Characterisation of antibiotic resistance gene clusters and their mobility within a collection of multi-drug resistant Salmonella spp

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Characterisation of antibiotic resistance gene clusters and their mobility within a collection of multi-drug resistant *Salmonella* spp.

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Declaration

I, Xiulan Liu, declare that the thesis submitted to the University of Wollongong in fulfillment of the degree of Doctor of Philosophy does not include any work published previously by another person unless appropriate reference is stated in the text. This material has not been submitted for qualifications at any other academic institution.

Signed: Xiulan Liu

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Ab = absorbance
Ap = ampicillin
APH = phosphotransferase
ARP = antibiotic resistance profile
attI = the gene cassette integration site
be = base element
bp = base pair
CFU = colony forming units
CIaa = chloroform/isoamylalcohol
Cm = chloramphenicol
CR = common region
CS = conserved segment
Cp = ciprofloxacin
Cp' = intermediate resistance to ciprofloxacin
CSPD = disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro) tricycle [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate
dfr = dihydrofolate reductase
DHPS = dihydropteroate synthase
dH_2O = distilled water
DIG = digoxigenin
DNA = deoxyribonucleic acid
dNTPs = deoxynucleotide triphosphates
DT104 = definitive type 104
ºC = degrees celsius
E. coli = *Escherichia coli*
EDTA = ethylenediaminetetraacetic acid
EMAI = Elizabeth Macarthur Agricultural Institute
ESBLs = extended spectrum beta lactamases
EU = European Union
NRA = National Registration Authority
Fl = florfenicol
FDA = the United States Food and Drug Administration
g = gram
Gm = gentamicin
GCK = gene construction kit
h = hour
HCl = hydrochloric acid
H_2O = water
Hg = mercury
HgS = mercury sulphide

In = integron
Int = integrase
Inc = incompatibility
IR = inverted repeat
IS = insertion sequence
kbp= kilo base pairs
kg = kilogram
Km = kanamycin
KOH = potassium hydroxide
kV = kilo volt
L = litre
LB = Luria Bertani
M = molar
mA = milliamp
$MgCl_2$ = magnesium chloride
MDR = multidrug resistance
MDU = Microbiological Diagnostic Unit (Melbourne)
MIC = minimum inhibitory concentration
min = minute
ml = millilitre
mm = millimetre
MQ water = Milli-Q water
µ = micro
n = nano
Na = nalidixic acid
NaCl = sodium chloride
NaOH = sodium hydroxide
NCBI = National Centre for Biotechnology Information
NEPSS = National Enteric Pathogens Surveillance System
NNDSS = National Notifiable Diseases Surveillance System
OD = optical density
ORF = open reading frame
% = percentage
PBS = phosphate buffered saline
PCR = polymerase chain reaction
pH = potential of Hydrogen
PCIaa = phenol/chloroform/isoamylalcohol
PT = phage type
PFGE =pulsed-field gel electrophoresis
QAC = quaternary ammonium compound
QLD = Queensland
R plasmid = resistance plasmid
r-det = resistance determinant
RDNC = results do not conform

RNA = ribonucleic acid
rpm = revolutions per minute
RT = room temperature
RTF = resistance transfer factor
sec = second
SDS = sodium dodecyl sulfate
SGI1 = Salmonella Genomic Island 1
Sm = streptomycin
Sp = spectinomycin
spp. = species
SRC = *Salmonella* reference collection
SSC = sodium citrate
sul = sulfonamide
TBE = tris-borate-EDTA
Tc = tetracycline
TE = tris-EDTA
Tn = transposon
Tp = trimethoprim
tra = transposition
tRNA = transfer RNA
U = units
UV = ultraviolet
USA = United States of America
UK = United Kingdom of Great Britain and Northern Ireland
V = volt
v/v = volume/volume
w/v = weight/volume
WHO = World Health Organisation

Abstract

One hundred and thirty-six *Salmonella enterica* strains, isolated from humans, animals, environmental and plant sources in Australia from 23 serovars, were examined for the streptomycin resistance gene *strA* and *strB*, the sulfonamide resistance gene *sul2*, and the tetracycline resistance gene *tetA*(A) and *tetA*(B). Thirteen strains were identified as containing the *strA-strB* genes located on the transposon Tn*5393*. *S. enterica* serovar Hadar accounted for 11 of these strains, 6 of which were isolated from humans and 5 were from ducks. This investigation is therefore the first report of the Tn*5393* transposon being detected in bacterial strains from a human source in Australia.

RSF1010 plasmids were identified and extracted from 4 *S. enterica* strains, and were further confirmed by restriction enzyme profiling using *Pst*I, *Ssp*I and *Eco*RV. Small non-conjugative plasmid p9123 was extracted and characterised from 3 of the *S. enterica* strains and also confirmed by restriction enzyme digestion. An RSF1010-like plasmid was also identified in 3 of the strains. This plasmid was found to be approximately 2.6 kb larger than RSF1010, and possibly derived from the RSF1010 plasmid via insertion of the tetracycline resistance gene *tetA*(A) between *strB* and *mobC* genes.

An IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* antibiotic resistance region was identified in 33 *S. enterica* strains, among these were 23 serovar Typhimurium isolates, 8 serovar

Bovismorbificans, 1 serovar Senftenberg and 1 isolate where the serovar could not be conclusively identified. The 23 Typhimurium strains were further characterised by PCR and Southern hybridisation analysis using a *bla*$_{TEM}$ gene probe. The analysis identified two classes of antibiotic resistance gene clusters. Eleven *S. enterica* serovar Typhimurium strains harboured an IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* antibiotic resistance gene cluster and another 10 *S. enterica* serovar Typhimurium strains contained an IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$ gene cluster, without the IS*26* element downstream of the *bla*$_{TEM-1}$ gene. Two strains contain elements of these gene clusters but further investigation is needed to fully identify these.

Further linkage PCR amplifications revealed that the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* antibiotic resistance gene cluster was possibly inserted into the 3′-CS of a class 1 integron (In4 type) and truncated the 3′-CS region. Three derivatives were identified, of which the *dfrA5*-*intI1* type was most commonly found downstream of the *bla*$_{TEM-1}$-IS*26* region. Southern hybridisation analysis using an IS*200* gene probe revealed that strains which contain different antibiotic resistance gene clusters also display different but related IS*200* profiles.

The antibiotic resistance gene clusters of 19 *S. enterica* serovar Typhimurium strains were transferred to an *E. coli* 294 Rif^{r} recipient either by direct mating or triparental mating methods. These experiments confirmed that the antibiotic resistance gene clusters were located on conjugative or mobilisable plasmids. The antibiotic resistance

gene clusters of 4 *S. enterica* serovar Typhimurium strains could not be transferred to the *E. coli* 294 Rif^r recipient. These experimental results suggest that the antibiotic resistance gene cluster of IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-bla_{TEM-1}-IS*26* might move as one genetic element between distinct plasmid backbones.

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Publications Arising from this Thesis

Paper 1

Characterisation of resistance genes in multiply antibiotic resistant Salmonella enterica serovar Typhimurium from human and bovine sources

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Paper 2

Persistence of RSF1010-like plasmids and origin of their sul2-strA-strB antibiotic resistance gene cluster

Sheree Yau, Xiulan Liu, Steven P. Djordjevic and Ruth M. Hall

Manuscript 1

Evolution of the Tn1696 transposon family

Amy K. Cain, Xiulan Liu, Steven P. Djordjevic and Ruth M. Hall

Chapter 1: Introduction

1.1 Use of antibiotics

With the discovery and development of antibiotics from the 1930's-1970's, the ability to treat infections advanced greatly. Penicillin, streptomycin, tetracycline and many other antibiotics were discovered and several synthetic classes of antibiotics were created during that time (Baker, 2006). Many antibiotics have a broad bacteriostatic or bactericidal spectrum and can affect both Gram-positive and Gram-negative bacteria. Antibiotics have been widely used in humans, animals and also plants for different purposes. In humans, antibiotics are widely used in clinical situations for the treatment of different infections, including those of the central nervous system, respiratory tract, gastrointestinal tract and urinary tract (Huovinen et al., 1995; Bryan et al., 2004). Since the early 1950's, antibiotics have been given to animals for therapeutic and non-therapeutic purposes (Pasquali et al., 2004; Kim et al., 2004). For therapeutic purposes, curative doses of antibiotics are used in clinically ill animals for a relatively short period of time (Shea et al., 2004). For example, in Australia sulfonamides including sulphadimidine, sulphadiazine, sulphatroxazole and sulphaquinoxaline are approved to use as mass medicants for the control of diseases in food producing species (NRA, 2000). For non-therapeutic purposes, antibiotics can be used as a prophylactic by adding to animal feed. Prophylactic antibiotics are used to prevent infectious disease that has not been clinically diagnosed and to control the dissemination of infectious diseases within a group of animals. Prophylactic antibiotics may be used at either

therapeutic doses or low doses (Shea et al., 2004). Some antibiotics are also used to promote animal growth by enhancing feed efficiency (Baker, 2006). Chlortetracycline was the first antibiotic introduced as a feed additive in 1950 (Gustafson, 1991). In Europe, the antibiotics used for animal growth promotion were approximately 15% of total antibiotic use in 1997 (Schwarz and Chaslus-Dancla, 2001). Antibiotics have also been used to control certain bacterial diseases of high-value fruits, vegetables and ornamental plants since the 1950's. The antibiotics most commonly used on plants are oxytetracycline and streptomycin (McManus and Stockwell, 2001). Both of these can be used to control the populations of phytopathogenic bacteria in apples, pears and other rosaceous plants (Palmer et al., 1997; Schnabel and Jones, 1999). In the USA, antibiotics applied to plants account for less than 0.1% of the total antibiotics produced annually (McManus and Stockwell, 2001).

According to their principal mechanisms of action, most antibiotics used for the treatment of bacterial infections can be categorised into 5 major modes: (1) interference with cell wall synthesis including β-lactams and glycopeptides; (2) inhibition of protein synthesis (macrolides, chloramphenicol, aminoglycosides and tetracyclines belong to this mode); (3) interference with nucleic acid synthesis (examples include fluoroquinolones and rifampin); (4) inhibition of a metabolic pathway (sulfonamides and folic acid analogues); and (5) disruption of bacterial membrane structure (polymyxins and daptomycin) (Table 1.1) (Tenover, 2006).

Table 1.1 Mechanisms of action of different antibiotics (adapted from Tenover, 2006).

(1) Interference with cell wall synthesis
β-Lactams: penicillins, cephalosporins, carbapenems, monobactams
Glycopeptides: vancomycin, teicoplanin
(2) Protein synthesis inhibition
Bind to 50S ribosomal subunit: macrolides, chloramphenicol, clindamycin, quinupristin-dalfopristin, linezolid
Bind to 30S ribosomal subunit: aminoglycosides, tetracyclines
Bind to bacterial isoleucyl-tRNA synthetase: mupirocin
(3) Interference with nucleic acid synthesis
Inhibit DNA synthesis: fluoroquinolones
Inhibit RNA synthesis: rifampin
(4) Inhibition of metabolic pathway: sulfonamides, folic acid analogues
(5) Disruption of bacterial membrane structure: polymyxins, daptomycin

1.2 Emergence of antibiotic resistance

In reality, the victory afforded by antibiotics over bacterial infectious diseases was short lived. It has been found that the use of antibiotics has been accompanied by the occurrence of bacteria, which are resistant to these particular substances (Schwarz and Chaslus-Dancla, 2001). Resistance to single antibiotics became prominent in organisms that encountered the first commercially produced antibiotics (Alekshun and Levy, 2007). Moreover, many bacterial isolates have been found resistant to daptomycin, quinupristin-dalfopristin, telithromycin and even purely synthetic agents such as ciprofloxacin. These antibiotics have been approved by the United States Food and Drug Administration (FDA) only within the last decade (D'Costa et al., 2006).

Bacteria resistant to multiple classes of antibiotics are designated multidrug resistant (MDR) strains traditionally. In a recently examined collection of soil-dwelling *Streptomyces* (the producers of many clinical therapeutic agents), every organism was

identified as multidrug resistant. Most were resistant to at least 7 different antibiotics, and the phenotype of some included resistance to 15-21 different drugs (D'Costa et al., 2006). Antibiotic resistance is now a global problem. Dispersion of successful clones of MDR bacteria is common, often via the global mobility of populations and food products (Hawkey and Jones, 2009). There are five models including the Natal Model, the Fisher Model, the Molarity Model, the Coregulation Model and the Selfish Operon Model that are offered to explain the formation of MDR strains. The Selfish Operon Model proposes that multiple antibiotic resistance genes clustering together enhance the transfer of these genes to other bacteria by mobile genetic elements (Lawrence, 1999).

Bacteria may manifest resistance to antibiotics through a variety of mechanisms including: (1) changes in bacterial cell wall permeability that restrict antibiotics access to target sites; (2) energy-dependent removal of antibiotics via membrane-bound efflux pumps; (3) inactivation or modification of the site of drug action; (4) introduction of a new drug resistant target; (5) hydrolysis of the antibiotic; (6) modification of the antibiotics; and (7) other 'by-pass' mechanisms (Davies, 1997; Barbosa and Levy, 2000). Some resistance mechanisms are distributed among a wide variety of bacteria, while others appear to be specific for certain bacterial species and genera. Up to six different mechanisms have been found to confer resistance to the same antibiotic (Schwarz and Chaslus-Dancla, 2001). For example, resistance to β-lactam antibiotics can be mediated by the first six of the above described mechanisms (Harbottle et al., 2006). This review will focus only on antibiotic resistance genes and the mercury resistance mechanisms

which are directly relevant to the experimental work conducted in this thesis.

1.2.1 Streptomycin resistance

Streptomycin, produced by *Streptomyces griseus*, was one of the first antibiotics to be used for disease control in humans, such as for the chemotherapy of tuberculosis (Schatz et al., 2005). The bactericidal mechanisms of streptomycin include misreading during protein translation, increased membrane permeability and irreversible uptake of the antibiotic (Davis, 1987). Streptomycin is also among the most commonly used antibiotic for food animal production worldwide (Pezzella et al., 2004). Since the late 1950's, streptomycin has been used commercially to control certain plant bacterial diseases in the United States, Canada and New Zealand (Chiou and Jones, 1993). Though currently streptomycin only has limited usage in human medicine, it is still important for animal infection therapy, growth promotion and plant bacterial disease control (Sundin, 2002).

Resistance to streptomycin was first identified and characterised in 1945 (Waksman et al., 1945). Resistance can be conferred via chromosomal mutations which alter the ribosomal binding site of streptomycin (Springer et al., 2001). Resistance can also be obtained by the action of enzymes, which can inactivate the antibiotic via either an adenylylation or phosphorylation process (Shaw et al., 1993). A large number of genes can confer streptomycin resistance. Among these, the phosphotransferase *aph(3")-Ib* gene (also named *strA*) and the *aph(6)-Id* gene (also named *strB*) appear to be widely

distributed in *Salmonella* and other Gram-negative bacteria (Pezzella et al., 2004). High-level streptomycin resistance in bacteria containing *strA* and *strB* is due to the combined action of these two enzymes. Apart from streptomycin resistance, genes *strA* and *strB* do not confer resistance to other related aminoglycosides such as spectinomycin, kanamycin and gentamicin (Shaw et al., 1993). A growing group of gene cassettes, designated *aadA* with an Arabic numeral to distinguish distinct genes, such as *aadA1* and *aadA2*, confer resistance to both streptomycin and spectinomycin (Gestal et al., 2005).

1.2.2 Sulfonamide resistance

Sulfonamides have been widely used to treat bacterial and protozoal infections since their clinical introduction in 1935. The combination of trimethoprim and sulfamethoxazole, for instance, is still commonly used in human medicine for the treatment of urinary tract infections (Perreten and Boerlin, 2003). Sulfonamide is among the most commonly used antibiotic for food animal production worldwide (Pezzella et al., 2004). In veterinary medicine, sulfonamides alone or in combination with other antibiotics are widely used to prevent and treat diarrhea and other infectious diseases in intensive animal husbandry (Perreten and Boerlin, 2003). Sulfonamides compete with the structural analog *p*-aminobenzoic acid for binding to the dihydropteroate synthase (DHPS), a catalytic enzyme in the folic acid biosynthesis pathway, thus inhibiting the formation of dihydrofolic acid (Skold, 2000).

Resistance to sulfonamides in *Escherichia coli* can result from mutations in the chromosomal DHPS gene (*folP*) (Swedberg et al., 1993; Vedantam et al., 1998). More frequently, sulfonamide resistance derives from the acquisition of an alternative DHPS gene (*sul*), the product of which has a lower affinity for sulfonamides (Swedberg and Skold, 1980; Radstrom and Swedberg 1988; Sundstrom et al., 1988). Sulfonamide resistance in Gram-negative bacilli generally arises from the acquisition of one of three genes, *sul1*, *sul2*, and *sul3*, encoding forms of dihydropteroate synthase that are not inhibited by the drug (Perreten and Boerlin, 2003; Skold, 2000). The *sul1* and *sul2* genes from *E. coli* share 57% DNA identity, while *sul3* (accession no. AJ459418) has 40.6% amino acid identity to *sul2* from *E. coli* plasmid RSF1010 (accession no. M28829), and 40.9% amino acid identity to *sul1* from *E. coli* plasmid R388 (accession no. X12869) (Radstrom and Swedberg, 1988; Guerra et al., 2004). The *sul1* gene is normally found linked to other resistance genes in class 1 integrons, while *sul2* is usually located on small non-conjugative plasmids or large transmissible multiresistance plasmids (Skold, 2000; Enne et al., 2001). The *sul3* gene was identified in a Swiss *E. coli* strain flanked by transposable elements (*sul3* domain) inserted into a conjugative plasmid (Perreten and Boerlin, 2003). Other studies have reported that *sul3* can be found on various large plasmids (Grape et al., 2003; Guerra et al., 2004). The dissemination and genetic background of the *sul3* gene has not been fully characterised (Antunes et al., 2007). The *sul1* and *sul2* genes are present in many different species and previously have been shown to be distributed equally among resistant isolates (Radstrom et al., 1991). Recently, the spread of *sul2* may have increased, as the gene

was reported to be more widespread among clinical isolates of *E. coli* than *sul1* in Denmark and the UK (Enne et al., 2001; Kerrn et al., 2002). The *sul3* gene is the most recently described gene, but has been increasingly identified in human isolates as well as principally among food animal isolates in the *Enterobacteriaceae* family (Perreten and Boerlin, 2003; Bischoff et al., 2005; Grape et al., 2003; Guerra et al., 2003; Hammerum et al., 2006). During the 1990's, the UK experienced a 97% reduction in clinical sulfonamide use. Nonetheless, co-selection of multidrug resistance genes and other factors will result in the maintenance of sulfonamide resistance in bacteria for the foreseeable future (Enne et al., 2004). It is therefore not surprising that increasing resistance to sulfonamide has been observed in *Salmonella* of animal origin worldwide (Lee et al., 1993; Frech and Schwarz, 2000).

1.2.3 Tetracycline resistance

Tetracyclines are inexpensive antibiotics that have been extensively used to treat a wide variety of diseases in humans, animals and plants since the late 1940's (Chopra and Roberts, 2001). Tetracyclines can inhibit bacterial protein synthesis through preventing aminoacyl-tRNA from binding to the bacterial ribosome (Roberts, 1996). A survey in the EU member states and Switzerland showed that tetracyclines were the most used antibiotics in veterinary medicine, accounting for almost two thirds of all antibiotics used (Frech and Schwarz, 2000). This widespread use of tetracyclines constitutes a high selective pressure and may have contributed to the successful spread of tetracycline resistance genes in zoonotic pathogens (Pezzella et al., 2004).

The first reported tetracycline resistance pathogen identified was *Shigella dysenteriae*, which was discovered in 1953 (Roberts, 1996). Since then, resistance to tetracycline has increased dramatically. Most tetracycline resistance determinants contain both structural and regulatory genes (Levy et al., 1989). Thirty-six classes of tetracycline resistance genes have been identified based on DNA-DNA hybridisation and DNA sequencing. Resistance to tetracycline is conferred by one or more of the 36 currently described *tet* genes plus the tetracycline regulatory gene *tetR* (Roberts, 2003). These *tet* genes encode one of three mechanisms of resistance: (1) an efflux pump; (2) a method of ribosomal protection; or (3) direct enzymatic inactivation of the drug (Chopra and Roberts, 2001). The efflux mechanism is more common among Gram-negative microorganisms and the tetracycline resistance genes *tetA*(A), (B), (C), (D), (E), (G), (I), (J) and (K) are all associated with this resistance mechanism. The ribosomal protection mechanism is more common among Gram-positive organisms and the tetracycline resistance genes *tetA*(M), (O), (S), (Q), (T) and (W) all belong to this group. The *tetA*(X) is the only tetracycline resistance gene causing the enzymatic alteration of tetracycline (Roberts, 1996; Levy et al., 1999; Chopra and Roberts, 2001). Tetracycline resistance genes have been found on resistance plasmids, transposons and integrons, which have lead to the rapid spread of tetracycline resistance among bacteria (Chopra and Roberts, 2001; Roberts, 2003).

