Only 2 out of 3 *ampC* gene sequences was successfully aligned with reference sequences. Both genes were grouped in one of the subgroups of group A. These two plasmids also grouped together with a bootstrap value of 100%. However, one of these plasmids formed a subgroup with other reference genes. High bootstrap value of 100% is shown, indicating a positive affiliation with reference genes.

In Figure 4.10 a phylogenetic relationship between *tetA* genes harboured by plasmids isolates from the Schoonspruit river and GenBank reference strains are illustrated. The identified *tetA* positive species can be closely affiliated with several reference strains as well as a broad-host-range vector, with strong bootstrap values of 99% and 100% respectively.

Table 4.6: GenBank identities of amplified (*IncP*, *ampC* and *tetA*) gene sequences for bacterial strains used in this study.

Sample name & gene	Description	Query cover	E-value	Identity	Accession
<i>IncP</i>					
pHR2	Escherichia coli strain 2009C-3133, complete genome	94%	2.00E-46	100%	CP013025.1
pHR3	Escherichia coli strain GB089, complete genome	100%	2.00E-156	99%	CP013663.1
pHR4	Escherichia coli strain GB089, complete genome	100%	3.00E-154	99%	CP013663.1
pHR5	Escherichia coli strain GB089, complete genome	99%	0	99%	CP013663.1
pHR6	Escherichia coli strain GB089, complete genome	100%	2.00E-156	99%	CP013663.1
<i>ampC</i>					
pHR2	Escherichia coli AmpC-EC26 (bla(ampc-EC26)) gene, complete cds	99%	0	99%	DQ092421.1
pHR5	Escherichia coli AmpC-EC66 (ampC-EC66) gene, complete cds	99%	0	99%	EF507686.1
pSR8	Escherichia coli AmpC-EC11 (bla(ampc-EC11)) gene, complete cds	99%	0	99%	DQ092430.1
<i>tetA</i>					
pSR3	Escherichia coli strain EPEC tetracyclin resistance protein (tetA) gene, partial cds	100%	0	100%	KU892720.1
pSR6	Escherichia coli pDEWT1 tet(A) gene for tetracycline efflux MFS transporter Tet(A), complete CDS	100%	0	100%	NG_048152.1
pSR7	Escherichia coli tet(A) gene for tetracycline efflux MFS transporter Tet(A), complete CDS	100%	1.00E-126	100%	NG_048150.1
pSR9	Escherichia coli strain MUM-57 TetA resistance protein gene, partial cds	100%	0	100%	JQ409109.1

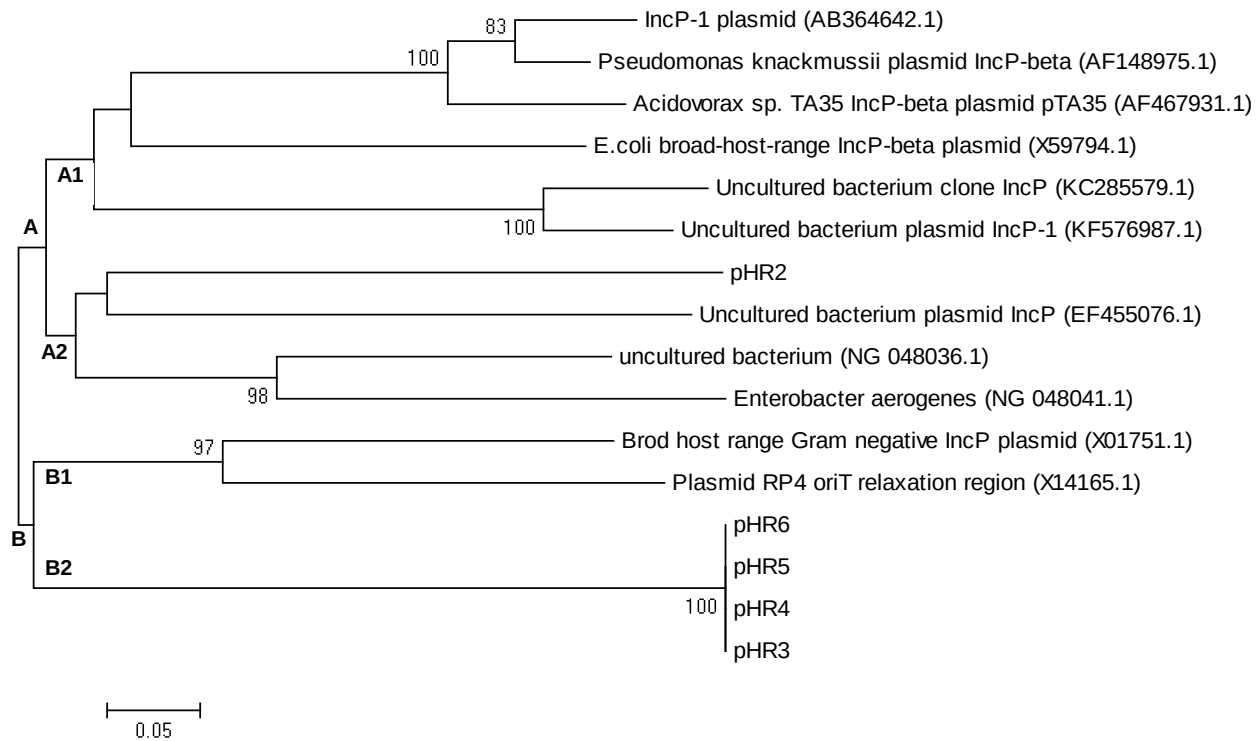


Figure 4.8: Neighbor-Joining phylogenetic tree representative of the IncP gene. Identities of plasmids belonging to the IncP group (Table 4.5) and reference strains was obtained from GenBank and used to construct the tree. Bootstrap values below 50% were not included.

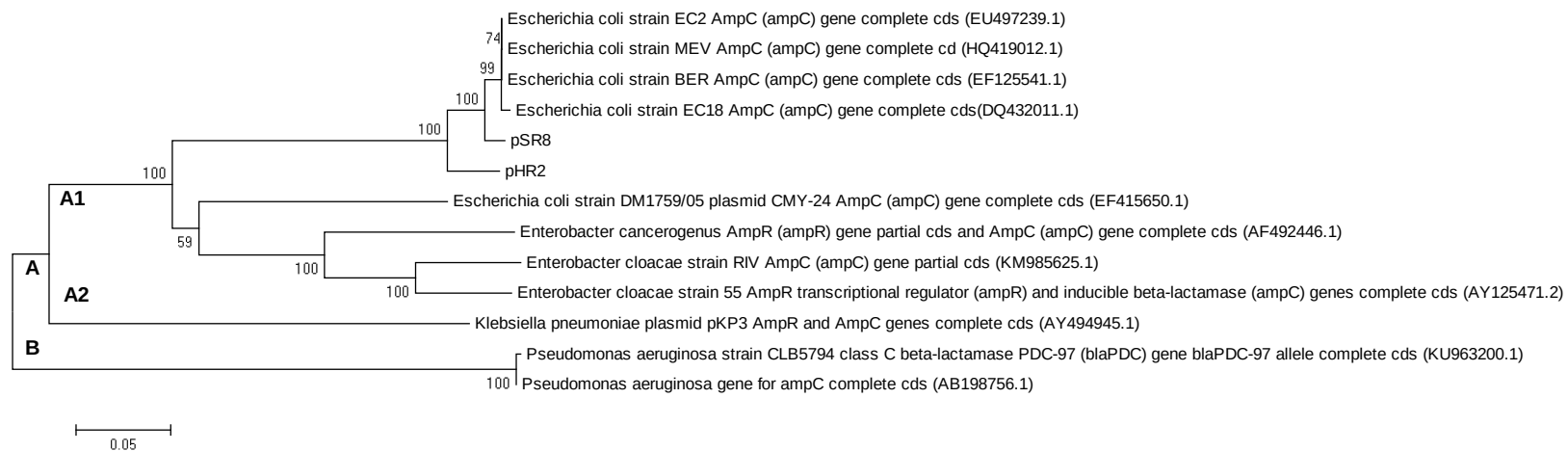


Figure 4.9: Neighbor-Joining phylogenetic tree representative of the nucleotide alignment of *ampC* gene detected in this work and reference nucleotide sequences obtained from GenBank. This data was used was used to construct the tree to illustrate a possible association between plasmids and the β -lactamase encoding gene.