1.2.4 β-lactam resistance

β-lactams consist of four major groups including penicillin, cephalosporins,

monobactams and carbapenems. These four major groups all have a β-lactam ring, which can be hydrolysed by the β-lactamases enzyme resulting in inactivation of the antibiotic (Gupta, 2007). The first β-lactamase was identified in *E. coli* prior to the release of penicillin for use in medical practice (Abraham and Chain, 1940). In Gram-negative pathogens, β-lactamase production remains the most important contributing factor to β-lactam resistance (Medeiros, 1997). So far, hundreds of β-lactamases have been discovered and characterised (Jacoby and Munoz-Price, 2005). According to the classification scheme of Ambler, the β-lactamases may be divided into four groups: class A, B, C, and D. Classes A, C and D enzymes act on many penicillins, cephalosporins and monobactams. Class B proteins act on penicillins, cephalosporins and carbapenems but not on monobactams (Gupta, 2007). AmpC, an inducible and usually chromosomally encoded enzyme found in many species of the *Enterobacteriaceae* and *P. aeruginosa*, is the prototype class C enzyme (Jacoby and Munoz-Price, 2005). The persistent exposure of bacterial strains to a multitude of β-lactams has led to overproduction and mutation of β-lactamases. Some β-lactamases are now capable of hydrolysing penicillins, broad-spectrum cephalosporins and monobactams and are called extended spectrum beta lactamases (ESBLs) (Bush, 2001). Although ESBLs have been reported most frequently in *E. coli* and *Klebsiella* species, they have been found in other bacterial species as well, including *Salmonella enterica*, *Citrobacter freundii* and *Serratia marcescens* (Paterson and Bonomo, 2005; Morosini et al., 1995; Palucha et al., 1999). Most ESBLs can be divided into three groups: TEM, SHV and CTX-M types (Paterson and Bonomo, 2005).

The TEM-1 enzyme was originally discovered in *E. coli* isolated from a patient named Temoniera, hence the designation as TEM (Gupta, 2007). A few years after it was first isolated, TEM-1, the first plasmid mediated β-lactamase found in Gram-negative organisms, spread worldwide and across many species (Pfaller and Segreti, 2006). TEM-3 (cefotaxime-hydrolysing enzyme type 1), was the first definitively characterised ESBL and was discovered in *Klebsiella pneumoniae* isolates in France (Sirot et al., 1987). Since the discovery of TEM-3, over 100 additional TEMs have now been isolated (Bradford, 2001).

The progenitor of the SHV class of enzymes, SHV-1, is universally found in *K. pneumoniae*. SHV-1 confers resistance to broad spectrum penicillins such as ampicillin, ticarcillin and piperacillin but not to cephalosporins (Livermore, 1995). In 1983, plasmid-borne SHV-2, derived from a mutation in SHV-1, was isolated from three isolates of *K. pneumoniae* and demonstrated resistance to cefotaxime as well as to other newer cephalosporins (Knothe et al., 1983). To date, over 50 SHV type β-lactamases have been described (Bradford, 2001).

CTX-M type enzymes, a new family of ESBLs, are rapidly spreading among *Enterobacteriaceae* worldwide. CTX-M-type ESBLs exhibit powerful activity against cefotaxime and ceftriaxone but generally not against ceftazidime (Rossolini et al., 2008). Over 50 different types of CTX-M β-lactamases have been found, which can be classified into six groups based on their amino acid identities (Bonnet, 2004; Rossolini

et al., 2008). Organisms that produce CTX-M enzymes have become the most prevalent type of ESBL-containing bacteria described during the past 5 years, in particular from European and South American countries (Canton and Coque, 2006).

The OXA type oxacillin hydrolysing enzymes are another family of β-lactamases and have been found predominantly in *P. aeruginosa* (Naas and Nordmann, 1999). OXA β-lactamases belong to Ambler class D48. These enzymes confer resistance to ampicillin and cephalothin (Bush et al., 1995). Many of the newer members of the OXA β-lactamase family have been found in bacterial isolates originating in Turkey and in France. Other unusual ESBL enzymes having also been described (*e.g.*, BES-1, CME-1, VE-B-1, PER, SFO-1 and GES-1), however these novel enzymes are only found infrequently (Bradford, 2001).

Since 1989, ESBL genes have been detected in non-typhoidal *Salmonella* isolated from humans in a number of hospitals in South America, North Africa and Eastern Europe (Bonnet, 2004). Broad-spectrum SHV-1, TEM-1 and OXA type β-lactamases have been frequently described in *E. coli* and *Salmonella* spp. from animals and food of animal origin in Spain, Germany, the USA and the UK. TEM-1 was the most common variant detected among these isolates (Carattoli, 2008).

1.2.5 Mercury resistance

Mercury is present in the environment as a result of natural processes and from

anthropogenic sources (Nascimento and Chartone-Souza, 2003). Mercury is present in the Earth's crust at concentrations ranging from 21 (lower crust) to 57 (upper crust) ppb in the elemental form and as a variety of HgS binary minerals (Barkay et al., 2003). Anthropogenic sources of Hg include burning fossil fuels and incineration or other disposal of products such as fluorescent light fixtures, batteries, dental restorations and electrodes used in the chlor-alkali process. Worldwide anthropogenic input is substantial, resulting in 75% of the global input of Hg into the environment (Barkay et al., 2003).

As a response to toxic mercury compounds globally distributed by both geological and anthropogenic processes, microorganisms have developed a surprising array of resistance systems to overcome the poisonous effect of this compound. Mercury resistance genes encoded by *mer* operons have been found in a wide range of Gram-positive and Gram-negative bacteria isolated from different environments (Barkay et al., 2003; Nascimento and Chartone-Souza, 2003). The *mer* operon allows bacteria to detoxify Hg(II) into volatile metallic mercury by enzymatic reduction (Nascimento and Chartone-Souza, 2003). *Mer* operons vary in structure and are constituted by genes encoding proteins for regulation (*merR*, *merD*), transport (*merT*, *merP*, and/or *merC*, and/or *merF*) and reduction (*merA*) (Nascimento and Chartone-Souza, 2003). Most *mer* operons contain the regulatory gene *merR*. *MerR* is a metal-responsive regulatory protein that binds the promoter-operator region, where it both positively and negatively regulates the expression of the divergently transcribed structural genes, and also negatively regulates its own expression (Caguiat et al., 1999;

Nascimento and Chartone-Souza, 2003). *MerD* is the secondary regulatory protein and also binds to the same operator-promoter region as *merR*, although very weakly (Nucifora et al., 1989; Mukhopadhyay et al., 1991). All the *mer* operons contain *merT* and *merP*, which are required for full expression of Hg(II) resistance, but loss of *merP* is less deleterious than loss of *merT*. Among *mer* operons that are encoded by transposons, Tn*21* was the first example found with *merC* gene (Nascimento and Chartone-Souza, 2003). Gene *merC* encodes inner membrane proteins which function in Hg(II) uptake, but deletion of *merC* from the Tn*21 mer* locus has no effect on Hg(II) resistance (Liebert et al., 2000). Gene *merF* was implicated in mercuric transport and found encoded by the plasmid pMER327/419 of *Pseudomonas fluorescens* between *merP* and *merA* (Wilson et al., 2000). Gene *merA* is a mercuric iron reductase that reduces reactive ionic Hg(II) to volatile, relatively inert, monoatomic Hg(0) vapor (Barkay et al., 2003).

Mer operons are often components of transposons and integrons and have been found widely in Gram-negative bacteria (Nascimento and Chartone-Souza, 2003; Barkay et al., 2003). One archetypal *mer* operon is carried by Tn*21*, which was originally on plasmid NR1 from *Shigella flexneri* identified in Japan (Nakaya et al., 1960). The *mer* operon was also found in Tn*501* from *Pseudomonas aeruginosa*, but this transposon lacks *merC* gene (Figure 1.1) (Barkay et al., 2003).

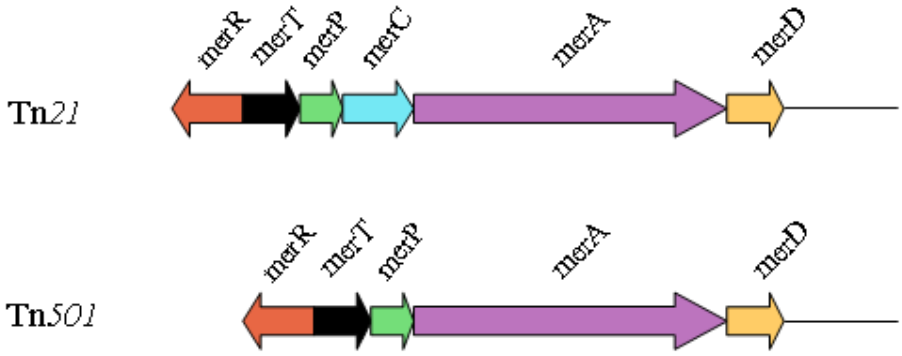


Figure 1.1 The *mer* operons from Gram-negative bacteria *Shigella flexneri* (Tn*21*) and *Pseudomonas aeruginosa* (Tn*501*). The genes and their transcription directions are indicated by arrows (adapted from Barkay et al., 2003).

1.3 Why antibiotic resistance is a concern?

There are a number of reasons why bacterial resistance should be a concern for physicians and regulatory authorities. (1) Resistant bacteria are becoming commonplace in healthcare institutions, where they often result in treatment failure and have serious consequences, especially in critically ill patients (Tenover, 2006). (2) Prolonged antibiotic therapy may lead to the development of low-level resistance that compromises therapy. This kind of low-level resistance is hard to detect by routine susceptibility testing methods used in hospital laboratories (Tenover et al., 2004). (3) Resistant bacteria may also spread and become broader infection-control problems in communities. Infected individuals including children, appear to have acquired their infections in a variety of community settings (Francis, et al., 2005; Herold et al., 1998). (4) Antibiotic resistance places an added burden on healthcare costs, although its full economic impact remains to be calculated (McGowan, 2001). The National Institute of Allergy and Infectious Disease of the USA estimates that the annual cost of treating antibiotic-resistant infections in the USA alone might be as high as $30 billion (NIAID,

1999). In Australia, the annual cost is estimated to be over one billion Australian dollars (DSRD, 2008).

1.4 How bacteria acquire antibiotic resistance?

Bacteria may be intrinsically resistant to ≥1 class of antibiotics, or may acquire resistance by *de novo* mutation or via the acquisition of resistance genes from other organisms (Tenover, 2006). Usually the original source of antibiotic resistance genes is the antibiotic producers, which usually harbour these resistance genes in chromosomal DNA and use them as a self-defense mechanism (Schwarz and Chaslus-Dancla, 2001). Another source of antibiotic resistance genes might come from mutations. In rare cases, a single mutation may be sufficient to confer high-level, clinically significant resistance on an organism, for example the high-level rifampin resistance in *Staphylococcus aureus* (Tenover, 2006). Through antibiotic selection, these resistance strains can survive and grow. Acquired resistance that develops due to chromosomal mutation and selection is termed vertical transfer. Bacteria also develop resistance through the acquisition of new genetic material from other resistant organisms and this is termed horizontal transfer. Horizontal transfer may occur between strains of the same species or between different bacterial species or genera (Tenover, 2006). It is now clearly established that horizontal transfer is the more common solution adopted by bacteria to escape antibiotic destruction, rather than mutation in a resident gene (Rowe-Magnus and Mazel, 1999).

Mechanisms of genetic exchange include transformation, transduction and conjugation. Transformation is the process whereby bacteria acquire and incorporate resistance genes from the environment. These DNA segments are released by other bacteria after cell lysis. During transduction, resistance genes are transferred between bacteria via bacterial viruses. This is now thought to be a relatively rare event. During conjugation, a Gram-negative bacterium transfers plasmid-containing resistance genes to an adjacent bacterium (Tenover, 2006). Figure 1.2 shows the three kinds of mechanisms. Through genetic exchange, many bacteria have become resistant to multiple classes of antibiotics (defined as resistance to ≥3 antibacterial drug classes) (Tenover, 2006).

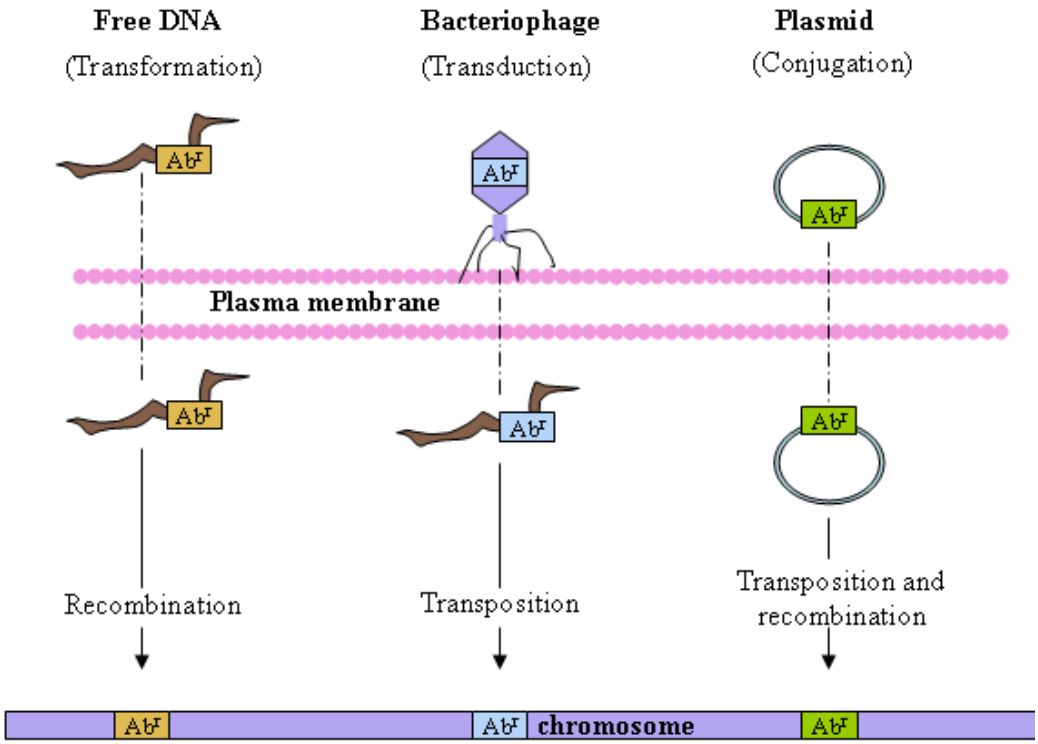


Figure 1.2 Acquisition of antibiotic resistance determinants by transformation, transduction and conjugation methods (adapted from Alekshun and Levy, 2007).

1.5 Mobile genetic elements

Mobile genetic elements including insertion sequences (IS), integrons, transposons, plasmids and phages are the tools used for genetic exchange. Access to antibiotic resistance genes coupled with the carriage of such genes on mobile genetic elements has facilitated their rapid dissemination into a wide variety of organisms (Sundin and Bender, 1996). Those mobile genetic elements which directly relate to the research conducted in this thesis will be the focus of the next section of this review.

1.5.1 IS elements

IS elements are a heterogeneous class of mobile elements and are common in prokaryotic genomes (Miriagou et al., 2006). The structure of an IS element contains two inverted repeats and a transposase gene. The two inverted repeats exert effects when the IS element inserts into a chromosome by RecA-dependent homologous recombination. Transposition is catalysed by an enzyme, which is encoded by the transposase gene (Figure 1.3) (Russell, 1998; Mollet et al., 1983). IS elements can mediate the transfer of resistance genes by forming composite transposons, where two flanking IS elements co-operate to mobilise the intervening DNA. IS elements are capable of promoting various types of genome rearrangements including deletions, inversions and replicon fusions. These attributes can lead to the assembly of gene clusters with specialised functions such as multiple antibiotic activities, virulence, symbiotic functions or new catabolic pathways (Mahillon et al., 1999). IS elements are also an integral part of many naturally occurring bacterial plasmids and can transfer

antibiotic resistance genes via plasmids (Chen et al., 2007).

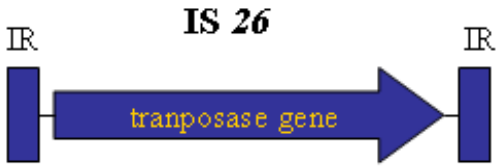


Figure 1.3 The structure of IS*26* element, which includes two inverted repeats and a transposase gene (adapted from Mollet et al., 1983).

1.5.2 Integrons

Integrons were defined by Hall and Collis as elements that recognise and capture mobile gene cassettes (Hall and Collis, 1995). Integrons have been reported in many Gram-negative and few Gram-positive bacteria (Martinez-Freijo et al., 1998). The essential components of an integron include the integrase gene (*intI1*), the gene cassette integration site (*attI*) and the promoter, which drives the expression of the integrated genes (Carattoli, 2001). Integron integrases can recombine different circularized resistance gene cassettes at the receptor *attI* site to permit expression of the encoded cassette proteins. Resistance cassettes normally contain a single gene associated with a specific recombination sequence, the 59 base element (59-be), which vary from 57 bp to 141 bp in length (Rowe-Magnus and Mazel, 1999). Integrons can be divided into two major groups: the resistance integrons and the superintegrons.

Resistance integrons carry mostly gene cassettes that encode resistance against antibiotics and disinfectants. Resistance integrons can be located either on the chromosome or on plasmids. The three classes of resistance integrons are designated class 1, class 2 and class 3 integrons (Hall and Collis, 1995; Carattoli, 2001). The class

1 integron, which was the first discovered integron, is now known to be important in the dissemination of antibiotic resistance genes in both Gram-positive and Gram-negative bacteria (Fluit and Schmitz, 2004). Class 1 integrons are typically found on plasmids or transposons that have been found in environments ranging from hospitals to poultry litter (Nandi et al., 2004; Giuliani et al., 2005). Class 1 integrons have a common structure including two conserved segments, the 5′-conserved segment (5′-CS) and 3′-conserved segment (3′-CS) (Figure 1.4). The 5′-CS contains a site-specific recombination system, which includes the *intI1* gene, the *attI1* site and promoters, and is capable of integrating and expressing gene cassettes. The gene cassettes or open reading frames containing a particular recombination site are incorporated at the *attI1* site (Carattoli, 2001). The majority of the cassettes identified encode antibiotic resistance genes (Radstrom et al., 1991). The 3′-CS codes for the *qacE△1* gene, which confers resistance to ethidium bromide and quaternary ammonium compounds, the *sul1* gene, which confers resistance to sulfonamide, and orf5, which encodes a protein of unknown function (Carattoli, 2001).

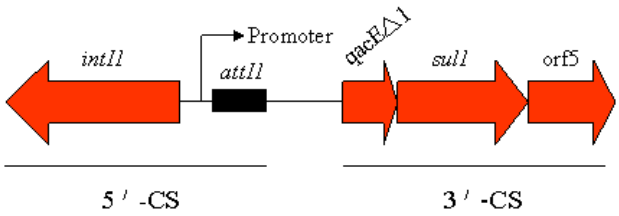


Figure 1.4 Schematic representation of a class 1 integron. Genes and the open reading frame of the 5′-CS and 3′-CS are indicated by arrows. *attI1*, recombination site (adapted from Harbottle et al., 2006).

Class 1 integrons are commonly associated with various transposons such as Tn*21*, Tn*1696* and Tn*1412* (Partridge et al., 2001). Tn*21*-derivative transposons carrying class

1 integrons have been described on IncF, IncFII, IncP-1 alpha, IncHI1 and IncHI2 plasmids (Patridge and Hall, 2004; Novais et al., 2006; Holt et al., 2007; Chen et al., 2007). Through incorporated into transposons and plasmids, integrons participate in the capture of resistance genes and dissemination of these genes among bacteria (Chen et al., 2004).