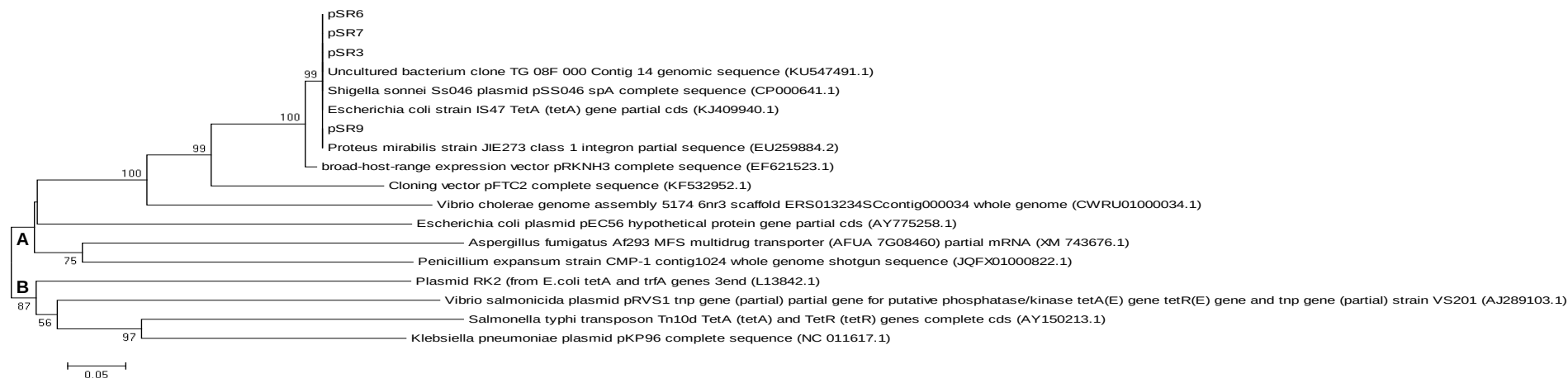


Figure 4.10: Neighbor-Joining phylogenetic tree representing the *tetA* gene nucleotide sequence alignments of genes detected in this work and those obtained from the NCBI database.

4.9 Summary of results

Plasmids were present in antibiotic resistant bacteria isolated from surface water sources in the North West province. Plasmids varied in size. The successfully transformed 10- β *Escherichia coli* strain with plasmid DNA showed different antibiotic resistance profiles as well as decreased levels of expression with low MIC values after transformation. Furthermore, plasmids were analysed pre- and post-transformation for the presence of antibiotic resistance genes that were possibly responsible for the specific phenotype. Only genes encoding for β -lactamase activity were amplified in both parental plasmids and the transformants. Attempts to select for plasmids associated with kanamycin and tetracycline resistance were unsuccessful. However, several genes encoding for efflux pump (*tetA*) were detected in parental plasmid strains.

All isolated plasmids were classified into the IncP group. Further identification showed that the latter were originally isolated from different *Escherichia coli* strains. Subsequently sequencing analysis confirmed that these antibiotic resistance genes encoding for β -lactamase (*ampC*) and efflux pumps (*tetA*) were indeed present on the plasmids extracted in this study. These, shared homology with relevant nucleotide sequences found in GenBank. Thus, plasmids can be associated with the occurrence of antibiotic resistance genes residing in bacteria in the aquatic systems of the North West province. Such plasmids may be widely distributed and transmitted by horizontal and vertical gene transfer and could have implications for human, animal and plant health.

Chapter 5

Discussion

5.1 Introduction

Prevalence of antibiotic resistance has become a global public health concern. Thus, increased interest exists in exploring the occurrence of antibiotic resistance genes in aquatic environments (Scwhartz *et al.*, 2003). These ecosystems provide an ideal setting for the acquisition and transfer of antibiotic resistance genes, due to the continuous antimicrobial pollution that results from anthropogenic activities (Rodriguez-Mozaz *et al.*, 2015). In addition, plasmids have been associated with the rapid development and spread of resistance genes in the environment (Strahilevitz *et al.*, 2009; Laroche-Ajzenberg *et al.*, 2014). Therefore, this study aimed to investigate the association of plasmids and the antibiotic resistance phenomenon in bacteria prevalent in aquatic surroundings. Plasmids were found in antibiotic resistant bacteria previously isolated from surface water systems (Harts and Schoonspruit rivers and Barbers Pan) in the North West province, South Africa. Furthermore, transformation through electroporation using a 10- β *E. coli* host strain was done. This was followed by susceptibility testing to compare the transformant profiles to those of the parental strains. Parental plasmid DNA were subjected to PCR amplification and sequencing to identify possible antibiotic resistance genes associated with plasmids.