Class 2 integrons are embedded in the Tn*7* family of transposons and consist of an integrase gene frequently followed by *aadA1*, *dfrA1* and *sat* gene cassettes (Radstrom et al., 1994; Gonzalez et al., 1998). Class 3 integrons have been described in some bacteria isolated from Japan such as *Pseudomonas aeruginosa* and *Serratia* spp. and the class 3 integron structure is comparable to that of class 2 integrons (Arakawa et al., 1995; Senda et al., 1996).

Large chromosomally-located integrons, which contain cassettes with a variety of functions, belong to the superintegrons group. The integron originally designated as class 4, is now named the *Vibrio cholerae* superintegron. Superintegrons have been described for many bacterial species (Fluit and Schmitz, 2004; Mazel, 2006). Until now, no evidence has been obtained to show that superintegrons are exchanged between different strains or bacterial species (Fluit and Schmitz, 2004).

1.5.3 Transposons

Transposons are transposable genetic elements. Like the IS elements, transposons also

contain genes for the insertion of the DNA segment into the chromosome and for the mobilisation of the element to other locations in the chromosome. Unlike the IS elements, transposons also contain genes of identifiable function such as antibiotic resistance genes (Russell, 1998). For example, Tn*3* contains 38 bp inverted repeat (IR) sequences at each end of the transposon and three known genes designated *tnpA*, *tnpR* and bla_{TEM}. The bla_{TEM} gene encodes β-lactamase and confers resistance to ampicillin. The other two genes are associated with transposition events: *tnpA* encodes the transposase, an enzyme that catalyses insertion of the transposon into new sites, whilst *tnpR* encodes the resolvase, which is involved in the particular recombinational event associated with transposition (Figure 1.5) (Russell, 1998; Partridge and Hall, 2005).

Figure 1.5 The structure of Tn*3* transposon, which includes two 38 bp inverted repeats, transposase gene *tnpA*, resolvase gene *tnpR* and β-lactamase gene bla_{TEM}. IR, inverted repeat (adapted from Partridge and Hall, 2005).

Two types of transposons, conjugative transposons and transposons harbouring integrons, have greatly contributed to the dissemination of antibiotic resistance genes in bacteria. Conjugative transposons have been found in both Gram-positive and Gram-negative species (Pembroke et al., 2002). Antibiotic resistance genes (including those encoded by integrons) captured by conjugative transposons can move from replicon to replicon and disseminate between different organisms, and even different genera, via the conjugation process (Rowe-Magnus and Mazel, 1999; Whittle et al.,

2002).

Transposons harbouring integrons are mainly observed in *Enterobacteriaceae* and *Pseudomonads* (Rowe-Magnus and Mazel, 1999). Tn*21* is the first recognised and most widely distributed transposon (Liebert et al., 1999). Many transposons encoding multiple antibiotic resistances in the *Enterobacteriaceae* belong to the Tn*21* subgroup of the Tn*3* family (Grinsted et al., 1990; Liebert et al., 1999). Transposon Tn*21* has three parts: a mercury resistance operon, an In2 integron and a transposition system (Figure 1.6). The *mer* operon encodes enzymes that convert toxic mercuric ions into less toxic metallic mercury (Misra, 1992). Tn*21* and its derivatives are major agents in the dissemination of mercury resistance in Gram-negative bacteria conferring resistance to both organic and inorganic mercuric compounds (Liebert et al., 1999). Tn*21* also carries a class 1 integron In2, which contains a conserved 5′-CS fragment and 3′-CS fragment. The gene cassette inserted is *aadA1*, which is one of the most prevalent resistance genes in the world, and confers resistance to streptomycin and spectinomycin. At the same time, Tn*21*-like transposons may confer resistance to various antibiotics through other resistance gene cassettes incorporated within the integron (Carattoli, 2001). In2 also harbours two IS elements IS*1326* and IS*1353*, where IS*1353* is inserted into IS*1326*. Upstream of the IS*1326* and IS*1353* locus, there is a deleted *tni* gene region, which consists only of *tniA* and part of *tniB* gene (Grinsted et al., 1990; Liebert et al., 1999). The function of this *tni* gene region is to transpose the In2 class 1 integron. In2 is flanked by two 25 base pair inverted repeats (IR) that suggest the potential for In2

mobility. However, no evidence of self-transposability of the In2 integron has been demonstrated (Carattoli, 2001). The Tn*21* transposition system includes the transposase gene *tnpA*, the resolvase *tnpR*, the putative transposition regulator *tnpM*, the resolution site *res* and inverted repeats (IR).

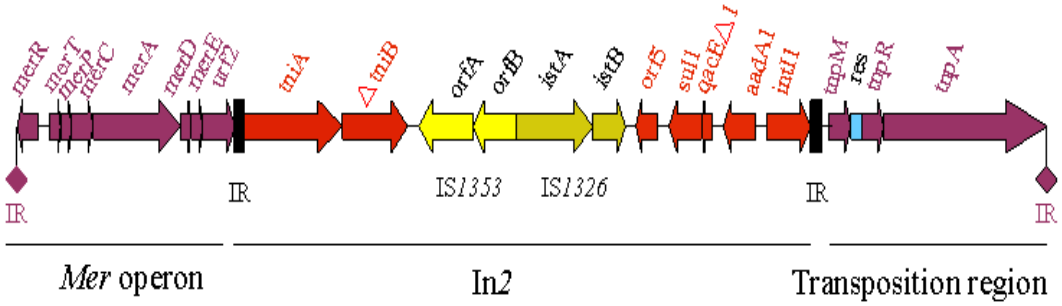


Figure 1.6 The structure of Tn*21* transposon includes a mercury resistance operon, a class 1 integron In2 and a transposition region. The black bars indicate the inverted repeats of the class 1 integron In2 (adapted from Carattoli, 2001).

The evolution of transposon Tn*21* is suggested to have occurred in three steps. The first step resulted in the formation of In2. Brown et al. (1996) have proposed that In2 evolved from a progenitor similar to Tn*402*. This step included the creation of the 3′-CS through the insertion of *sul1* and other gene cassettes. The insertion of IS*1326* then caused the partial deletion of the adjacent 3′-CS and *tni* module (Brown et al., 1996). The second step in this progress resulted in the insertion of In2 into the hypothetical ancestor of Tn*21* (‘Tn*21*△’). Finally, the third step resulted in the insertion of IS*1353* into the Tn*21* transposon (Figure 1.7) (Liebert et al., 1999).

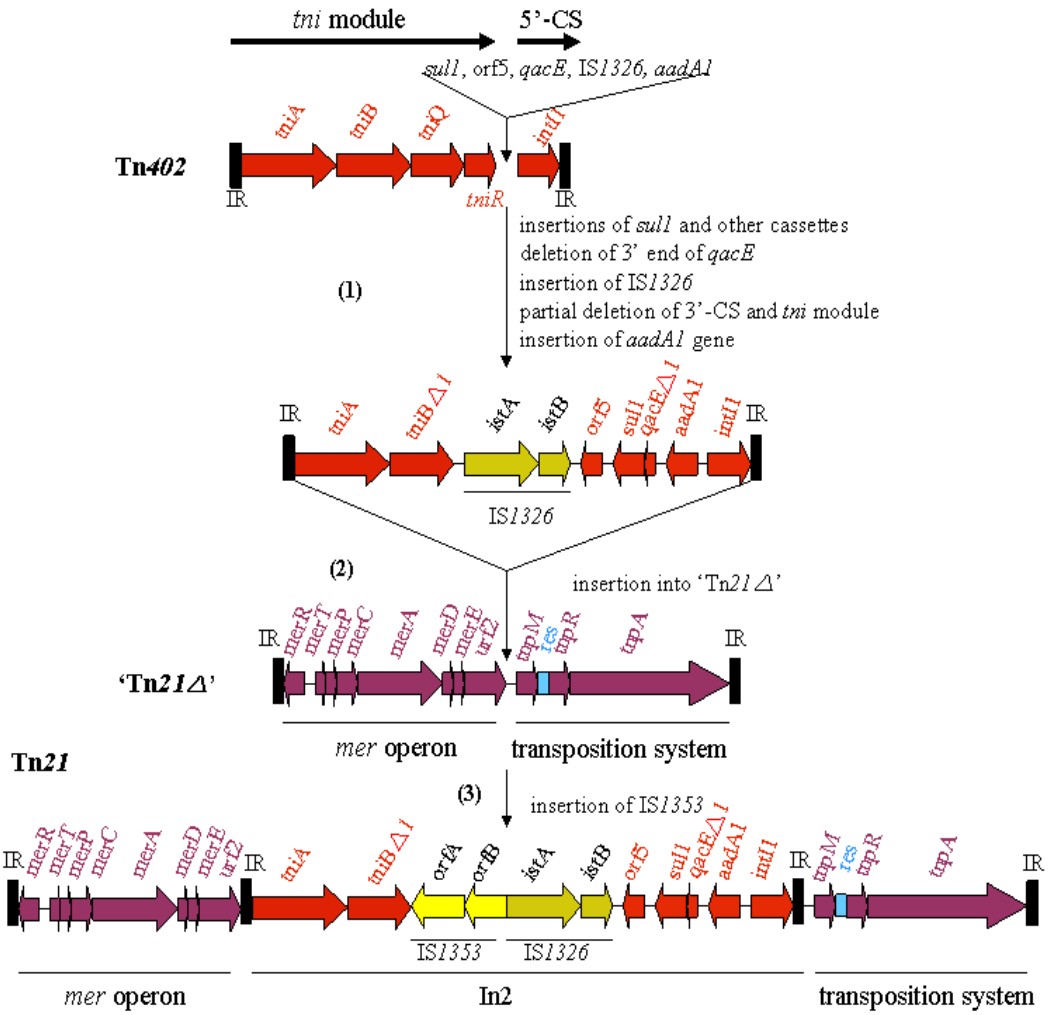


Figure 1.7 The hypothetical process for the formation of Tn*21*. Step (1) indicates the formation of In2 from a progenitor similar to Tn*402* after several genetic events including insertion of *sul1* and other gene cassettes, deletion of the 3′ end of *qacE*, insertion of IS*1326*, partial deletion of the 3′-CS and *tni* module, and insertion of the *aadA1* gene cassette. Step (2) shows the insertion of In2 into the hypothetical ancestor of Tn*21* transposon. Step (3) shows the insertion of IS*1353* into In2 to form the Tn*21* transposon. All the genes and their transcriptional directions are indicated by arrows. Black vertical bars indicate inverted repeats (modified from Liebert et al., 1999).

The Tn*21* transposon family is classified on the basis of phylogenetic analysis of *tnp* genes, *res* sites and IR ends. However, the Tn*21* family varies considerably in the numbers and types of additional genes carried (Partridge and Hall, 2004; Szczepanowski et al., 2005). The Tn*21*-derivative transposon harboured on plasmid

pRMH760 carries a Tn*1* ampicillin resistance transposon, a Tn*4352B* kanamycin/neomycin resistance transposon, two gene cassettes *dfrA10* and *aadB*, extra *sul1* and *qacE*△*1* genes, but has lost the IS*1353* element (Figure 1.8) (Partridge and Hall, 2004). The Tn*21* derivative on plasmid R100 lost the IS*1353* element and a *yahA* gene seems to have been inserted instead. Also, the open reading frame orf5 has been replaced by the *ybbA* gene (Figure 1.8) (Szczepanowski et al., 2005). For the Tn*21* derivative on Tn*SF1*, two transposons Tn*1* and Tn*4352B* are inserted, while the IS*1353* and IS*1326* elements are missing. In addition, a macrolide-resistance operon *mph*(A)-*mrx*-*mphR*(A) is inserted upstream of the 3′-CS segment of the class 1 integron (Figure 1.8) (Chen and Chen, 2005).

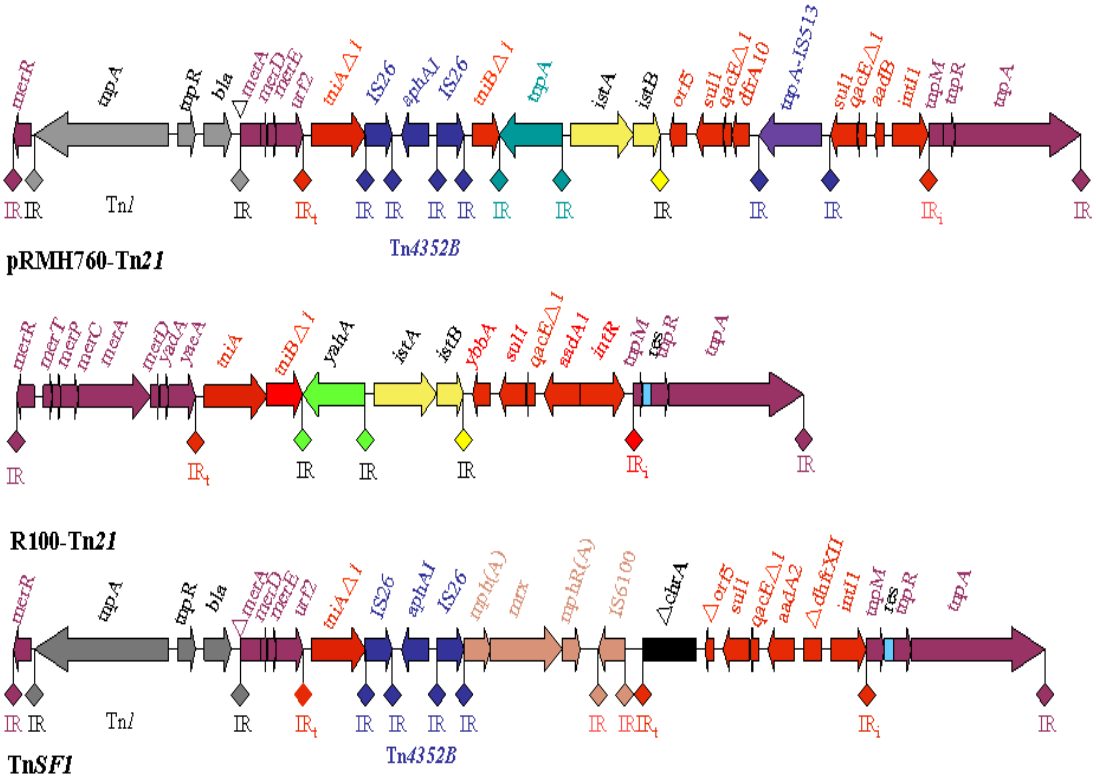


Figure 1.8 The members of Tn*21*-like transposon family found on plasmid pRMH760, R100 and Tn*SF1* (adapted from Szczepanowski et al., 2005).

1.5.4 Plasmids

Plasmids are extra-chromosomal segments of DNA that replicate independently of the chromosome and can be exchanged among various bacteria (Frost et al., 2005; Harbottle et al., 2006). Plasmids contain genes that are essential for the initiation and control of their own replication. Some plasmids also contain genes that ensure stable inheritance, such as equipartitioning during cell division or conjugal transfer (Carattoli, 2003). Plasmids are usually classified by incompatibility (Inc) groups, defined as the inability of two plasmids to be propagated stably in the same cell. Incompatibility is a manifestation of relatedness, sharing common replication controls or equipartitioning elements (Couturier et al., 1988). Four major incompatibility groups have been defined on the basis of genetic relatedness and pilus structure: the IncF group, including IncF, IncS, IncC, IncD and IncJ; the IncP group, including IncP, IncU, IncM and IncW; the Ti plasmid group, including IncX, IncH, IncN and IncT; and the IncI group, including IncI, IncB and IncK (Waters, 1999). Plasmids are not essential to bacterial survival, but typically carry genes that impart some selective advantage to the host bacterium, such as virulence determinants, adherence and antibiotic resistance genes (Harbottle et al., 2006). Levin (1995) made two predictions regarding the evolution of multi-resistance plasmids. For two incompatible plasmids simultaneously under selection pressure, new plasmids will arise by transposition. For compatible resistance plasmids, new multi-resistance plasmids will arise through co-integration (Sherley et al., 2004).

Plasmids that carry resistance genes are called R plasmids or R factors. The R plasmid

NR1 (R100) was originally isolated from *Shigella flexneri* in Japan in the later 1950's (Nakaya et al., 1960). There were two components in this plasmid, identified as a resistance determinant (R-det) and a resistance transfer factor (RTF). R-det contains a composite Tn*9*-like transposon carrying the *catA1* gene and transposon Tn*21*. RTF carries the genes for self-transmission (*tra* gene) and autonomous replication (*rep* gene). Transposon Tn*10* is also located in the RTF region, which confers antibiotic resistance to tetracycline (Figure 1.9) (Liebert et al., 1999). Since the discovery of the first R plasmid in the 1950's, antibiotic resistance plasmids have been increasingly associated with both Gram-positive and Gram-negative bacterial pathogens and commensal organisms (Watanabe and Fukasawa, 1961; Harbottle et al., 2006). Plasmid-encoded antibiotic resistance encompasses most classes of antibiotics currently used in clinical practice (Bennett, 2008). It is common for a single plasmid to simultaneously mediate resistance to multiple antibiotics and be shared among different bacterial genera (Sherley et al., 2004). Many resistance plasmids are conjugative because they encode genes which promote cell-to-cell DNA transfer, particularly their own transfer (Bennett, 2008). Conjugative plasmids in Gram-positive bacteria tend to be smaller than those in Gram-negative bacteria, reflecting a somewhat different mechanism of cell-to-cell coupling, which requires less genetic information (Bennett, 2008).

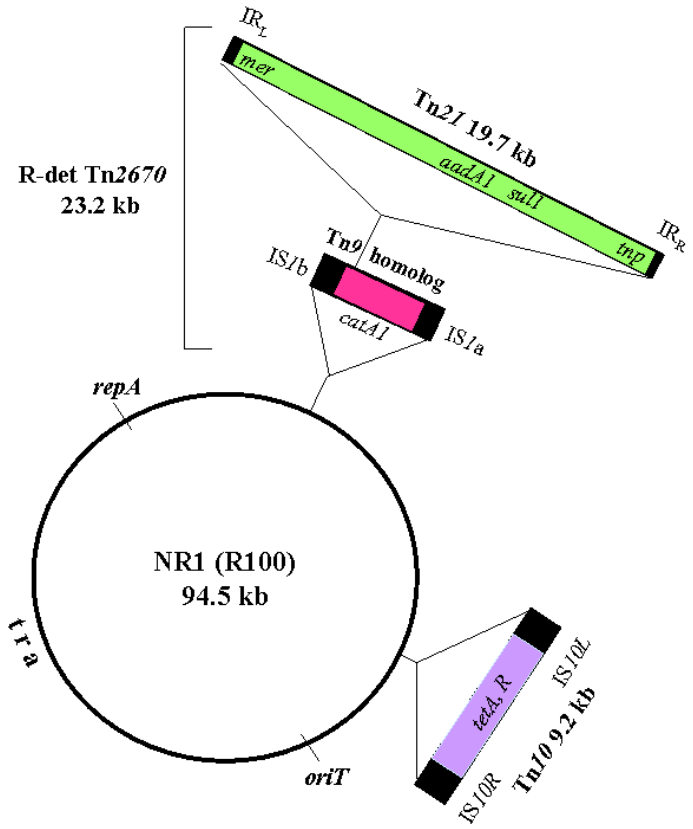


Figure 1.9 The structure of R plasmid NR1 (adapted from Liebert et al., 1999).

1.6 *Salmonella*

The bacterial isolates under investigation in this thesis uniformly belong to the Genus *Salmonella*. Therefore, this bacterial Genus will be briefly reviewed.

1.6.1 What is *Salmonella*?

Salmonella is a Genus of zoonotic, Gram-negative bacteria, which belong to the rod-shaped *Enterobacteriaceae* family. *Salmonella* infection can cause salmonellosis. The most common form of salmonellosis is a self-limited, uncomplicated gastroenteritis. The symptoms include diarrhea, abdominal cramps and fever. *Salmonella* infection can also cause enteric fevers such as typhoid fever. In some patients, *Salmonella* infection may spread from the intestines to the blood stream, then to other body sites and cause

death unless the person is treated properly with antibiotics.