5.2 Prevalence of antibiotic resistant-bacteria in aquatic sources

In this study, MAR-bacteria previously isolated from the Harts river (site situated before WWTP at Lichtenberg), Barbers Pan (North of Goosepan) and the Schoonspruit river (downstream from a WWTP) were selected for further analysis. These were all Gram negative bacterial isolates from all three water sources and were all resistant to ampicillin and tetracycline. Results also revealed that bacterial strains from the Schoonspruit river, collected downstream from a wastewater treatment plant had the most diverse resistance phenotype. Similar findings were documented by Molale (2012) who investigated the water quality of these specific surface water systems in the North West province but focussed on the Gram positive *Enterococcus* sp. These results of the prevalence of MAR bacteria in species previously isolated from Barbers Pan, the Harts- and Schoonspruit rivers thus correspond with results reported in the present study. The occurrence of multiple-antibiotic resistant bacteria is not surprising, as these river systems are frequently impacted by anthropogenic activities such as surface runoff from mining and agriculture and effluent from wastewater treatment plants (NWDAC-SoER, 2008). This may contribute to the introduction of antibiotics and antibiotic resistant bacteria into environmental surface water systems (Awofolu *et al.*, 2007).

Moreover, the predominant occurrence of ampicillin and tetracycline resistant bacteria in aquatic environments have been reported (Carvalho *et al.*, 2014; Molale and Bezuidenhout, 2016). The presence and frequency of these resistance phenotypes agreed with findings of Nsofor and Iroegbu (2013a), Aggarwal *et al.* (2015) and Wose Kinge *et al.* (2010). It has been suggested that prevalence of multiple antibiotic resistant bacteria might have originated from selective pressure caused by the presence of antibiotics in the water (Suzuki *et al.*, 2010). Additionally, the overuse of antibiotics in clinical, veterinary practices and agriculture may contaminate environmental water sources (Suzuki *et al.*, 2010).

Thus, the widespread use of broad-spectrum antibiotics may select for resistance in a wide range of bacteria, as these antibiotics are active against a great number of bacteria. Limited antibiotic treatment options are available for infections caused by β -lactamase-producing pathogens (WHO, 2012; Paterson and Bonomo, 2005). This is cause for concern since we have reached a post antibiotic era and new antibiotics that are effective against these infections may take up to 10 years to develop (WHO, 2012). Considering the MAR phenotypes and specifically the resistance to ampicillin and tetracycline that the rest of the analyses were conducted.

5.3 Plasmids DNA profiles

Plasmids were isolated (Figure 4.1) from the Gram negative, antibiotic resistant bacterial strains mentioned in 5.2. Similar results were obtained in another study where plasmids were isolated from multiple resistant bacteria in aquatic environments (Laroche-Ajzenberg *et al.*, 2014). In the current study plasmid sizes ranged from 1,500 to 13,995 bp. In a previous study by Saranathan *et al.* (2014) plasmids have been isolated with sizes ranging from 500 bp up to 25 kb. These plasmids harboured antibiotic resistance. Thus finding plasmids associated with MAR bacteria from the surface water sources of the North-West province in the mentioned size ranges is not uncommon.

Antibiotic resistant bacteria containing plasmids in the water environment creates a unique opportunity for the evolution and transfer of antibiotic resistance genes (Manjusha and Sarita, 2013). The prevalence antibiotic resistant plasmids is cause for concern, as it can be transferred to possible pathogenic bacteria (Nsofor and Iroegbu, 2013b).

5.4 Bacterial transformation and susceptibility profiles

Three of the plasmids could be successfully transformed into 10- β competent *E. coli* host strain using electroporation. Results also showed high transformation efficiencies ranging from 3.1×10^8 to 7.1×10^8 transformants per microgram DNA. This finding is consistent with those of Janjua *et al.* (2014) and Yildirim *et al.* (2016) who also reported the same range of transformation efficiency. In addition, differences found in transformation efficiency may be influenced by plasmid size. Plasmids of smaller sizes have been reported to deliver greater efficiencies (Sheng *et al.*, 1995). This technique has proven to deliver sufficient copies of DNA that may be used for further analyses such as cloning and protein expression (Liu *et al.*, 2014).

The transformation of these plasmids into 10- β competent *E. coli* could suggest that the plasmids are of broad-host range. Similar findings were reported by Manjusha and Sarita (2013), where plasmids isolated from MAR-*Vibrio* species were transferred into the *E. coli* DH5 α host strain. Therefore, the transfer of these resistance plasmids may cause non-pathogenic bacteria to develop resistance to antibiotics (Schlüter *et al.*, 2007a).