Salmonella infections in humans often result from two causes. Handling or consuming contaminated food products, particularly foods of animal origin such as poultry, beef, pork, eggs, milk, seafood and fresh produce may result in infection. Alternatively, infections may be acquired by direct and indirect contact with farm animals, reptiles, chicks and pets. This is because infected animals usually shed *Salmonella* organisms in their faeces (Miko et al., 2005). Humans can become infected when they place contaminated food, hands or other objects in their mouths (Todd et al., 2008). Pathogenic salmonellae are ingested in food and survive passage through the gastric acid barrier. These then invade the mucosa of the small and large intestine and also produce toxins. Invasion of epithelial cells stimulates the release of proinflammatory cytokines, which induce an inflammatory reaction. The acute inflammatory response causes diarrhea and may lead to ulceration and destruction of the mucosa. The bacteria can also disseminate from the intestines to cause systemic disease (Zhao et al., 2003).

Salmonella are currently classified into two species: *S. bongori* and *S. enterica. S. enterica* encompasses six different subspecies I, II, IIIa, IIIb, IV and VII. *S. enterica* subspecies I is responsible for 99% of all known salmonellosis in warm-blooded animals. All other subspecies and *S. bongori* are mostly restricted to cold-blooded hosts such as reptiles and snakes (Porwollik and McClelland, 2003). *S. bongori* or *S. enterica* subspecies II to VII are able to infect humans, colonise the intestine and cause disease, but such cases are rare and are usually the result of contact with reptiles (Kingsley et al.,

2000). Over 2,500 serovars of *S. enterica* have been identified by the agglutination method. These serovars have very different host ranges including broad-host ranges and host-specialist ranges. For example, *S. enterica* serovar Typhimurium is a broad-host range serovar, which can cause disease in humans, cattle, pigs, horses, poultry, rodents and sheep. In comparison, *S. enterica* serovar Typhi is a host-specialist serovar, which causes disease only in humans and higher primates (Porwollik and McClelland, 2003). Additionally, the virulence and epidemiology of these serovars may vary. For instance, most serovars cause only gastroenteritis, while specific serovar such as *S. enterica* serovar Typhi, Paratyphi A and C, and Sendai can cause enteric fever. A few nontyphoid serovars such as *S. enterica* serovar Choleraesuis and Dublin are more likely to cause bacteremia than diarrhea (Fierer and Guiney, 2001).

Among the many serovars, *S. enterica* serovars Typhimurium and Enteritidis are the most common causes of human salmonellosis (Herikstad et al., 2002). In the United States, *S. enterica* serovar Typhimurium is one of the most prevalent serovars and nearly 10% of *Salmonella* infections among humans are caused by it (Glynn et al., 2004). In Germany, *S. enterica* serovar Typhimurium and Typhimurium var. Copenhagen caused approximately 50% of the annually reported outbreaks of bovine salmonellosis between 1995 and 2002 and therefore represent the most important serovars (Methner, 2005). *S. enterica* serovar Typhimurium also has a growing importance in other regions such as Southeast Asia, Africa and the Western Pacific (Herikstad et al., 2002). The numbers of infections caused by *S. enterica* serovar Enteritidis increased since the 1970's in the

United States and further investigations showed that eggs were the major vehicle for the infections in humans. Though the incidence of infections caused by *S. enterica* serovar Enteritidis decreased greatly after 1996 in humans, many cases and outbreaks continue to occur (Braden, 2006). *S. enterica* serovar Newport has emerged as the third most common *Salmonella* serovar causing human salmonellosis in the United States (Zhao et al., 2003). *S. enterica* serovar Agona was first identified in Ghana (Guinee et al., 1961). Since then, this serovar has been reported in many countries worldwide in both humans and animals (Clark et al., 1973). In Brazil, *S. enterica* serovar Agona was the fourth most common *Salmonella* serovar isolated from non-human sources and one of the top 10 serovars associated with human disease (Tavechio et al., 2002). *S. enterica* serovar Dublin isolates mainly cause severe infections in cattle (Akiba et al., 2007). Although *S. enterica* serovar Dublin is considered to be a cattle-adapted pathogen, it can cause severe systemic infections in humans (Fang and Fierer, 1991). *S. enterica* serovar Hadar is normally isolated from poultry (Limawongpranee et al., 1999), while *S. enterica* serovar Derby is associated with pigs (Davies et al., 1998; Letellier et al., 1999). In Australia, the top ten serovars isolated from humans and some animals in 2003 are given in Table 1.2. *S. enterica* serovar Typhimurium caused 70% of reported salmonellosis (NEPSS, 2003).

Table 1.2 The top 10 *Salmonella* serovars isolated from humans and some animals including cattle, sheep, pigs and poultry in 2003 (NEPSS, 2003).

Serovars	Numbers of isolates
S. enterica serovar Typhimurium	3113
S. enterica serovar Vichow	380
S. enterica serovar Bovismorbificans	215
S. enterica serovar Dublin	183
S. enterica serovar Muenchen	182
S. enterica serovar Anatum	158
S. enterica serovar Zanzibar	73
S. enterica serovar Give	64
S. enterica serovar Bredeney	30
S. enterica serovar Senftenberg z27 phase	26

1.6.2 Antibiotic resistance in *Salmonella* and its cost

During the early 1970's, plasmids of the IncFI group, encoding multiple antibiotic resistances, were frequently reported in *Salmonella* isolated in Europe, the Middle East and North Africa (Anderson et al., 1977). Resistance to nalidixic acid and ciprofloxacin also was found. Full ciprofloxacin resistance has been described in faecal isolates of *Salmonella* serovar Typhimurium in China (Cui et al., 2008). In middle and low-income countries, the spread of ESBL resistance genes has also been recorded (Zaidi et al., 2007; Usha et al., 2008). Resistance to ciprofloxacin, extended spectrum cephalosporins and the older antibiotics in non-typhoid *Salmonella* will severely limit treatment options and cause treatment failures (Collard et al., 2007). Of particular concern was the isolation of ceftriaxone- and ciprofloxacin-resistant *Salmonella*, because these two agents are very important in treating *Salmonella* infections in children and adults respectively (Casin et al., 2003; Dunne et al., 2000; White et al., 2001). A case of treatment failure due to ceftriaxone resistant *S. enterica* serovar Anatum has been reported in Taiwan (Su et al.,

2003). As assessed in the last few years, *Salmonella* shows increasing antibiotic resistance rates in isolates obtained from both food animals and humans. *S. enterica* strains belonging to different serovars and showing multiple antibiotic resistances (to four or more antibiotics) are now widespread in both developed and developing countries (Threlfall, 2002).

Multidrug-resistant *S. enterica* serovar Typhimurium Definitive Type 104 (DT104) commonly exhibits resistance to seven antibiotics including ampicillin, florfenicol, chloramphenicol, streptomycin, spectinomycin, sulfamethoxazole and tetracycline. In DT104, resistance genes were all located within the boundaries of a large class 1 integron In104 (Levings et al., 2005). The genetic make-up of In104 comprises the *floR* and *tet* genes bracketed by two integron-derived regions called InC and InD, respectively (Levings et al., 2005). InC encodes the aminoglycoside resistance gene *aadA2* and InD encodes the β-lactamase *pse-1* gene. The *floR* gene confers resistance to florfenicol-chloramphenicol and the *tet* genes including *tetR* and *tetA*(G) confer resistance to tetracycline (Figure 1.10) (Carattoli et al., 2002; Randall et al., 2004). *S. enterica* serovar Typhimurium DT104 has increased from 0.6% prevalence in human infections in the United States in 1979 to 34% in 1996 (Dechet et al., 2006; Glynn et al., 1998). In recent years, some isolates of this phage type have also been found to be resistant to fluoroquinolones and higher generation cephalosporins (Fey et al., 2000; Winokur et al., 2000). Apart from *S. enterica* serovar Typhimurium DT104 phage type, there have been various clusters of resistance genes reported that formed “antibiotic

resistance islands" in other *Salmonella* isolates (Carattoli et al., 2002; Chen et al., 2007).

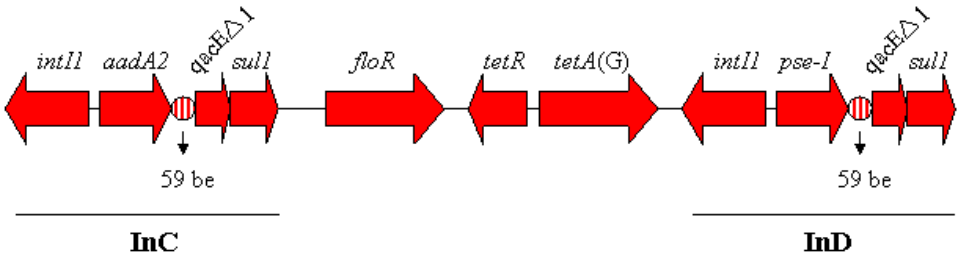


Figure 1.10 Schematic representations of genes within and between InC and InD regions in *S. enterica* serovar Typhimurium DT104. InC and InD are indicated by black lines. All the genes and their transcriptional directions are indicated by arrows. 59 be, 59 base pairs (adapted from Carattoli et al., 2002).

Food-borne diseases caused by non-typhoid *Salmonella* represent an important public health problem and an economic burden in many parts of the world (Miko et al., 2005). According to estimates by the Centers for Disease Control and Prevention, food-borne *Salmonella* infections are responsible for 1.4 million illnesses annually worldwide, which lead to 16,000 hospitalisations and 600 deaths (Zhao et al., 2003). It is estimated that the annual economic costs due to food-borne *Salmonella* infections in the United States alone may be as high as $2.3 billion (Kennedy et al., 2004).

1.7 Aim of this research

This thesis has three main aims:

1. To characterise the common antibiotic resistance genes in a *Salmonella* collection of various serovars including:

   -streptomycin resistance genes *strA* and *strB*

   -sulfonamide resistance gene *sul2*

-tetracycline resistance genes *tetA*(A) and *tetA*(B)

-ampicillin resistance gene bla_{TEM}

-class 1 integron specific integrase gene *intI1*

-mercury reductase gene *merA*

-other antibiotic resistance gene clusters

2. To explore where the antibiotic resistance gene clusters are located within *Salmonella* spp. which contain such resistance genes.

3. To determine if and how antibiotic resistance gene clusters transfer between different *Salmonella* strains.

Chapter 2: Materials and Methods

2.1 Bacterial strains

2.1.1 *Salmonella* strains

One hundred and thirty-six *Salmonella enterica* strains containing 23 serotypes were used for this project (Table 2.1). These strains were isolated in Australia between 1999 and 2001 and serotyped by the Microbiological Diagnostic Unit (MDU) (Melbourne). The standard procedure according to the Kauffman and White scheme was used to serotype these strains (Popoff et al., 2003). The antibiotic resistance profiles of these strains were determined using the method described by Bettelheim et al. (2003). Among them, 39 strains were isolated from humans including 23 local residents and 16 recently returned overseas travelers. Ninety-two isolates were from Australian animals and these isolates were cultured from bovine, equine, avian, chicken, porcine and canine sources. Three strains were isolated from environmental sources such as waste effluent, abattoir effluent and fish tank water. The last isolate was identified in imported spice. There are some data for some strains which is unknown including the source of strain SRC137, the serovar of strain SRC121, and the isolation dates of strains SRC56, SRC117 and SRC135.

The concentrations of different antibiotics used for examining resistance were ampicillin (Ap, 32 μg/ml), chloramphenicol (Cm, 10 μg/ml), gentamicin (Gm, 2.5 μg/ml), kanamycin (Km 10 μg/ml), streptomycin (Sm, 25 μg/ml), spectinomycin (Sp,

50 μg/ml), sulfathiazole (Su, 550 μg/ml), tetracycline (Tc, 20 μg/ml), or trimethoprim (Tp, 50 μg/ml) (Amavisit et al., 2001; Bettelheim et al., 2003). Resistance to nalidixic acid (Na, 50 μg/ml) and ciprofloxacin (Cp, 2 μg/ml) was also recorded for some isolates, mainly from humans. Among the 136 strains, 13 were resistant to only one of these antibiotics, 25 were resistant to two antibiotics, 98 were multiple drug resistant (MDR) strains and showed resistance to three or more of these antibiotics.

Table 2.1 The 136 *Salmonella enterica* isolates used in this study

Isolate no.	Serovar/Phage Type	Source/Country/Year	Resistance profile
SRC12	Typhimurium PT135	human, F (99)	ApSu
SRC13	Typhimurium PT135	human, F (99)	ApSmSuTcTp
SRC14	Typhimurium PT44	human, F (99)	ApSmSuTcTp
SRC15	Typhimurium PT9	human, F (99)	SmSu
SRC16	Typhimurium Ptu	human, M (99)	ApCmGmSpSuTcTp
SRC26	Typhimurium PT1var2	bovine (99)	ApKmSmSuTcTp
SRC27	Typhimurium PT135	equine (99)	ApCmGmKmSmSpSuTcTp
SRC28	Typhimurium PT135	equine (99)	ApCmGmKmSmSpSuTcTp
SRC29	Typhimurium PT141	porcine (99)	ApGmKmSmSuTcTp
SRC30	Typhimurium PT141 v4	porcine (99)	ApGmKmSmSuTcTp
SRC31	Typhimurium PT44	bovine (99)	ApSmSuTcTp
SRC32	Typhimurium PT68	bovine (99)	ApKmSmSuTcTp
SRC33	Typhimurium PT9	bovine (99)	SmSu
SRC56	Typhimurium 104L	human, M Timor (ND)	ApCmGmKmSmSpSuTcTp
SRC113	Typhimurium PT12	bovine (00)	Tc
SRC114	Typhimurium PT135	avian (00)	Sm
SRC115	Typhimurium PT135	chicken (00)	TcTp
SRC116	Typhimurium PT135	bovine (00)	ApSmSuTcTp
SRC117	Typhimurium PT141	porcine (ND)	ApGmKmSmSuTp
SRC118	Typhimurium PT141	porcine (00)	ApGmKmSmSuTcTp
SRC119	Typhimurium PT208 v1	porcine (00)	ApCmGmKmSmSpSuTc
SRC120	Typhimurium PT29	porcine (00)	ApKmSmSuTp
SRC122	Typhimurium PT29	porcine (00)	ApKmSmSuTp
SRC123	Typhimurium PT29	porcine (00)	ApKmSmSuTcTp
SRC124	Typhimurium PT44	bovine (00)	ApSmSuTcTp
SRC125	Typhimurium PT44	bovine (00)	KmSuTcTp
SRC126	Typhimurium PT44	bovine (00)	ApSmSuTp

Isolate no.	Serovar/Phage Type	Source/Country/Year	Resistance profile
SRC127	Typhimurium PT44	bovine (00)	KmSuTcTp
SRC128	Typhimurium PT44	bovine (00)	ApSmSuTcTp
SRC129	Typhimurium PT44	bovine (00)	ApKmSmSuTcTp
SRC130	Typhimurium PT64	bovine (00)	SmSu
SRC131	Typhimurium PT9	bovine (00)	ApSmSuTp
SRC132	Typhimurium PT9	bovine (00)	ApSmSu
SRC133	Typhimurium PT9	bovine (00)	ApKmSmSu
SRC134	Typhimurium PT99	pheasant (00)	Tc
SRC135	Typhimurium RDNC	porcine (ND)	ApKmSmSuTcTp
SRC136	Typhimurium RDNC	porcine (00)	ApKmSmSuTp
SRC137	Typhimurium	ND/ND	Tc
SRC17	Bovismorbificans	bovine (99)	ApCmSmSuTcTp
SRC36	Bovismorbificans PT13	human, F (01)	ApKmSmSuTcTp
SRC59	Bovismorbificans PT13	bovine (01)	ApKmSmSuTcTp
SRC60	Bovismorbificans PT13	bovine (01)	ApCmKmSmSuTcTp
SRC61	Bovismorbificans PT11	bovine (01)	ApKmSmSuTcTp
SRC62	Bovismorbificans PT32	bovine (01)	ApCmKmSmSuTcTp
SRC79	Bovismorbificans PT14	bovine (00)	ApCmKmSmSuTcTp
SRC80	Bovismorbificans PT24	bovine (00)	ApCmKmSmSuTcTp
SRC81	Bovismorbificans 24	abattoir effluent (00)	ApGmTc
SRC82	Bovismorbificans 32	bovine (00)	ApSmSuTp
SRC83	Bovismorbificans u	chicken (00)	CmSmSpSuTc
SRC24	Sofia	chicken (99)	SmSpSuTp
SRC25	Sofia	chicken (99)	ApTc
SRC52	Sofia	human,M (01)	Tp
SRC53	Sofia	human, F (01)	Km
SRC75	Sofia	chicken (01)	Tp
SRC76	Sofia	chicken (01)	SuTp
SRC77	Sofia	chicken (01)	SuTcTp
SRC105	Sofia	canine (00)	Tc
SRC106	Sofia	chicken (00)	ApSmSuTc
SRC107	Sofia	chicken (00)	SmSu
SRC108	Sofia	chicken (00)	SuTcTp
SRC109	Sofia	chicken (00)	SmSuTcTp
SRC110	Sofia	chicken (00)	ApSmSuTc
SRC111	Sofia	chicken (00)	SuTcTp
SRC07	Hadar	human, M Indo (99)	ApSmTc
SRC08	Hadar PT10	human, M o/s (99)	ApTc (NaCp')
SRC09	Hadar PT2	human, F (99)	SmTc
SRC39	Hadar PT10	human, M Indo (01)	SmTc (NaCp')
SRC40	Hadar PT11	human, F Bali (01)	Tc (NaCp')
SRC41	Hadar PT11	human, M (01)	CmKmSmSpSuTc (NaCp')
SRC42	Hadar PT14	human, F Bali (01)	Tc (Na)

Isolate no.	Serovar/Phage Type	Source/Country/Year	Resistance profile
SRC43	Hadar PT2	human, F (01)	SmTc (Na)
SRC44	Hadar PT22	human, F (01)	SmTc (Na)
SRC64	Hadar PT11	duck (01)	SmTc
SRC65	Hadar PT14	duck (01)	SmTc
SRC66	Hadar PT2	duck (01)	SmTc
SRC67	Hadar PT22	duck (01)	SmTc
SRC68	Hadar PT33	duck (01)	SmTc
SRC20	Infantis	chicken (99)	SmSpSuTc
SRC46	Infantis	human, F (01)	SmSpSuTp
SRC47	Infantis	human, M (01)	ApTcTp
SRC70	Infantis	feline (01)	SmSpSuTc
SRC71	Infantis	chicken (01)	SmSpSuTc
SRC72	Infantis	chicken (01)	ApSmSpSuTc
SRC92	Infantis	canine (00)	SmSpTcTp
SRC93	Infantis	chicken (00)	ApCmSpSuTc
SRC94	Infantis	chicken (00)	CmSmSpSuTc
SRC95	Infantis	chicken (00)	SmSpSuTc
SRC96	Infantis	chicken (00)	SmSuTc
SRC10	Kiambu	human, M (99)	ApCmSmSpSuTcTp
SRC11	Kiambu	human, M (99)	ApCmSmSpSuTcTp (NaCp')
SRC21	Kiambu	chicken (99)	SuTcTp
SRC97	Kiambu	chicken (00)	SuTp
SRC98	Kiambu	chicken (00)	SuTcTp
SRC99	Kiambu	chicken (00)	ApCmSmSpSuTcTp
SRC100	Kiambu	chicken (00)	SmSuTcTp
SRC34	Agona	human, F Africa (01)	CmSmSpSuTcTp
SRC35	Agona	human, M Malay (01)	SmSuTc (Na)
SRC57	Agona	porcine (01)	ApGmTc
SRC58	Agona	porcine (01)	Tc
SRC04	Derby	human, M Malay (99)	CmGmSmSpSuTcTp
SRC18	Derby	porcine (99)	ApTc
SRC37	Derby	human, F (01)	SuTc
SRC63	Derby	porcine (01)	SmTc
SRC85	Derby	porcine (00)	SmTc
SRC86	Derby	porcine (00)	ApCmTc
SRC87	Derby	porcine (00)	SmTcTp
SRC88	Derby	porcine (00)	ApGmTc
SRC45	Havana	human, M (01)	SmSpSuTc
SRC69	Havana	porcine (01)	ApGmKmSmSpTc
SRC90	Havana	porcine (00)	ApCmSuTcTp
SRC91	Havana	porcine (00)	GmSmSpSuTc
SRC23	Senftenberg	porcine (99)	ApCmSmSpSuTcTp
SRC102	Senftenberg	porcine (00)	ApGmKmSmSpSuTcTp

Isolate no.	Serovar/Phage Type	Source/Country/Year	Resistance profile
SRC103	Senftenberg	porcine (00)	ApGmKmSmSpSuTc
SRC104	Senftenberg	meat, bone meal (00)	Sm
SRC49	Paratyphi B dT^+	human, F (01)	ApCmSmSpSuTc
SRC50	Paratyphi B dT^+	human, M (01)	ApCmSmSpSuTc
SRC101	Paratyphi B dT^+	fish tank water (00)	ApCmSmSpSuTc
SRC54	Stanley	human, M Thai (01)	CmGmKmSmSpSuTc (Cp')
SRC55	Stanley	human, F Thai (01)	SmSuTc
SRC112	Stanley	porcine (00)	ApGmSuTcTp
SRC02	Blockley	human, F Bali (99)	KmSmTc (NaCp')
SRC03	Blockley	human, M Thai (99)	CmKmSmSpSuTcTp (NaCp')
SRC22	Ohio	porcine (99)	CmGmKmSmSpSuTc
SRC74	Ohio	porcine (01)	ApCmGmKmSmSpSuTc
SRC01	Singapore	human, F (01)	CmSmSpSuTcTp (Na)
SRC51	Singapore	human, M (01)	CmSmSpSuTcTp (NaCp')
SRC05	Cerro	human, F Thai (99)	ApCmSuTcTp (NaCp')
SRC06	Enteritidis PT1	human, F HK (99)	SmSuTc (NaCp')
SRC19	Emek	waste effluent (99)	CmSuTcTp (NaCp')
SRC38	Dusseldorf	human, M Malay (01)	ApCmSuTcTp (Na)
SRC48	Montevideo	human, M (01)	GmKmSmSpTc (NaCp')
SRC73	Kentucky	imported spice, (01)	ApGmSmSpSuTc (Na)
SRC78	Anatum	porcine (00)	Tc
SRC84	Bredeney	porcine (00)	SuTcTp
SRC121	ND	porcine (00)	ApGmKmSmSuTp

ND- no isolation time was available for SRC56, SRC117 and SRC135. The isolation source and time was not available for SRC137. The serovar and phage type of SRC121 were not available either. Indo, Indonesia.