Resistance phenotypes expressed in the parental strains were not the same as those of the transformants. Furthermore, results also showed that two of the *E. coli* transformed with plasmids pHR2 and pHR5 were not able to express the ampicillin resistance phenotype even though they were selected using ampicillin containing media, but showed resistance to streptomycin. A study done by Molale and Bezuidenhout (2012) found that differences in susceptibility profiles were observed before and after plasmid curing. This may suggest that the specific phenotype was not entirely plasmid encoded. Genes encoding for β -lactamases have been found in both chromosomal (Schwartz *et al.*, 2003) and mobile genetic elements such as plasmids (Peter-Getzlaff *et al.*, 2011). It is possible that these genes might have a synergistic effect, expressing a greater resistance against ampicillin together than when separate (Pérez-Pérez *et al.*, 2009). In addition to this it has been found that β -lactam resistance is expressed at low levels even when these genes are detected (Jacoby, 2009). This could possibly explain the contrasting resistance/susceptibility phenotypes of the parental strains and the strains transformed with the plasmids.

However, the 10- β *E. coli* transformed with plasmid pSR8 showed resistance to ampicillin. This may possibly indicate the presence of genes encoding for antibiotic resistant mechanisms suggesting that the plasmid was responsible for the transfer of ampicillin resistance (Schlüter *et al.*, 2007b). In a study by Saranathan *et al.* (2014) it was shown that four of their isolates, initially showed resistance to specific antibiotics, but were susceptible after plasmid curing. Linking the resistance phenotype to the plasmids.

5.5 PCR amplification of Incompatibility group

Plasmid DNA samples were subjected to incompatibility classification groups as previously described by Götz *et al.* (1996) using PCR. The specific incompatibility groups namely IncP, IncQ and IncW were initially selected because they were found to occur in bacteria isolated from aquatic environments (Götz *et al.*, 1996). All plasmids were negative for IncW and IncQ. However, all plasmids were positive for the IncP group. These results were consistent with the reports of Haines *et al.* (2006) Schlüter *et al.* (2007a) and Moura *et al.* (2012). This group harbour conjugative plasmids and represent a broad host range (Schlüter *et al.*, 2007a).

The presence of these IncP plasmids in environmental water systems are commonly associated with multiple antibiotic resistance (Popowska and Krawczyk-Balska, 2013). This is similar to findings of Laroche-Ajzenberg *et al.* (2014), who also detected a multiple antibiotic resistance plasmid pME8 that were responsible for the resistance shown to several antibiotic classes including β -lactams, tetracyclines and aminoglycosides amongst others. Furthermore, some of the bacterial hosts that IncP plasmids were isolated from are known opportunistic pathogens (Adamczyk and Jagura-Burdzy, 2003). Finding such plasmids in aquatic systems could mean that they may largely contribute to the dissemination of antibiotic resistance genes among bacterial species in such aquatic habitats (Schlüter *et al.*, 2007a). The detection of this group of plasmids in our aquatic systems is an illustration of their widespread occurrence in the aquatic environment of the North West province (Dröge *et al.*, 2000).

5.6 PCR amplification of antibiotic resistance genes

5.6.1 β -lactams (*ampC*)

Three of the plasmids tested positive for the detection of the β -lactam *ampC* gene. These plasmids were from bacterial strains isolated from both the Harts and Schoonspruit rivers. Similar results were reported by Schwartz *et al.* (2003) who also detected the *ampC* gene in aquatic environmental isolates. However, one of the bacterial strains (see Table 4.1 HR2) that harboured a plasmid tested positive for the *ampC* gene, even though reduced resistance to ampicillin was observed. Karami *et al.* (2007) also reported results indicating intermediate resistance linked to the presence of the *ampC* gene.

Results from this study may imply that β -lactam resistance (*ampC* gene) is ubiquitous in the aquatic environment of the North West province but may not always be expressed at high levels for phenotypic detection

Plasmid mediated resistance to β -lactamases may be responsible for newly acquired resistances to cephalosporins and carbapenems (Drawz and Bonomo, 2010) Increased

prevalence of nosocomial outbreaks due to this resistance mechanism have been reported worldwide and limits available treatment options (Philippon *et al.*, 2002). The detection of plasmids harbouring genes responsible for the β -lactam resistance phenotype may be transferred between microbes in different environments. These antibiotics work by altering target enzymes, inactivation of antibiotics, alterations in membrane permeability.

After transformation of these parental plasmids into the new host (10- β *E. coli*) transformants were also positive for β -lactamase activity. The *ampC* gene was also detected in the plasmids that were re-isolated from the transformants. These re-extracted plasmids were also positive for the IncP genetic element.

5.6.2 Tetracyclines (*tetA*)

High prevalence of tetracycline resistance phenotypes was reported in this study. This is linked to the detection of the *tetA* gene in parental plasmids from bacterial strains isolated from both Barbers Pan and the Schoonspruit river. These genes that encode for efflux pumps (Chopra and Roberts, 2001; Beceiro *et al.*, 2013) were the most prevalent harboured by plasmids.

The occurrence of these resistance genes in aquatic systems may be due to the widespread utilization of tetracycline in veterinary, agriculture and medicine practices (Chopra and Roberts 2001; Gilchrist *et al.*, 2007). The bacterial strains that carried the *tetA* gene were associated with high MIC values (≥ 256 $\mu\text{g/ml}$). Thumu and Halami (2012) showed elevated MIC levels for tetracycline in the range of 256 and 512 $\mu\text{g/ml}$. Furthermore, the presence of these genes have previously been associated with plasmids (Chopra and Roberts, 2001; Beceiro *et al.*, 2013). Findings of Tennstedt *et al.* (2005) have shown that these genes were the most common found on IncP plasmids. This is also substantiated by our results, suggesting that these genes may be plasmid bound and can spread accordingly. This raises concern, since these genes are widely distributed in the aquatic environment and may confer resistance to several antibiotics (Chopra and Roberts, 2001).

5.6.3 Aminoglycosides (*nptII*)

In this work, kanamycin was one of the selected antibiotics used to screen for presence of antibiotic resistance plasmids. However, no *nptII* genes were detected in plasmids associated with kanamycin resistance. In similar studies (Zhu, 2007; Woegerbauer *et al.*, 2014) it was shown that a larger quantity of samples should be included to obtain overall distribution of the *nptII* gene in the aquatic environments. This could possibly explain the negative results obtained in this study, seeing that a small number of isolates were used. However, the

presence of the gene has been found in various water systems (Smalla *et al.*, 1993; Zhu, 2007; Woegerbauer *et al.*, 2014).

Whole genome sequencing of a broad host range IncP plasmid revealed otherwise (Tennstedt *et al.*, 2005). In that study it was shown that the backbone of plasmid pTB11 have specific regions for kanamycin resistance. This may possibly suggest that other antibiotic resistance genes may be encoded on plasmids that code for aminoglycoside resistance (Juan *et al.*, 2009). These authors suggested that the plasmids in a *P. aeruginosa* strain contained additional aminoglycoside modifying enzymes in addition to β -lactamases. Possibly resulting in the co-selection of both these resistance genes (Beceiro *et al.*, 2013) which might have complicated the detection of the *nptII* gene encoding resistance to aminoglycosides. This may further stress treatment options as the aminoglycosides are commonly used to treat numerous infections instigated by both Gram positive and Gram negative microbes (Beceiro *et al.*, 2013).

5.7 Sequencing data analysis

Findings in this work showed that 12 amplicons from plasmids that originated from the parental species were sequenced and included five IncP, three *ampC* and four *tetA* genes. The identities of genes were successfully obtained using BLASTn searches and were then compared to known reference genes from GenBank (Table 4.6). Sequence similarities of $\geq 99\%$ were obtained in most of the identified sequences, where in some instances several sequences were a definite match (100%). Expected (E) values were 0 and below.

Results showed that these specific antibiotic resistance genes (*ampC*, *tetA* and IncP) were indeed present on the parental plasmids. The identities of bacterial strains from which parental plasmids were originally isolated from were *E. coli* strains. Plasmids of the IncP group are widespread in Gram negative bacteria such as *E. coli* and *Pseudomonas spp.* (Shintani *et al.*, 2010). Similar results were reported by Tauch *et al.* (2003) that showed the presence of genes encoding for both multidrug resistance efflux pumps and β -lactamases on a conjugative antibiotic resistance plasmid. Furthermore, results of this study also correspond with findings of Tennstedt *et al.* (2005) who reported that the *tetA* gene was most common in all of the sequenced IncP plasmids.