Abbreviations: Ap, ampicillin resistance; Cm, chloramphenicol resistance; Cp', intermediate resistance to ciprofloxacin; Gm, gentamicin resistance; Km, kanamycin resistance; Na, nalidixic acid resistance; Sm, streptomycin resistance; Sp, spectinomycin resistance; Su, sulphathiozole resistance; Tc, tetracycline resistance; Tp, trimethoprim resistance.

2.1.2 *E. coli* strains

The *E. coli* DH5α strain was grown on LB plates or LB broth and made competent for electroporation. The *E. coli* 294 strain was grown on LB plates with 100 μg/ml rifampicin to select for a rifampicin resistant spontaneous mutant (*E. coli* 294 Rif^r),

which was subsequently used as a recipient strain in the mating experiment. The *E. coli* strain HB101 containing the pRK600 helper plasmid was grown on LB plates with 10 µg/ml chloramphenicol and used in triparental mating experiments. Recipes for all media and buffers are listed in Appendix I.

2.2 Bacterial media

2.2.1 MacConkey agar

MacConkey agar (Oxoid, Basingstoke, Hampshire, United Kingdom) is a selective medium for Gram-negative bacteria and inhibits the growth of Gram-positive bacteria. *Salmonella* strains were streaked out on MacConkey agar plates and incubated at 37°C for 18-24 h for single colonies.

2.2.2 Luria Bertani (LB) medium

LB medium is a nutrient medium, allowing for the culture of aerobic Gram-positive and Gram-negative bacteria. In this study, LB medium (agar and broth) was used to culture *Salmonella* and *E. coli* strains at 37°C for 18-24 h.

2.3 DNA preparation

2.3.1 Boiled DNA preparations

Salmonella strains were grown on MacConkey agar (Oxoid) overnight at 37°C. Then, 3-4 single colonies from the streaked plates were suspended in 1 ml of sterile MQ water and centrifuged at 16,000 *g* for 5 min. The supernatant was removed, the pellet

resuspended in 400 µl of sterile MQ water and the solution heated at 105°C for 10 min. Following centrifugation at 16,000 *g* for 5 min, the supernatant was decanted and used as a crude DNA template for PCR amplification.

2.3.2 DNA extraction by the phenol/chloroform method

High quality DNA for PCR amplification and Southern hybridisation was extracted by the phenol/chloroform method (Sambrook et al., 1989). *Salmonella* strains were grown on MacConkey agar (Oxoid) at 37°C overnight. Then, 5 ml sterile PBS was added to the plate and swirled around gently. Colonies were gently scraped off the MacConkey plate with a sterile L shaped glass rod. The culture was transferred with a sterile transfer pipette to a pre-weighed sterile vacutainer tube. Following centrifugation at 2,500 *g* for 30 min, the supernatant was discarded. An 8 ml aliquot of sterile phosphate buffered saline (1 X PBS) buffer was used to fully resuspend the pellet, which was centrifuged at 2,500 *g* for another 30 min and the supernatant discarded.

The weight of the pellet was calculated, an aliquot of 10% sucrose/TE buffer was added (ratio of 1:10 weight/volume). To 2 ml of slurry, a 250 µl aliquot of lysozyme solution (50 mg/ml, freshly made) and 750 µl EDTA (0.5M, pH 8.0) was added. The solution was mixed and incubated at 37°C for 15 min. Then, 500 µl of 10% SDS/TE and 50 µl RNaseA (10 mg/ml) (Sigma-Aldrich, St Louis, Missouri, USA) was added, mixed with gentle inversion and incubated at 37°C for 30 min. A 25 µl proteinase K (10 mg/ml) (Roche Molecular Biochemicals, Mannheim, Germany) aliquot was added, mixed by

gentle inversion and incubated at 37°C for 1 h. The tube was then incubated at 56°C overnight.

The solution was transferred to SST Gel and Clot Activator Vacutainer tubes (Becton Dickinson Vacutainer Systems, NJ, USA) and 4 ml of TE saturated phenol/chloroform/isoamylalcohol (PCIaa) solution (25:24:1) added, mixed gently but thoroughly, then centrifuged at 2,800 *g* for 15 min. The PCIaa extraction was repeated two more times. The solution was transferred to a new SST tube and 4 ml of TE saturated chloroform/isoamylalcohol (CIaa) solution (24:1) was added, mixed gently but thoroughly, then the solution centrifuged at 2,800 *g* for 15 min. The CIaa extraction was repeated. The aqueous phase was transferred to dialysis bags (around 20 cm) and dialysed against 1 L of sterile TE buffer (pH 8.0) for 24-48 h with 2-3 buffer changes. The dialysed DNA was transferred to 5 ml sterile tubes. The concentration of DNA was measured at OD 600 nm using an Ultrospec 3300 Pro UV/visible spectrophotometer (Amersham Biosciences, Buckinghamshire, United Kingdom). The extracted chromosomal DNA was stored at -20 °C prior to use.

2.3.3 DNA extraction using the Corbett Robotics X-tractor Gene^{TM} instrument

E. coli transconjugants were streaked out on LB plates with appropriate antibiotics and incubated at 37°C overnight. A single colony from the LB plate was inoculated into 1 ml of LB broth and incubated at 37°C overnight with vigorous agitation (200 rpm). The cells were pelleted by centrifugation at 16,000 *g* for 5 min. The pellet was washed with

1 ml of 1 X PBS, and then 1 ml of sterile MQ water was used to resuspend the pellet. A 180 µl aliquot of the solution was loaded into one well of a 96-well lysis block tray housed on the Corbett Robotics X-tractor Gene^{TM} instrument (Corbett Robotics, QLD, Australia). The DNA was isolated by the robot following the manufacturer's instructions.

2.4 PCR amplification

Polymerase Chain Reaction (PCR) was used to amplify specific chromosomal DNA segments from *Salmonella* strains and *E. coli* transconjugants. Each PCR reaction mixture included 5 µl of 10 X PCR reaction buffer (Roche), 160 µM each dNTP (Bioline, London, UK), 20 pmole of each primer (Sigma-Aldrich), 5 µl (10-50 ng) of DNA template, 1 U of Taq DNA polymerase (Roche), and added sterile MQ water to a 50 µl final volume. Oligonucleotide primers were purchased from Sigma (Sigma-Aldrich) and dissolved in 100 µl sterile MQ water to make working stock solutions. MQ water was used as a negative control in each PCR batch. PCR was performed using thermocycler PC-960 or thermocycler FTS-960 (Corbett Research, Mortlake, Australia). The programs used were usually 94°C for 3-5 min followed by 35 cycles of denaturation (94°C for 30-45 s), annealing (52-62°C for 30-60 s) and extension (72°C for 30 s-2.5 min), and a final extension at 72°C for 10-15 min. When required, the reaction mix was optimised by altering the concentrations of template DNA, primers, or $MgCl_2$.

2.5 Agarose gel electrophoresis

A 10 µl sample of PCR product was mixed with 3 µl of loading dye (Appendix I) and loaded onto a 1-1.5% agarose gel (Bio-Rad, Hercules, CA, USA) dissolved in a 0.5 X TBE buffer (Appendix I). Electrophoresis was carried out in a 0.5 X TBE buffer with Bio-Rad electrophoresis equipment. The gels were run under constant electrophoretic conditions of 90 V, 500 mA for 2 h. However, for Southern hybridisation which requires a high level of resolution, the gels were run at 110 V, 500 mA for 3 h. The gel was stained by immersion in a 0.5 X TBE buffer with 5 µg/ml ethidium bromide (Bio-Rad) for 30 min, then distained with 0.5 X TBE buffer for another 15-20 min. The gel was visualised with ultraviolet light (wavelength, 254 nm), and imaged using a GelDoc 1000 image analysis station (Bio-Rad). The sizes of DNA fragments were estimated using a 100 bp plus DNA ladder or 1 kb DNA ladder (Fermentas, Vilnius, Lithuania). The marker used to estimate the sizes of bands in Southern hybridisation were digoxigenin-labeled molecular mass marker II or VII (Roche).

2.6 Restriction endonuclease analysis of DNA

The various restriction endonucleases used in this study were purchased from Roche (Roche Diagnostics), Fermentas (Fermentas, Vilnius, Lithuania) or Biolabs (New England Biolabs, Beverly, MA, USA). Digestions were conducted in 1.5 ml microfuge tubes and consisted of 10 µl (100-200 ng) of PCR product, 3 µl of 10 X restriction buffer (specific to the enzyme and supplied by the manufacturer), 10 U of the required restriction enzyme, and finally dH_2O added to 30 µl. Digestions with a combination of

two enzymes usually were performed in a reaction mixture containing 10 µl (100-200 ng) of PCR product, 6 µl 10 X reaction buffer (specific to both enzymes), 10 U of each required restriction enzyme, and dH_2O to 30 µl. The reaction mixture was incubated at 37°C on a Thermoline ^{TM} dry cell heating block (Scientific Equipment Pty Ltd, Smithfield, Australia) overnight to allow digestion. Finally, the reaction mixture was heated at 65°C for 10 min to denature the enzyme. The digestion mixture was analysed using a 2.0% (w/v) agarose gel (Bio-Rad) and run at 110 V, 500 mA for 2 h.

2.7 Purification of PCR products

PCR products were purified using a QIAquick PCR purification kit (Qiagen, California, USA) following the manufacturer's protocols. Briefly, 50 µl of PCR product was added to a Qiagen spin column microfuge tube, 300 µl PB buffer (Qiagen) was added and then the tube centrifuged at 16,000 *g* for 1 min, after which the supernatant was discarded. Then, 0.75 ml PE buffer (Qiagen) was added, and centrifuged at 16,000 *g* for 1 min, after which the supernatant was discarded. The solution was centrifuged at 16,000 *g* for another min to completely remove PE buffer. To elute the purified DNA, 50 µl of MQ water was added and centrifuged for 1 min at 16,000 *g*.

2.8 DNA sequence analysis

DNA sequencing was performed using an ABI 3130 automated DNA instrument and BigDye terminator kit (Applied Biosystems, Foster City, CA, USA). Each DNA sequencing reaction contained 2 µl of 5 X sequencing buffer, 3.2 pmol of forward or

reverse primer, 50-100 ng purified PCR product, with sterile MQ water added to a final volume of 10 µl. The amplification was performed in a Perkin Elmer GeneAmp 9600 thermocycler (Applied Biosystems). The program consisted of 25 cycles of denaturation (95°C, 30 s), annealing (50°C, 15 s) and extension (60°C, 240 s). After amplification, 2.0 µl of 3 M sodium acetate (pH 4.6) (Appendix I) and 50 µl of 95% ethanol were used to precipitate the extension product. The mix was put on ice for 10 min, and then centrifuged at 16,000 *g* for 30 min. The supernatant was removed and 250 µl of 70% ethanol was added to wash the pellet. The tube was then centrifuged at 16,000 *g* for 10 min. The ethanol was aspirated carefully with a micropipette and air dried in a fume hood. Each PCR product was completely sequenced on both strands. Sequence alignments were carried out using ClustalW. BLAST searches were used to identify similarities with known DNA sequences deposited in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.9 Southern Hybridisation

2.9.1 DNA probe preparation

A hybridisation probe was labeled with digoxigenin using the digoxigenin Easy Hyb kit (Roche) following the manufacturer's instructions. The PCR amplification of probes was performed as normal except for the standard dNTPs being replaced with DIG-labeled dNTPs (Roche). The amplified DIG-labeled PCR product was purified with a QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions (see section 2.7). The DIG-labeled probe was denatured by heating at

100°C for 10 min, cooled on ice, and stored at -20°C prior to use in Southern hybridisations.

2.9.2 Gel preparation

Chromosomal DNA was digested with a selected enzyme at 37°C overnight. A solution of 0.7%-0.8% agarose (w/v) dissolved in 0.5 X TBE was used to separate the digested chromosomal DNA. The gel was run at 110 V, 500 mA for 2-3 h and the result was documented. The gel was soaked in 0.2% HCl (Appendix I) with gentle agitation for 5 min and this process was repeated. The gel was next soaked in denaturation buffer (Appendix I) for 15 min with gentle agitation to denature the DNA. This operation was repeated twice with a change of solution each time. The gel was soaked in neutralisation buffer (Appendix I) for 7.5 min with gentle agitation to neutralise the DNA. This process was repeated with a change of buffer.

2.9.3 Preparation of hybridisation apparatus

While the gel was running for Southern hybridisation, some preparation work was undertaken. Three 15 cm x 33 cm wicks were cut and soaked in 20 x SSC buffer (3M NaCl, 300mM sodium citrate) (Appendix I) for 10 min. One 12.8 cm x 14 cm N^+ nylon membrane (Amersham Biosciences) was cut and soaked in MQ water for approximately 5 min until it was wetted thoroughly. The nylon membrane was then soaked in 20 X SSC buffer for at least 5 min. Three 12.8 cm x 14 cm wicks were cut and soaked in 2 X SSC buffer (0.3M NaCl, 30mM sodium citrate) (Appendix I) for at least 5 min. A stack

of paper towels were cut to approximately 5-8 cm high.

2.9.4 The membrane transfer

The Southern hybridisation reservoir was filled with 20 X SSC buffer (Appendix I) to just below the platform. Three 15 cm X 33 cm wicks were put on the platform individually and the ends were hung well into each end of the reservoir. Any air bubbles under the sheets were gently removed with a rolling pin. The pre-treated gel was placed bottom-up on the wick in the reservoir. A mask was placed onto the reservoir and a hole was cut in the mask approximately 2 mm smaller than the gel on all sides. A prepared N^+ nylon membrane (Amersham Biosciences) was placed over the gel. Then, 3 pieces of 12.8 cm X 14 cm wicks previously soaked in 2 X SSC buffer (Appendix I) were positioned on the top of the membrane. Again any air bubbles under the sheets were gently removed as mentioned above. A stack of absorbent paper approximately 5-8 cm high was placed on the top of the wicks, followed by a glass plate and a 500 g weight, and left overnight. The DNA was transferred to the N^+ nylon membrane by capillary action. The next day, the membrane was removed, rinsed with MQ water and left to air dry. The DNA was fixed to the membrane by baking in an oven at 80°C for 2 h.

2.9.5 Hybridisation

The membrane was placed in a Hybaid bottle and 25 ml Boehringer EasyHyb solution (Roche) added. The membrane was pre-hybridised at 58°C for 2 h using the Hybaid Hybridisation Oven (Hybaid). To make the hybridisation solution, 5 µl of denatured

probe (see section 2.9.1) was added to 25 ml Boehringer EasyHyb solution. The pre-hybridisation solution was replaced with 25ml hybridisation solution and the membrane was hybridised at 65°C for 1 h. The membrane was incubated in the Hybridisation Oven overnight at 42°C.

2.9.6 The membrane development

The hybridisation solution was removed the next day and the membrane was washed with 35 ml 2 X SSC/0.1%SDS buffer (Appendix I) at room temperature for 15 min. This step was repeated. Next, the membrane was more stringently washed with 35 ml 2 X SSC/0.1% SDS buffer (Appendix I) at 68°C for 15 min. This wash was repeated and all washes discarded. The membrane was then equilibrated with 50 ml Boehringer Wash Buffer (Roche) for 1 min and blocked for 1 h at room temperature with Boehringer Blocking Buffer (Roche). An anti-DIG antibody solution was prepared by adding 5 µl anti-digoxigenin-AP Fab fragment (Roche) to 50 ml of Boehringer Blocking Buffer. The Boehringer Blocking Buffer was discarded and the anti-DIG antibody solution was added to the membrane, and incubated for another 30 min at room temperature. The membrane was then washed with 50 ml Boehringer Wash Buffer (Roche) twice, each time for 15 min, at room temperature. The wash buffer was discarded and the membrane transferred to a plastic bag, and allowed to equilibrate for more than 2 min with 5 ml Boehringer Detection Buffer (Roche). Then, 50 µl CSPD (calf spleen phosphodiesterase) chemiluminesence substrate (Roche) was added to the bag and mixed well using a rolling sponge. The membrane was kept in the dark for 30 min. The CSPD (Roche)

solution was removed from the membrane, and the plastic bag was sealed around the membrane edge.

In the dark room under red light, two pieces of X-ray film (Amersham Biosciences) were placed above and below the membrane in an X-ray cassette and left for 30 min. The films were then developed by immersion in Kodak developer (Eastman Kodak Company, Rochester, NY, USA) for 2 min, followed by briefly rinsing in water. The films were immersed in Kodak fixer (Eastman Kodak Company) for 2 min and rinsed thoroughly in water before being air dried. The X-ray films were then scanned using a GS-800 Calibrated Densitometer (Bio-Rad).

2.10 Small plasmid extraction

Small plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's protocol. Briefly, *Salmonella* or *E. coli* strains were grown on LB plates with an appropriate antibiotic at 37°C overnight. A single colony was taken and grown in 3 ml of LB broth with the same antibiotic selection overnight. Centrifugation of 1 ml of culture at 16,000 *g* for 5 min formed a pellet. The pellet was resuspended in 250 µl Buffer P1 (Qiagen) and transferred in the solution to a microcentrifuge tube with a micropipette. A 250 µl sample of Buffer P2 (Qiagen) was added and mixed gently by inverting the tube 4-6 times. Then, 350 µl Buffer N3 (Qiagen) was added and the tube was inverted immediately but gently 4-6 times. The tube was centrifuged at 16,000 *g* for 10 min and the supernatant was applied to the QIAprep column (Qiagen) by decanting.

The column was then centrifuged at 16,000 *g* for 1 min and the flowthrough discarded. The column was washed using 0.75 ml Buffer PE (Qiagen) and centrifuged at 16,000 *g* for 1 min, then additionally centrifuged for another minute to remove the residual wash buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute the DNA, 50 µl of MQ water was added and the tube was centrifuged at 16,000 *g* for 1 min. Plasmids were analysed by 1.2% agarose dissolved in 0.5 X TBE buffer. The electrophoresis was carried out at 90 V, 500 mA for 2-3 h.

2.11 Large plasmid extraction

Large plasmid DNA was extracted and analysed by a procedure modified from the method of Kado and Liu (1981). A loop of glycerol stock culture (in 20% glycerol, at -80°C) was grown overnight in 3 ml of LB broth with appropriate antibiotic selection. Then, 1 ml of this culture was harvested by centrifugation at 16,000 *g* for 5 min at room temperature. The cell pellet was suspended in 20 µl TE buffer, mixed gently with 100 µl of lysis solution (Appendix I), and incubated at 58°C for 30 minutes. Afterwards, 100 µl of phenol/chloroform (1:1) solution (Appendix I) was added and mixed gently, followed by centrifugation at 16,000 *g* for 30 minutes. The upper aqueous phase was analysed by 0.8% agarose (w/v) dissolved in 1 X TBE buffer (Appendix I). The electrophoresis buffer used was 1 X TBE buffer and the gel was run at 100 V, 500 mA for 3 h.