Neighbor-joining phylogenetic trees for each of the three genes were constructed to illustrate phylogeny of the gene sequences. Sequences with high sequence similarities were used to construct the trees (Figures 4.8-4.10). The neighbour-joining technique identifies operational taxonomic units (OUT's) which minimizes the branch length at the point where OUT's cluster. This approach can be employed in evolutionary distance data sets, providing the correct tree

topology (Saitou and Nei, 1987). A previous study used phylogenetic analyses to illustrate the association of plasmids from the IncP group and antibiotic resistance genes (Dröge *et al.*, 2000).

Figure 4.8 illustrate the evolutionary relationship of IncP genes. Results showed that one of the identified parental plasmids (pHR2) from the Harts river grouped together with the IncP plasmid from an uncultured bacterium with a bootstrap value below 50%. This may indicate that these plasmids are not closely related. The remaining plasmid isolates from the Harts river showed a close affiliation to each other. This is supported by high bootstrap values of 100% and may suggest that these plasmids may be different from other IncP plasmids, as they were not closely grouped with other relevant plasmids. However, in another study fully analysed plasmids were compared to nucleotide sequences of known IncP plasmids (Tennstedt *et al.*, 2005) and findings showed that the origin of replication of their plasmids were 99.9% identical to that of the reference plasmids. They further revealed that the backbone modules of these plasmids were similar and shared a close affiliation. The findings of the present study thus require further investigation to elucidate the phylogeny of the IncP plasmids from the North West province.

The *ampC* sequences from plasmid isolated from both the Harts and Schoonspruit rivers (Figure 4.9) showed close association with other reference nucleotide sequences on Genbank. This is supported by bootstrap values of 100%. Both these *ampC* positive plasmids (pHR2 and pSR8) shared close homology with each other and pSR8 formed a subgroup with reference nucleotide sequences.

Phylogenetic relationship between *tetA* genes (Figure 4.10) carried by parental plasmids and reference genes grouped together and with a broad-host range vector. This is substantiated by high bootstrap values of 99% and 100%, respectively. In a similar study a multi-resistance plasmid pB10 were compared to a degradative plasmid pJP4 based on their genomic composition (Trefault *et al.*, 2005). Findings revealed that these two plasmids shared a close homology with each other on nucleotide sequence level (99.9-100%). Furthermore, phylogenetic analyses have shown that both degradative and antibiotic resistance IncP plasmids share a common ancestor (Dröge *et al.*, 2000; Schlüter *et al.*, 2003; Heuer *et al.*, 2004).

5.8 Concluding remarks

Plasmids were present in multiple antibiotic resistant bacteria isolated from water sources in the North West province. Not all of them were able to be transformed into the *E. coli* host

strain. Differences in the susceptibility profiles of the parental plasmids and transformants were recorded. All the plasmids were subjected to PCR and were classified into the IncP group. Furthermore, *ampC* gene that encode resistance to β -lactams were detected in both parental plasmids and transformants. The *tetA* gene encoding for efflux pumps were also present in parental strains.

Most of the tetracycline resistance genes were prevalent in both Barbers Pan (before WWTP at Lichtenburg) and the Schoonspruit river downstream from a wastewater treatment plant. It has previously been suggested that bacteria isolated from the effluent of WWTP's, carry plasmids that belong to the IncP group commonly show resistance to broad spectrum antibiotics (Szczepanowski *et al.*, 2009). Supplementary to sequencing data, phylogenetic analyses illustrated a positive association between plasmid bound genes and the presence of antibiotic resistance phenotype in bacterial strains.

Chapter 6

Conclusions and Recommendations

6.1 Conclusions

The present study was aimed to characterize plasmids associated with antibiotic resistant bacteria previously isolated from surface water sources in the North West province, South Africa. In achieving the aim, the following objectives were completed:

6.1.2 MAR-bacteria in surface water systems

Multiple antibiotic resistant bacterial strains were present in the surface water sources in the North West province, South Africa. High incidences of resistance to broad spectrum antibiotics were evident. Most of the bacteria showed resistance to two or more antibiotics from different classes. Common resistance patterns were against ampicillin and tetracycline. These antibiotics are generally used for the prevention of bacterial infections in human health and in agriculture. The prevalence of these antibiotic resistant microbes in water environments may have originated from effluent of agriculture settings or/and from WWTPs (NWDAC-SoER, 2008).