2.12 Preparing competent *E. coli* DH5α cells

Overnight *E. coli* DH5α cells were grown on a LB plate for single colonies. Three to

four single colonies from this plate were inoculated into 20 ml of LB broth and grown with vigorous agitation (250 rpm) overnight. Then, 20 ml of overnight culture was inoculated into 200 ml LB broth and grown with vigorous agitation (250 rpm) until the absorbance (OD_{600}) reached 0.6. The *E. coli* DH5α cells were harvested by centrifugation at 4,000 *g* for 10 min at 4°C. The pellet was washed with 130 ml of chilled sterile MQ water and centrifuged at 4,000 *g* for 10 min at 4°C. Using 70 ml of chilled sterile MQ water, the pellet was washed then centrifuged at 4,000 *g* for 10 min at 4°C. Then, 5 ml of cold 10% glycerol (Appendix I) was used to resuspend the cells, which were centrifuged at 4,000 *g* for 5 min at 4°C. The cells were resuspended in 0.75 ml of cold 10% glycerol solution, aliquoted into 50 µl stocks, and stored at -80°C.

2.13 Electroporation of *E. coli* DH5α cells

For electroporation, approximately 50 ng of plasmid DNA was mixed with 50 µl of thawed DH5α competent cells. The mixture was transferred to a sterile prechilled Gene Pulsar cuvette with an interelectrode distance of 0.2 cm (Bio-Rad). The pulse was delivered by a Gene Pulsar (Bio-Rad) under 2.5 kV as suggested by the manufacturer. The cells were immediately resuspended to a final volume of 1 ml LB broth, which was preheated to 37°C. The mixture was incubated at 37°C for 1 h after which 200 µl was spread onto selective LB plates with appropriate antibiotics and grown overnight at 37°C. The colonies from the selective LB plates were patched onto LB plates with selective antibiotics. DNA boiled preps were made from single colonies and used as templates for PCR confirmation (see section 2.4). The colonies containing the expected

antibiotic resistance genes were suspended in 10% glycerol/1% casamino solution (Appendix I) and stored in -80°C for future use. In this experiment, *E. coli* DH5α competent cells were also used as a negative control and plated onto selective LB plates. Plasmid pUC19 (Invitrogen, Mount Waverley, VIC, Australia) was used as a positive control and the transformants were selected using ampicillin (32 µg/ml) LB plates.

2.14 Chemical transformation of *E. coli* JM109 cells

Chemically competent *E. coli* JM109 cells (Promega, Madison, Wisconsin, USA) were thawed on ice and mixed gently. Then, 100 µl of competent JM109 cells was mixed with 50 ng of plasmid preparation in a pre-chilled sterile tube and the mixture kept on ice for 10 min. The cell mixture was temperature-shocked at 42°C for 50 sec then kept on ice for 2 min. The cells were then added to 900 µl of LB broth, which was preheated to 37°C, and incubated for 1 h at 37°C. 200 µl aliquots of the incubated culture were plated onto LB selective plates with the appropriate antibiotics and grown overnight at 37°C. The colonies with the expected antibiotic resistance profile were patched onto LB plates with selective antibiotics. Plasmid pUC19 was used as a positive control with colonies selected on ampicillin (32 µg/ml) LB plates. The *E. coli* JM109 competent cells (Promega) were also used as a negative control and plated onto selective LB plates.

2.15 Conjugation experiments

Salmonella spp. donor strains were grown on selective LB plates with the appropriate

antibiotics whilst the *E. coli* 294 Rif^r recipient was grown on rifampicin (100 µg/ml) LB plates. A single colony from the *Salmonella* spp. donor plate was inoculated into 3 ml of LB broth with the appropriate antibiotics and grown at 37°C overnight at 200 rpm. A single colony from the *E. coli* 294 Rif^r recipient plate was inoculated into 3 ml of LB broth with 100 µg/ml rifampicin and grown at 37°C overnight at 200 rpm. Then, 100 µl of overnight donor cells was mixed gently with 100 µl of overnight *E. coli* 294 Rif^r recipient cells. The mixture was spread onto a LB plate at 37°C and grown overnight. The cell growth was washed from the LB plate using 1 ml of sterile 1 X PBS (Appendix I). A serial dilution (1:10. 1:100, 1:1000) was undertaken for the suspension in 1 X PBS buffer. Then, 200 µl of each diluted culture was spread onto selective LB plates, and grown overnight at 37°C. A single colony was picked from selective LB plates and patched onto another selective LB plate. For each mating experiment, a total of 10 single colonies were patched to obtain 10 replicates. Then, single colonies from the selective LB plates were patched onto other selective LB plates as required. Colonies with the expected antibiotic resistance profile were chosen as potential *E. coli* transconjugants. Boiled DNA preparations were made from potential transconjugants and used as templates for *E. coli* specific *usp*A gene and *Salmonella* specific *invA* gene confirming PCR. Only those colonies confirmed to be *E. coli* and not *Salmonella* and which had the expected antibiotic resistance profile and gene complement were stored in 10% glycerol/1% casamino acid solution at -80°C for future use.

2.16 Triparental mating experiments

For the conjugation experiment with *E. coli* HB101 containing the pRK600 helper plasmid, all steps were undertaken as for the conjugation experiment. However instead of using 100 µl of donor and recipient cultures mixed together, only 80 µl of each donor, recipient and *E. coli* HB101 (pRK600) cultures were mixed together.

2.17 Antibiotic resistance testing

E. coli strains were grown on selective LB plates with appropriate antibiotics at 37°C overnight. The antibiotic testing plates were made with Sensitest Agar (Oxoid). One colony at least 2 mm in diameter was picked from an LB plate and resuspended in 1.5 ml of sterile saline. The Sensitest agar plates were flooded with the saline solution, and then the solution was spread uniformly over the agar surface and excess fluid removed. The plates were allowed to dry for 45 min in a containment cabinet. Different antibiotic discs (Oxoid) were loaded using the disc applicator (Oxoid) on top of the Sensitest Agar plates and then incubated at 37°C overnight. The radius of zones of inhibition were measured from the center of the disc and recorded for each antibiotic. If the radius of the antibiotic zone of inhibition was larger than 6 mm, it was recorded as sensitive. If the radius of the antibiotic clearing zone was less than 6 mm, it was recorded as resistant (Figure 2.1). The antibiotic discs used included amikacin 30 µg/ml (AK30), gentamicin 10 µg/ml (CN10), kanamycin 30 µg/ml (K30), netilimicin 30 µg/ml (NET30), spectinomycin 25 µg/ml (SH25), streptomycin 25 µg/ml (S25) and tobramycin 10 µg/ml (TOB10).

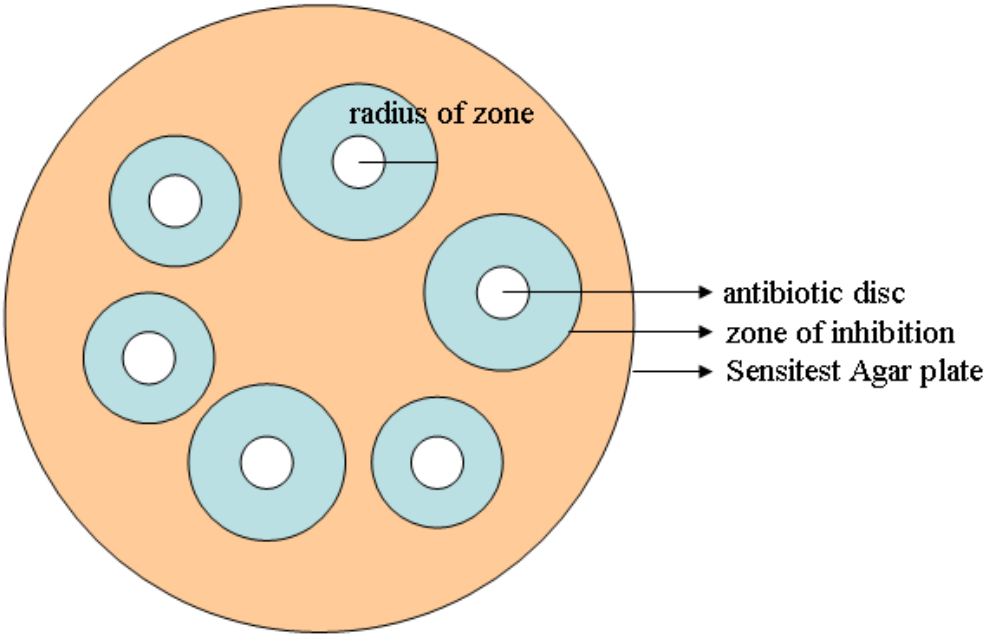


Figure 2.1 The antibiotic disc, the zone of inhibition and the Sensitest Agar plate used in antibiotic resistance testing experiment.

Chapter 3: Primary PCR screening for *strA*, *strB*, *sul2*, *tetA*(A) and *tetA*(B) genes

3.1 Introduction

3.1.1 Tn*5393*-like transposons

In bacterial isolates from plants, *strA* and *strB* genes are often encoded on the Tn*5393* transposon, which is approximately 6.7 kb in size. Tn*5393* is usually born on conjugative plasmids and belongs to the Tn*3* transposon family (Chiou and Jones, 1993). Unlike plasmid RSF1010, the *strA-strB* genes on Tn*5393* are not linked with the *sul2* gene (Sundin and Bender, 1996). Derivatives of the Tn*5393* transposon from different sources can be discriminated by the presence of insertion sequences. Tn*5393* isolated from the apple pathogen *Erwinia amylovora* contains the IS*1133* element (Sundin and Bender, 1996). The basic structure of Tn*5393* includes a transposase (*tnpA*), a resolvase (*tnpR*), the IS*1133* element, linked *strA-strB* genes and inverted repeats at each end of the transposon (Figure 3.1) (Sundin and Bender, 1996). The Tn*5393*-like transposon from *Pseudomonas syringae* without an IS element is called Tn*5393a* (Figure 3.1) (Sundin and Bender, 1993; Sundin and Bender, 1995). The Tn*5393*-like transposon from *Xanthomonas campestris* with an IS*6100* element is called Tn*5393b* (Figure 3.1) (Sundin and Bender, 1995). In Norway, a Tn*5393*-like transposon was located in the fish pathogen *Aeromonas salmonicida* and named Tn*5393c* (Figure 3.1) (L'Abee-Lund and Sorum, 2000). There is no structural difference between Tn*5393a* and Tn*5393c* except that these originated from different sources. Isolated from *Alcaligenes faecalis*,

Tn*5393d* is a derivative of the original Tn*5393*, with additional resistance determinants, including the *bla*$_{PER-1}$ gene and *aphA6b* gene (Figure 3.1) (Mantengoli and Rossolini, 2005). The *bla*$_{PER-1}$ gene confers resistance to β-lactams, while *aphA6b* is a new allelic variant of the *aphA6* aminoglycoside phosphotransferase gene and confers resistance to kanamycin, streptomycin and amikacin. There are two *strA-strB* genes in the Tn*5393d* transposon and one of the *strB* genes is truncated at the 3′ end (Mantengoli and Rossolini, 2005). Tn*5393d* apparently originated from the consecutive insertion of two composite transposons carrying the *aphA6b* and *bla*$_{PER-1}$ genes into the Tn*5393* backbone. The putative composite transposon carrying *bla*$_{PER-1}$ was named Tn*4176* (Mantengoli and Rossolini, 2005).

The IS elements in Tn*5393* and Tn*5393b* are involved in the expression of *strA-strB* genes, and can increase the expression of *strA-strB* in their bacterial hosts (Sundin and Bender, 1995). Tn*5393a* conferred streptomycin resistance to *P. syringae* at 75 mg/ml. A putative promoter, located within *res* in Tn*5393a*, was involved in the expression of *strA-strB* (Sundin and Bender, 1995). The streptomycin resistance in *X. campestris* was increased to 250 mg/ml by the insertion of IS*6100* in Tn*5393b*. Except the putative promoter identified within *res* site, another sequence located within IS*6100* functioned as a promoter (Sundin and Bender, 1995). Similarly, a promoter sequence located within IS*1133* was presumed to provide a promoter for the high levels of streptomycin resistance (500 to 1,000 mg/ml) observed in Tn*5393* from *E. amylovora* (Chiou and Jones, 1993). The IS*1133* element is inserted between the *strA* and *tnpR* genes in

Tn*5393*, while the IS*6100* is inserted within the *tnpR* gene and inactivates this gene in Tn*5393b*, so the increased MIC associated with the IS*1133* element is much higher than that of IS*6100* (LA'bee-Lund and Sorum, 2000).

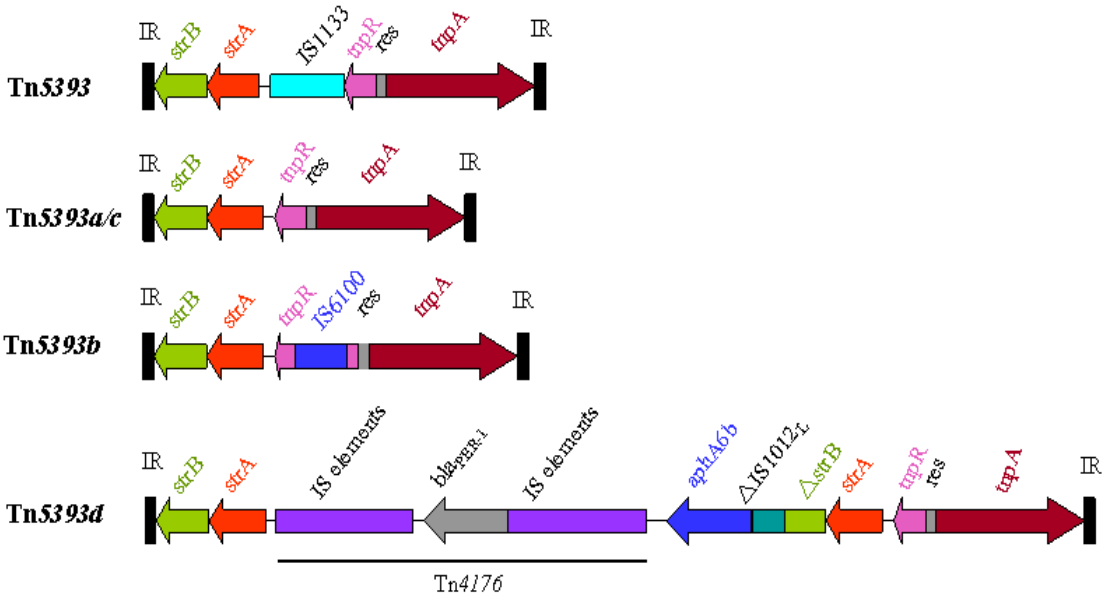


Figure 3.1 The structures of Tn*5393*-like transposons including Tn*5393* from *Erwinia amylovora*, Tn*5393a* from *Pseudomonas syringae*, Tn*5393b* from *Xanthomonas campestris*, Tn*5393c* from *Aeromonas salmonicida*, and Tn*5393d* from *Alcaligenes faecalis*. The genes and their transcriptional directions are indicated by arrows. Abbreviations: IR, inverted repeat; *strA* and *strB* confer resistance to streptomycin; *⊿strB*, truncated *strB* gene; *tnpR*, resolvase; *res*, recombination site; *tnpA*, transposase gene; bla_{PER-1} confers resistance to β-lactam; *aphA6b* confers resistance to kanamycin, streptomycin and amikacin; IS*1133* and IS*6100*, two IS elements (modified from LA'bee-Lund and Sorum, 2000; Mantengoli and Rossolini, 2005).

3.1.2 RSF1010 and other small plasmids

In 14 Genera of human- and animal-associated bacteria, *strA-strB* are often linked with the *sul2* gene and located on the small non-conjugative plasmid RSF1010 (Palmer et al., 1997). RSF1010 is approximately 8.7 kb in size and belongs to the IncQ plasmid group (Scholz et al., 1989). The structure of RSF1010 includes streptomycin resistance genes *strA* and *strB*, the sulfonamide resistance gene *sul2*, replication genes including *repC*,

repA, and *repB*, mobilisation genes including *mobB*, *mobA* and *mobC*, the repressor gene *repF*, and a gene encoding protein E for which a function has yet to be elucidated (Figure 3.2) (Scholz et al., 1989). Plasmid RSF1010 is rarely reported in bacteria from plants or the soil, but a related RSF1010 plasmid has been detected in *Erwinia amylovora* from apple orchards in Michigan (Palmer et al., 1997). The *strA-strB* genes linked with *sul2* were also found on the small IncQ-like plasmid pMS260, which was isolated from *Actinobacillus pleuropneumoniae* and is approximately 8 kb in size (Ito et al., 2004). This plasmid has the same replication and mobilisation genes as RSF1010 plasmid. It differs from RSF1010, in that it contains a *cac* gene, whose function is to regulate the expression of *repA* and *repC* genes (Figure 3.2) (Ito et al., 2004). The plasmid pIE1115 from an uncultured *Eubacterium* was reported by Smalla et al. (2000). This plasmid has the same *strA-strB* linked with *sul2* genes, mobilisation and replication genes as seen for the RSF1010 plasmid. It is different from RSF1010, because it has an orf1 open reading frame with unknown function, lincosamide nucleotidyltransferase *linB*-like gene and two iterons (Figure 3.2) (Smalla et al., 2000). Another 6.2 kb small plasmid designated p9123 was isolated by Enne et al. (2004) from a clinical *E. coli* isolate. Apart from containing *strA-strB* linked with *sul2* genes, this small plasmid also harbours five open reading frames with unknown function including orf1, orf2, orf3, orf4 and orf5 (Figure 3.2) (Enne et al., 2004). Plasmid pBP1 is another broad-host-range, non-conjugative small plasmid. It is more widely distributed than RSF1010 among *E. coli* and its size is approximately 6.3 kb (Korfmann et al., 1983).

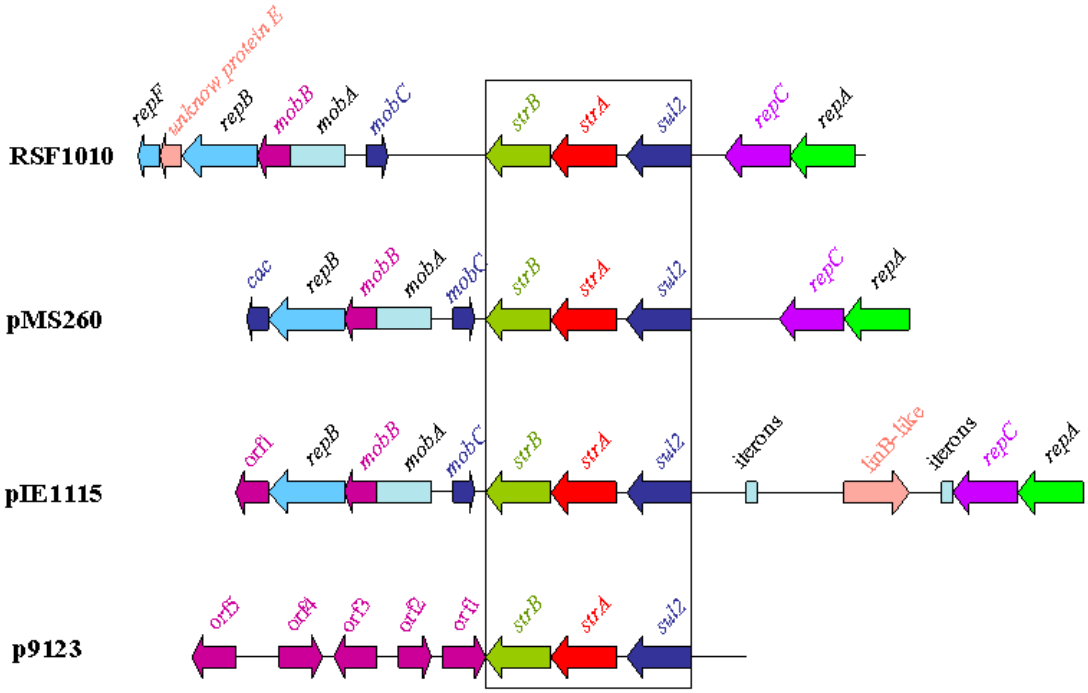


Figure 3.2 The maps of plasmid RSF1010 (accession no. M28829), pMS260 (accession no. AB109805), pIE1115 (accession no. NC_002524) and p9123 (accession no. NC_005324). The genes and their transcriptional directions are indicated by arrows. The common region of *strA*, *strB* and *sul2* is indicated by a black box. *strA* and *strB* confer resistance to streptomycin; *sul2* confers resistance to sulfonamide; *repC*, *repA* and *repB* encode replication functions; *mobB*, *mobA* and *mobC* encode mobilisation functions; *repF* encodes a repressor function; *cac* encodes a regulation function on the *repC* and *repA* genes; orf1, orf2, orf3, orf4 and orf5 are open reading frames; *linB*-like encodes lincosamide nucleotidyltransferase (modified from Scholz et al. 1989; Ito et al., 2004; Smalla et al., 2000; Enne et al., 2004).

3.1.3 *tetA*(A), *tetA*(B), *tetA*(G) tetracycline resistance genes and the Tn*1721* transposon

The widespread use of tetracycline constitutes a high selective pressure and may have contributed to the successful spread of tetracycline resistance determinants in zoonotic pathogens such as *Salmonella* (Robert, 1996; Pezzella et al., 2004). Several different *tet* genes have been described as conferring resistance to tetracycline in *Salmonella*. Among these *tet* genes, classes A, B, C, D, and G are the most frequent types (Frech and Schwarz, 1998). The gene *tetA*(A) was found on plasmids as well as on the chromosome,

while the genes *tetA*(B), *tetA*(C), and *tetA*(D) were detected on the chromosomes of *S. enterica* strains of different serotypes including Typhimurium, Enteritidis, Hadar, Saintpaul and Choleraesuis (Frech and Schwarz, 2000). Recently, *tetA*(B) genes have been detected on large conjugative plasmids such as pAKU_1 and pHCM1 (Holt et al., 2007; Parkhill et al., 2001).

The *tetA*(A) gene is often found located on transposon Tn*1721* in *Salmonella* isolates (Frech and Schwarz, 1998; Frech and Schwarz, 1999). Tn*1721* is approximately 11 kb in size and belongs to the Tn*21* subgroup of Tn*3*-like transposons (Schoffl et al., 1981; Allmeier et al., 1992). The Tn*1721*-borne genes can be grouped into three clusters: the transposition loci *res*, *tnpR* and *tnpA*, the open reading frame orf1, and the resistance genes *tetA*(A) and *tetR* (Figure 3.3) (Waters et al., 1983). Tn*1721* is bound by the 38 bp IRL and IRRII inverted repeats at each end. This transposon is subdivided by the IRRI inverted repeat into two almost identically sized parts, one containing the genes required for transposition and the other containing functional tetracycline resistance genes (Schoffl et al., 1981; Allmeier et al., 1992). The *tetA*(A) gene, which is located on a Tn*1721*-like transposon has been found in several different *Salmonella* plasmids. In *S. enterica* serovar Dublin, a 47 kb plasmid pGFT1 was found, which mediated tetracycline resistance via a *tetA*(A) gene located on a Tn*1721*-analogous transposon (Frech and Schwarz, 1998). In another isolate from *S. enterica* serovar Typhimurium var. Copenhagen, a 9.5 kb plasmid pGFT4 was found to contain a truncated Tn*1721*, which lacked the entire transposase region (Frech and Schwarz, 1999).

The *tetA*(B) gene is usually located on Tn*10*, a 9.3 kb transposon which is found inserted at various positions in chromosomal DNA (Frech and Schwarz, 2000). The *tetA*(G) gene has been detected as part of the chromosomal multidrug resistance gene cluster SGI1 in *S. enterica* serovar Typhimurium DT104 (Briggs and Fratamico, 1999). Two variants of *tetA*(G) with 92% sequence identity were detected in *Pseudomonas* species (Schnabel and Jones, 1999).

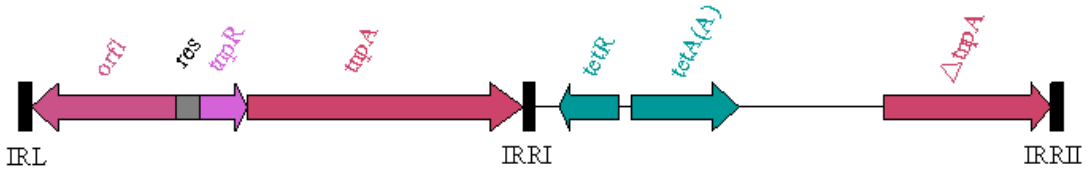


Figure 3.3 The structure of Tn*1721*. The genes and their transcription directions are indicated by arrows. orf1, open reading frame 1; *res*, recombination site; *tnpR*, resolvase; *tnpA*, transposase; *△tnpA*, truncated transposase; *tetR*, tetracycline regulatory gene; *tetA*(A), tetracycline resistance class A gene; IRL, left inverted repeat; IRRI and IRRII, two right inverted repeats (adapted from Allmeier et al., 1992).

This chapter will focus on the screening of streptomycin resistance genes *strA* and *strB*, sulfonamide resistance gene *sul2*, and tetracycline resistance genes *tetA*(A) and *tetA*(B). Transposon Tn*5393*, plasmid RSF1010 and transposon Tn*1721* were screened as well.

3.2 Materials and Methods

3.2.1 PCR amplification

PCR and the following primer sets were used to amplify fragments of *strA* (strA-F/strA-R), *strB* (strB-F/strB-R), *sul2* (sulII-F/sulII-R), *sul2-strA* (sulII-F/strA-out), *strA-strB* [strA-F(L)/strB-R(L)] and *mobB-repB* regions (RSF1010-F/RSF1010-R) respectively. The primers used are indicated in Figure 3.4.

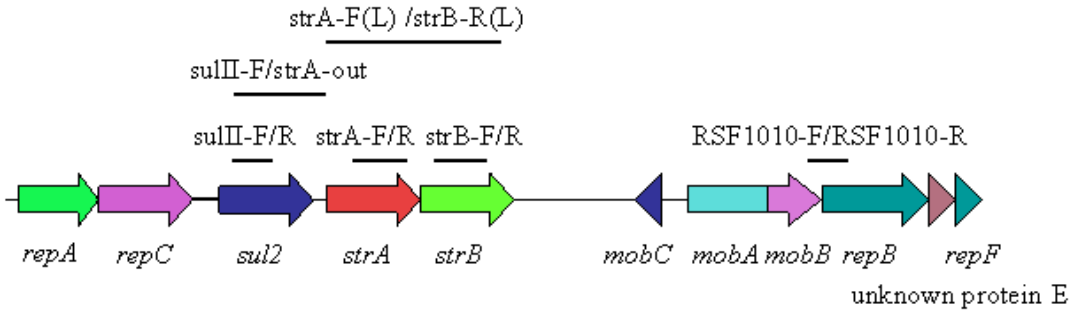


Figure 3.4 The primer positions of sulII-F/sulII-R, strA-F/strA-R, strB-F/strB-R, sulII-F/strA-out, strA-F(L)/strB-R(L), and RSF1010-F/RSF1010-R on plasmid RSF1010 (accession no. M28829). The *repA*, *repC*, *sul2*, *strA*, *strB*, *mobC*, *mobB*, *repB*, unknown protein E and *repF* genes are shown as arrows with the arrowhead indicating their transcriptional directions. The *mobA* gene is truncated and indicated as a box. Fragments amplified by PCR including *sul2*, *strA*, *strB*, *sul2-strA*, *strA-strB* and *mobB-repB*, are shown as lines with the primer pairs indicated above.

Primers smAR and IS1133F were used to identify transposon Tn*5393* and the primer pair is indicated in Figure 3.5A. Primers Tn5393-3 and Tn5393-1 were used to identify Tn*5393b* transposon (Figure 3.5B).

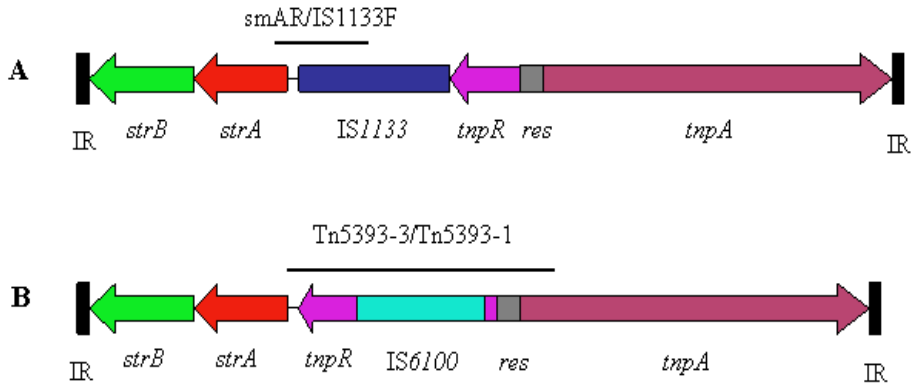


Figure 3.5A The primer position of smAR/IS1133F on transposon Tn*5393* (accession no. M95402). **B** The primer position of Tn5393-3/Tn5393-1 on Tn*5393b* transposon (accession no. U20588). The *strB*, *strA*, *tnpR*, *tnpA* genes are shown as arrows with the arrowhead indicating their transcriptional directions. IS*1133* and IS*6100* elements are indicated as boxes. *res*, recombination site. Vertical bars indicate inverted repeats (IR). The fragments amplified by PCR are shown as lines with the primer pairs indicated above.

Primer pair TAF/TetAR3 was used to amplify the right arm of Tn*1721* transposon

(Figure 3.6).

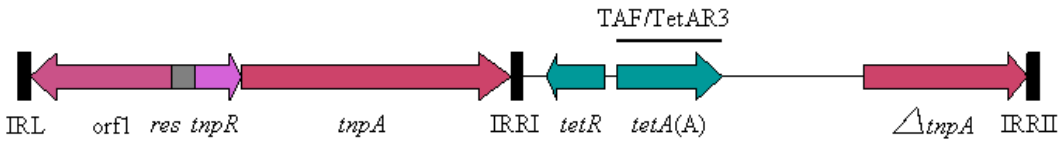


Figure 3.6 The primer position of TAF/TetAR3 on Tn*1721* transposon (accession no. X61367). The orf1, *tnpR*, *tnpA*, *tetR*, *tetA*(A) and *△tnpA* genes are shown as arrows with the arrowhead indicating their directions of transcription. *res*, recombination site. Three 38 bp inverted repeats are shown as vertical bars. The fragment amplified by PCR is shown as a line with the primer pair indicated above.

All primer sequences are listed in Table 3.1. Except for the Tn5393-3/Tn5393-1 PCR, the program used for all other PCR amplifications was: 94°C for 5 min, followed by 35 cycles 94°C for 30 sec, 53°C-60°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. The annealing temperature for each PCR is listed in Table 3.2. Amplifications were performed as described in Chapter 2 (section 2.4) using a thermocycler PC-960 (Corbett Research).

For the Tn5393-3/Tn5393-1 PCR amplification, the program used was: 94°C for 5 min, followed by 35 cycles 94°C for 1 min, 57°C for 1 min, 72°C for 2 min, and finally 72°C for 10 min. The amplification was performed using a thermocycler FTS-960 (Corbett Research). All PCR products were analysed by using 1% (w/v) agarose gel as described in Chapter 2 (section 2.5).

Table 3.1 The primers used for PCR and multiplex PCR amplifications.

Primer	Primer sequence (5′-3′)	Location	Nucleotide positions	Accession no.	Reference
strA-F	CTT GGT GAT AAC GGC AAT TC	*strA*	5196-5215	M95402	Gebreyes and Altier, 2002
strA-R	CCA ATC GCA GAT AGA AGG C	*strA*	5743-5725	M95402	Gebreyes and Altier, 2002
strB-F	ATC GTC AAG GGA TTG AAA CC	*strB*	5877-5896	M95402	Gebreyes and Altier, 2002
strB-R	GGA TCG TAG AAC ATA TTG GC	*strB*	6385-6366	M95402	Gebreyes and Altier, 2002
sulII-F	GGC AGA TGT GAT CGA CCT CG	*sul2*	7997-8016	M28829	Leverstein-van Hall et al., 2002
sulII-R	ATG CCG GGA TCA AGG ACA AG	*sul2*	8401-8382	M28829	Leverstein-van Hall et al., 2002
strA-F(L)	TGAATCGCATTCTGACTGGTT	*strA*	89-109	M28829	Palmer et al., 1997
strB-R(L)	GCTAGATCGCGTTGCTCCTCT	*strB*	1659-1639	M28829	Palmer et al., 1997
strA-out	AAC CAG TCA GAA TGC GAT TCA	*strA*	109-89	M28829	Palmer et al., 1997
IS1133F	GATTGGCTGGGCAACAGGTGA	*tnpA* of IS*1133*	4465-4486	M95402	Pezzella et al., 2004
smAR	TCCTCCTGCCAGTTGATCAC	*strA* of Tn*5393*	5180-5161	M95402	Pezzella et al., 2004
Tn5393-1	CGGCAAGATGCTCGTGCCGTG	*tnpA of* Tn*5393*	2604-2624	M95402	Sundin, 2002
Tn5393-3	AGTCAGAATGCGATTCACC	*strA* of Tn*5393*	4984-5002	M95402	Sundin, 2002
RSF1010-F	GGT GGC ACT GGA AGC TAT GG	*mobB* of RSF1010	4266-4285	M28829	Palmer et al., 1997
RSF1010-R	AGG TCA AAC TCG CTG AGG TCG	*repB* of RSF1010	4658-4638	M28829	Palmer et al., 1997
tet(A)-F	GCT ACA TCC TGC TTG CCT TC	*tetA*(A)	7562-7581	X61367	Ng et al., 2001
tet(A)-R	CAT AGA TCG CCG TGA AGA GG	*tetA*(A)	7771-7752	X61367	Ng et al., 2001
tet(B)-F	TTG GTT AGG GGC AAG TTT TG	*tetA*(B)	2003-2022	J01830	Ng et al., 2001
tet(B)-R	GTA ATG GGC CAA TAA CAC CG	*tetA*(B)	2661-2642	J01830	Ng et al., 2001
tet(G)-F	CAG CTT TCG GAT TCT TAC GG	*tetA*(G)	681-700	S52437	Zhao and Aoki, 1992
tet(G)-R	GAT TGG TGA GGC TCG TTA GC	*tetA*(G)	1524-1505	S52437	Zhao and Aoki, 1992
TAF	GTA ATT CTG AGC ACT GTC GC	*tetA*(A)	6718-6737	X61367	Waters et al.,1983
TetAR3	GGC ATA GGC CTA TCG TTT CCA	*tetA*(A) of Tn*1721*	7917-7897	X61367	Hartman et al., 2003

Table 3.2 The annealing temperatures used for different PCR amplifications.

PCR name	Primers	Annealing temperature
strA	strA-F/strA-R	53°C
strB	strB-F/strB-R	53°C
sul2	sulII-F/sulII-R	60°C
sul2-strA region	sulII-F/strA-out	57°C
strA-strB region	strA-F(L)/strB-R(L)	58°C
mobB-repB region	RSF1010-F/RSF1010R	58°C
Tn*5393*	smAR/IS1133F	59°C
Tn*5393b*	Tn5393-3/Tn5393-1	57°C
Tn*1721*	TAF/TetAR3	57°C

3.2.2 Multiplex PCR amplification

Multiplex PCR was used to amplify *tetA*(A), *tetA*(B) and *tetA*(G) genes. The primer sequences are listed in Table 3.1. Each reaction unit included 1µl of each primer, Taq 0.2 µl, dNTPs (2 mM) 6 µl, $MgCl_2$ (25 mM) 2 µl, 10 X reaction buffer 5 µl, DNA templates 5 µl (10-50 ng), with sterile MQ water to make up to a 50 µl volume. The program used for the amplification was: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2.5 min, and finally 72°C for 10 min. The amplification was performed using a thermocycler PC-960 (Corbett Research).

3.2.3 Sequence analysis of each PCR product

The PCR products of *strA*, *strB*, *sul2*, *sul2-strA* region, *strA-strB* region, *mobB-repB* region, Tn*5393* (IS*1133*), *tetA*(A), *tetA*(B) and the right arm of Tn*1721* were purified with a QIAquick PCR purification kit (Qiagen) as described in Chapter 2 (section 2.7). The PCR products were sequenced using an ABI 3130 automated DNA instrument (Applied Biosystems) as described in Chapter 2 (section 2.8).

3.2.4 Small plasmid extraction of 11 *Salmonella* strains

Small plasmids were prepared for 11 *Salmonella* strains including SRC6, SRC15,

SRC33, SRC35, SRC55, SRC56, SRC106, SRC107, SRC109, SRC110 and SRC130 using a QIAprep Spin Miniprep Kit (Qiagen) as described in Chapter 2 (section 2.10).

3.2.5 Electroporation of 8 *Salmonella* plasmids

Small plasmids extracted from 8 *Salmonella* strains (SRC15, SRC33, SRC55, SRC56, SRC130, SRC106, SRC107 and SRC109) were electroporated into *E. coli* DH5α competent cells according to the method described in Chapter 2 (section 2.13).

3.2.6 Chemical transformation of plasmids from *Salmonella* strains SRC35 and SRC110

Two plasmid preparations from *Salmonella* strains SRC35 and SRC110 were transformed into *E. coli* JM109 competent cells (Promega) using a chemical transformation method as described in Chapter 2 (section 2.14).

3.2.7 Conjugation of a plasmid from *Salmonella* strain SRC6

A *Salmonella* strain SRC6 plasmid was conjugated into *E. coli* 294 Rif^r by the mating method as described in Chapter 2 (section 2.15). To designate transformants/transconjugants, the prefix 'P' was used followed by the designation of the original host strain. For example, a transformant for strain SRC15 was indicated as pSRC15. This designation was used for both transformation and conjugation experiments.

3.2.8 Antibiotic resistance testing for 11 *Salmonella* plasmids

Antibiotic resistance profiles were tested for 11 *Salmonella* plasmids harboured in *E. coli* (pSRC6, pSRC15, pSRC33, pSRC35, pSRC55, pSRC56, pSRC106, pSRC107, pSRC109, pSRC110 and pSRC130) according to the antibiotic resistance testing

method described in Chapter 2 (section 2.17).

3.3 Results

3.3.1 Screening for *strA*, *strB* and *sul2* genes

To screen 136 *Salmonella* strains, PCR was used to amplify the *strA*, *strB* and *sul2* genes with the primer pair strA-F/strA-R, strB-F/strB-R and sulII-F/sulII-R (see Figure 3.4 for the positions). Three PCR amplicons for *strA*, *strB* and *sul2* genes were obtained at the expected sizes of 548 bp, 509 bp and 405 bp, respectively (Figure 3.7). Among the 136 strains, 68 contain *strA* genes (50.0%), and 70 contain *strB* genes (51.5%). In total, 67 strains were confirmed to contain both *strA* and *strB* genes (49.3%). In this collection, one strain was detected containing *strA* without the *strB* gene and three strains harboured *strB* but without the *strA* gene. For *sul2* screening, 59 strains were found to harbour *sul2* genes (43.4%). Among them, 48 strains contained *sul2*, *strA* and *strB* genes together. One strain harboured the *sul2* gene and also the *strA* gene but without *strB*. Ten strains contained only *sul2*, without *strA* or *strB* genes. 19 strains contained both *strA* and *strB* genes, but without the *sul2* gene. 55 strains contained neither *strA*, *strB* nor the *sul2* gene (40.4%).