6.1.3 Plasmids DNA isolated from antibiotic resistant bacteria

Plasmids were present in the antibiotic resistant bacteria isolated from the three surface water systems in the North West province, South Africa. Plasmids ranging from 1,500 to 13,995 bp were detected. Their occurrence was associated with the antibiotic resistance phenotype revealed by the multi resistance profiles shown by the parental bacterial strains. Plasmids have been reported to harbour genes that encode resistance to most classes of antibiotics such as β -lactams, tetracyclines, aminoglycosides, macrolides and fluoroquinolones (Bennett, 2008; Martínez, 2009). In this study, most of the latter resistance phenotypes were observed in the parental strain potentially associated with the plasmid.

6.1.4 Bacterial transformation and susceptibility profiles

Three plasmids were successfully transformed into 10- β *E. coli* through electroporation and were associated with ampicillin resistance. In addition, plasmids that were possibly responsible for tetracycline and kanamycin resistance were not transformed. Plasmid DNA of successful ampicillin resistant transformants were re-extracted and were subjected to PCR for the detection of the same phenotype of resistance and incompatibility group marker genes. Findings presented for the susceptibility profiles of the transformants revealed that these profiles differed from their parental strains. High incidences of resistance of up to five different antibiotics were shown in the parental strains. However, after transformation it was reported otherwise, showing resistance to one or two antibiotics only. This implies that the plasmids

may not be carrying all the genes that cause the resistance phenotype. However, all three transformants harboured the *ampC* genes. Two of these transformants carried resistance markers for streptomycin but not kanamycin (*nptII*). Since the *ampC* was detected among all the parental plasmids as well as the re-isolated plasmids from the transformants it may suggest that this specific *ampC* gene that encode resistance to β -lactams may be ubiquitous in aquatic environment of the North West province.

6.1.5 Identification of Incompatibility group and resistance genes

Using a PCR based approach plasmids were categorized into the IncP groups. Variations in amplicon sizes for the IncP amplicons were illustrated through agarose gel electrophoresis. This may be due to different evolutionary history of these group of plasmids in aquatic environments of the North West province. These IncP group plasmids were associated with *ampC* and *tetA* antibiotic resistance genes.

In addition, further identifications illustrated that nucleotide sequences of the amplified IncP plasmids and associated resistance genes share similarities with those present on the NCBI database. In comparing plasmid sequences with reference strains provide more knowledge on evolutionary history of plasmids, accessory modules and antibiotic resistance genes they may contain. Moreover, the identified plasmids and related antibiotic resistance genes all occurred in Gram negative, (*E. coli*) strains. Several of these genes (*tetA*) code for efflux pumps and β -lactamases (*ampC*) rendering antibiotics ineffective against bacterial infections. This is cause for concern, as plasmids may be responsible for the quick dispersal of these virulence genes in the aquatic environment.

Finally, it can be concluded that the overall aim and the specific objectives of this study were successfully achieved.

6.2 Recommendations

In this study a few discrepancies were experienced that gave rise to the following recommendations:

- It is important to refer back to the parental plasmids to determine which genes were responsible for the resistance phenotypes. Whole genome sequencing and bioinformatics analysis can be employed to fully sequence and analyse these genetic elements. This approach may aid in shedding more light on the origin of these degradative elements and its association with the antibiotic resistance phenomenon.

- Screen parental plasmids for other genes; *aphA*, *aadA1*, *aadA2*, *aadA4*, *aacA4*, *strAB* that encode for aminoglycoside resistance, since incidences of antibiotic resistance plasmid have been reported (Schlüter *et al.*, 2007a).

It is evident from findings in this study that further optimization of the electroporation protocol needs to be done. In transforming plasmids, maybe include other *E. coli* host strains. Several authors have proposed ways of transforming a wide range of bacteria on selected media containing appropriate antibiotics (Aune and Aachmann, 2010; Janjua *et al.*, 2014; Yildirim *et al.*, 2016).

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Appendix A

Sampling Date	Water Source	Sampling site	Sample name	GPS-Coordinates. S	GPS-Coordinates. E
2014	Harts river	Before WWTP at Lichtenburg	HR1-R8	S26°12'54.7"	E26°12'30.3"
2014	Barbers Pan	North of Goosepan	Br1-Br3	S26°56'76.9"	E25°59'49.9"
2016	Schoons pruit river	Downstream from WWTP	SR1-R9	S26°53'53.5"	E26°38'30.4"