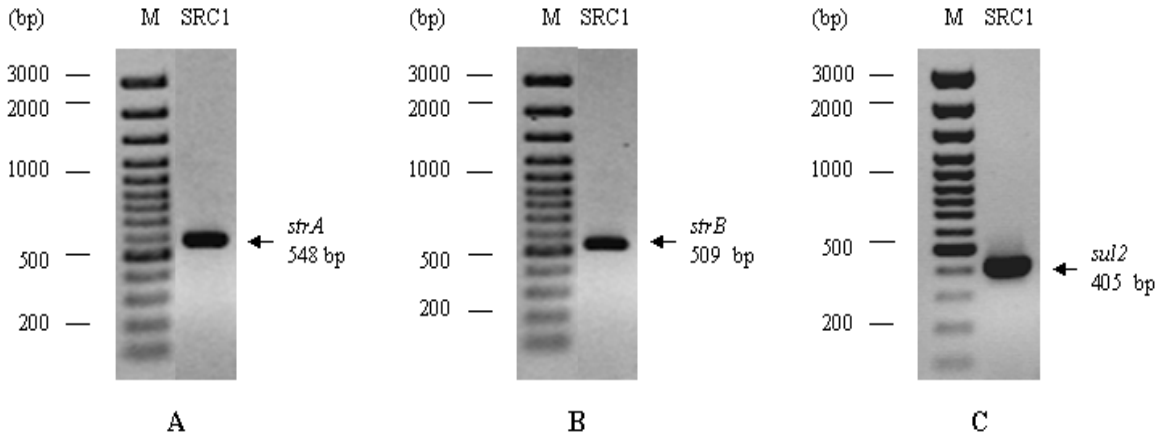


Figure 3.7 Gel electrophoresis of three different PCR amplicons representing *strA*, *strB* and *sul2* genes. **A** strain SRC1 is a representative for the 548 bp *strA* amplicon; **B** strain SRC1 is a representative for the 509 bp *strB* amplicon; and **C** strain SRC1 is a

representative for the 405 bp *sul2* amplicon. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

The streptomycin resistance genes *strA* and *strB* can occur adjacently, but can also be found separately. Thus, the *strA-strB* linkage PCR (see Figure 3.4) amplification was performed on 67 *Salmonella* strains containing both *strA* and *strB* genes. A PCR amplicon was obtained as expected with a size of 1571 bp (Figure 3.8A). All the 67 *Salmonella* strains were positive for this PCR. DNA sequence analysis of the PCR amplicon from strain SRC13 revealed that it was 99% identical to the *strA-strB* region from RSF1010 plasmid (accession no. M28829). DNA sequence analysis of the *sul2* gene from strain SRC136 revealed that this amplicon was identical to the *sul2* gene from RSF1010 (accession no. M28829).

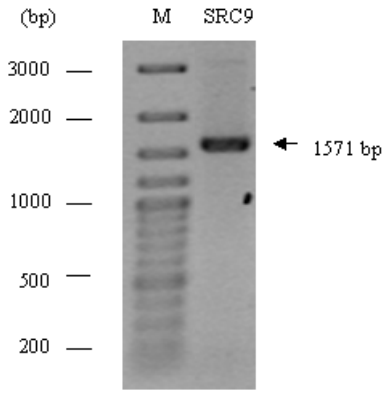


Figure 3.8A Gel electrophoresis of the *strA-strB* linkage PCR amplicon. Strain SRC9 was chosen as a representative for the 1571 bp *strA-strB* PCR amplicon and was visualised on a 1.0% (w/v) agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

All the PCR amplicons were further characterised by digestion with *Rsa*I + *Hae*III restriction enzymes and 4 fragments were obtained with expected sizes of 564 bp, 510 bp, 237 bp and 153 bp (Figure 3.8B). Two fragments of 64 bp and 43 bp in size could not be visualised from the agarose gel.

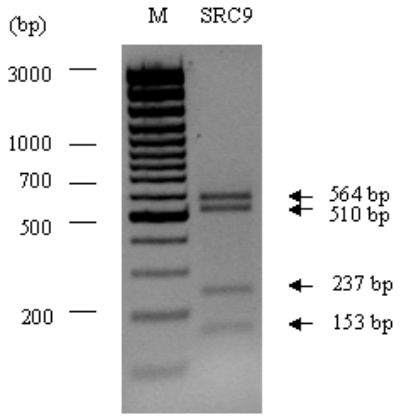


Figure 3.8B Restriction endonuclease analysis of the *strA-strB* linkage PCR amplicon digested with *Rsa*I + *Hae*III. The SRC9 amplicon yielded fragments of 564 bp, 510 bp, 237 bp and 153 bp visualized on a 2.0% (w/v) agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

In order to determine whether the *sul2* gene was linked to the *strA* gene, a pair of primers sulII-F /strA-out (see Figure 3.4), was used to link these two genes together for 49 strains containing the *strA* and *sul2* genes. A PCR amplicon of the expected size of 797 bp was obtained (Figure 3.9A) and 47 strains were positive for this PCR. DNA sequence analysis of this amplicon from strain SRC126 confirmed that it was 99% identical to the *sul2-strA* region from RSF1010 plasmid (accession no. M28829). All the PCR amplicons were further confirmed and characterised by digestion with the restriction enzyme *Pst*I, and two fragments were obtained with expected sizes of 576 bp and 221 bp (Figure 3.9B). The restriction endonuclease analysis using *Pst*I confirmed that all the *sul2-strA* linkage PCR amplicons had the same RFLP profile.

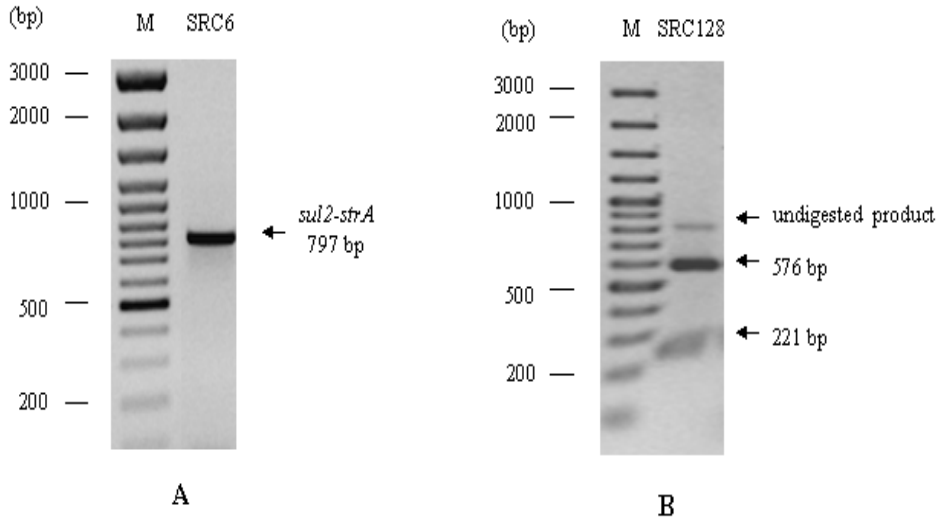


Figure 3.9A Gel electrophoresis of the *sul2-strA* linkage PCR amplicon. Strain SRC6 was chosen as a representative for the 797 bp PCR amplicon (1.0% agarose gel). **B** Gel electrophoresis of *Pst*I digested *sul2-strA* linkage PCR amplicon. Strain SRC128 was chosen as a representative to show the 576 bp and 221 bp digested fragments on a 2.0% agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

Among the 49 strains examined, 47 harboured linked *sul2-strA-strB* genes. One strain contained *strA-strB* and *sul2* genes, where *sul2* was not linked to the *strA-strB* genes. All strains that contained *sul2* and *strA-strB* conferred resistance to SmSu. One strain contained *sul2* and *strA*, without the *strB* gene, where the *sul2* gene again did not link to the *strA* gene (Table 3.3).

Table 3.3 A summary of the PCR screening results of *strA*, *strB*, *sul2*, *strA-strB* and *sul2-strA* for 136 *Salmonella* strains.

Antibiotic resistance structure	Numbers of strains	Strain SRC numbers
strA-strB	19	7, 8, 9, 39, 43, 44, 64, 65, 66, 67, 68, 73, 2, 3, 52, 54, 100, 125, 127
sul2-strA-strB	47	6, 15, 33, 35, 55, 56, 130, 106, 107, 109, 110, 1*, 13*, 14*, 17*, 23*, 26*, 27*, 28*, 29*, 30*, 31*, 32*, 36*, 51*, 60*, 61*, 62*, 79*, 80*, 82*, 116*, 117*, 118*, 120*, 121*, 122*, 123*, 124*, 126*, 128*, 129*, 131*, 132*, 133*, 135*, 136*
sul2, *strA-strB*	1	34
sul2, *strA*	1	16
strB only	3	40, 41, 42
sul2 only	10	12, 22, 37, 69, 74, 76, 91, 102, 103, 119
No *strA*, *strB*, *sul2*	55	4, 5, 10, 11, 18, 19, 20, 21, 24, 25, 38, 45, 46, 47, 48, 49, 50, 53, 57, 58, 59, 63, 70, 71, 72, 75, 77, 78, 81, 83, 84, 85, 86, 87, 88, 90, 92, 93, 94, 95, 96, 97, 98, 99, 101, 104, 105, 108, 111, 112, 113, 114, 115, 134, 137

Strains containing transposon Tn*5393* are highlighted in green. Strains containing RSF1010 or RSF1010-like plasmids are highlighted in yellow. Strains containing other small plasmids are highlighted in pink. An asterisk indicates strains used for further research in Chapter 4.

3.3.2 Tn*5393* and Tn*5393b* transposon detections

The *strA-strB* genes are normally located on the transposon Tn*5393* (Sundin, 2000). A primer pair smAR/IS1133F located in *strA* and IS*1133* respectively (see Figure 3.5A) was used to screen for the Tn*5393* transposon in 71 *Salmonella* strains, which contained the *strA* or *strB* genes. PCR amplicons of the expected size of 715 bp for 13 strains indicating that they contained Tn*5393* were obtained (Figure 3.10). These 13 strains are highlighted in green in Table 3.3. DNA sequence analysis of the PCR amplicon from strain SRC39 confirmed that it was identical to the fragment of the *strA*-IS*1133* region from *Erwinia amylovora* plasmid pEa34 (accession no. M95402). Among the 13 strains with Tn*5393* transposon, 11 were *S. enterica* serovar Hadar, 1 *S. enterica* serovar Typhimurium, and 1 *S. enterica* serovar Kentucky. For the 11 Hadar strains, 6 strains were isolated from humans and 5 were isolated from ducks. In addition to harbouring Tn*5393*, the *S. enterica* serovar Typhimurium strain SRC27 also contained *sul2*.

As mentioned above (Table 3.3), in this collection there were 19 isolates harbouring linked *strA-strB* without the *sul2* gene, 12 of which were identified to contain the Tn*5393* transposon, but Tn*5393* transposon was not identified in the remaining 7 strains. This result was confirmed by another screening PCR for the Tn*5393* transposon with a different set of primers Tn5393-3/Tn5393-1 (Figure 3.5B). This set of primers was also used to screen for transposon Tn*5393b* within the 71 *Salmonella* strains mentioned above. The PCR amplicon was expected to be 2.1 kb in size and all strains were negative for this PCR.

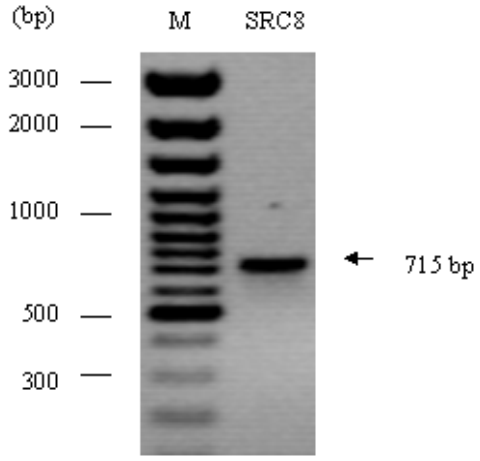


Figure 3.10 Gel electrophoresis of a Tn*5393* PCR amplicon. Strain SRC8 was chosen as a representative for the 715 bp PCR product on a 1.0% agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

3.3.3 Detection of RSF1010 and related plasmids

The screening results using *strA*, *strB*, *strA-strB*, *sul2* and *sul2-strA* PCR confirmed that 47 strains contained *sul2-strA-strB* antibiotic resistance genes in this *Salmonella* collection (Table 3.3). Because the *sul2-strA-strB* genes associated with human and animal sources are often located on small non-conjugative plasmids such as RSF1010 (Sundin and Bender, 1996), a RSF1010 specific PCR amplification (see Figure 3.4) was performed for these 47 strains. PCR amplicons of an expected size of 393 bp were obtained for 7 strains from both human and animal sources (Figure 3.11). These strains

are highlighted in yellow in Table 3.3. DNA sequence analysis of the PCR amplicon from strain SRC15 confirmed that it was identical to the corresponding region of plasmid RSF1010 (accession no. M28829).

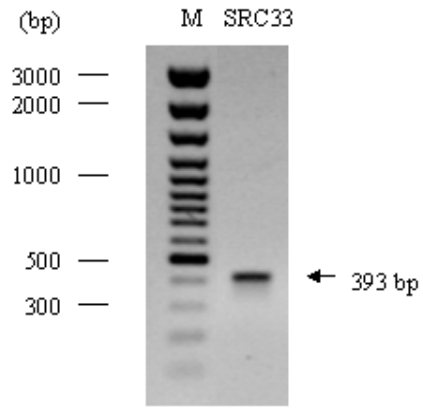


Figure 3.11 Gel electrophoresis of RSF1010 PCR amplicon. Strain SRC33 was chosen as a representative for the 393 bp PCR product on a 1.0% agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

3.3.4 Detection of other small plasmids

Southern hybridisation using a *strB* probe was performed on 47 strains harbouring the *sul2-strA-strB* genes to collect more information about their structures. Enzyme *Bgl*II was used to digest the chromosomal DNA for each strain. The outcome of the *strB* Southern hybridisation did not provide any further information except for confirming the existence of the *strB* gene, because the *Bgl*II fragments were too big to estimate their sizes (data not shown). However through the *strB* Southern hybridisation, another four strains isolated from chickens, including SRC106, SRC107, SRC109 and SRC110 (strains highlighted in pink in Table 3.3) were found to contain the *strB* gene on small plasmids.

3.3.5 Transformation or conjugation of small plasmids

Small plasmid preparations were extracted from 11 strains containing RSF1010 or other small plasmids using the QIAprep Spin Miniprep Kit (Qiagen). The plasmid

preparations from 8 strains, including SRC15, SRC33, SRC55, SRC56, SRC106, SRC107, SRC109 and SRC130, were transformed into *E. coli* DH5α competent cells by electrotransporation. The plasmid preparations from strains SRC35 and SRC110 were transformed into *E. coli* JM109 competent cells (Promega) via chemical transformation, and the plasmid from strain SRC6 was conjugated into *E. coli* 294 Rif^r recipient cells (Table 3.4). The resistance profiles were tested for these transformants and transconjugants by the antibiotic resistance testing method. The results showed that 7 transformants/transconjugants, including those containing pSRC6, pSRC15, pSRC33, pSRC35, pSRC55, pSRC107 and pSRC130, had the same antibiotic resistance profiles as the original *Salmonella* strains. Apart from streptomycin and sulfonamide resistance, plasmid pSRC56 did not harbour other antibiotic resistance genes. Plasmid pSRC106, did not possess ampicillin and tetracycline resistance, while plasmid pSRC109, did not confer tetracycline and trimethoprim resistance. Plasmid pSRC110 did not confer tetracycline resistance (Table 3.4). These antibiotic resistance genes were not associated with these small plasmids, and are most likely located on the chromosome or other plasmids.

Table 3.4 Methods used to transfer the small plasmids from *Salmonella* to *E. coli* competent cells for resistance profiling.

Strain	Serovar	Resistance profile of the original strain	Transfer method	Transformant/ transconjugant resistance profile
SRC15	Typhimurium PT9	SmSu	Electroporated into *E. coli* DH5α	SmSu
SRC33	Typhimurium PT9	SmSu	Electroporated into *E. coli* DH5α	SmSu
SRC130	Typhimurium PT64	SmSu	Electroporated into *E. coli* DH5α	SmSu
SRC56	Typhimurium 104L	ApCmGmKm SmSpSuTcTp	Electroporated into *E. coli* DH5α	SmSu
SRC6	Enteritidis PT1	SmSuTc(NaCp')	Conjugated into *E. coli* 294 Rif^r	SmSuTc
SRC35	Agona	SmSuTc	Transformed into JM109	SmSuTc
SRC55	Stanley	SmSuTc	Electroporated into *E. coli* DH5α	SmSuTc
SRC106	Sofia	ApSmSuTc	Electroporated into *E. coli* DH5α	SmSu
SRC107	Sofia	SmSu	Electroporated into *E. coli* DH5α	SmSu
SRC109	Sofia	SmSuTcTp	Electroporated into *E. coli* DH5α	SmSu
SRC110	Sofia	ApSmSuTc	Transformed into JM109	ApSmSu

3.3.6 Restriction endonuclease analysis of small plasmids

Plasmid preparations from the 11 transformants/transconjugants were extracted (Figure 3.12).

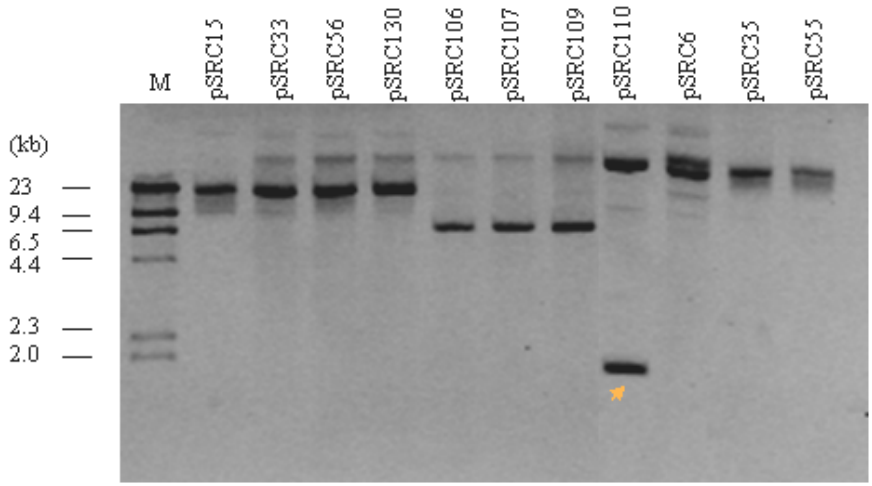


Figure 3.12 Gel electrophoresis of plasmid extracts from 11 transformants/transconjugants. The numbers above the gel represent plasmid numbers. The 1.7 kb plasmid from strain pSRC110 is indicated by an arrow. The bacteriophage lambda DNA digested with *Hin*dIII restriction enzyme (Roche) is labeled M and the corresponding sizes are given on the left.

These plasmids were digested with different restriction enzymes including *Pst*I, *Ssp*I and *Eco*RV. Figure 3.13 shows the restriction digestion map of the RSF1010 plasmid (accession no. M28829) with enzymes *Pst*I, *Ssp*I and *Eco*RV. Figure 3.14 shows the restriction digestion map of the p9123 plasmid (accession no. NC_005324) with enzymes *Pst*I, *Ssp*I and *Eco*RV.

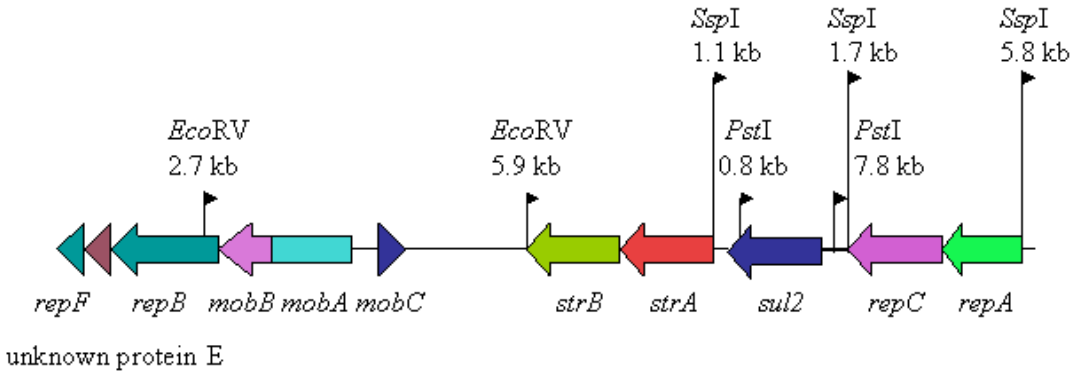


Figure 3.13 The restriction digestion map of RSF1010 plasmid (accession no. M28829)

with enzymes *Pst*I, *Ssp*I and *Eco*RV. The genes and their transcriptional directions are indicated by arrows. When digested with enzyme *Pst*I, two fragments of 0.8 kb and 7.8 kb in sizes are obtained. When digested with *Ssp*I, three fragments of 1.1 kb, 1.7 kb and 5.8 kb in sizes are obtained. When digested with *Eco*RV, two fragments of 2.7 kb and 5.9 kb in sizes are obtained. The restriction sites are indicated by black flags with the enzymes and fragment sizes indicated.

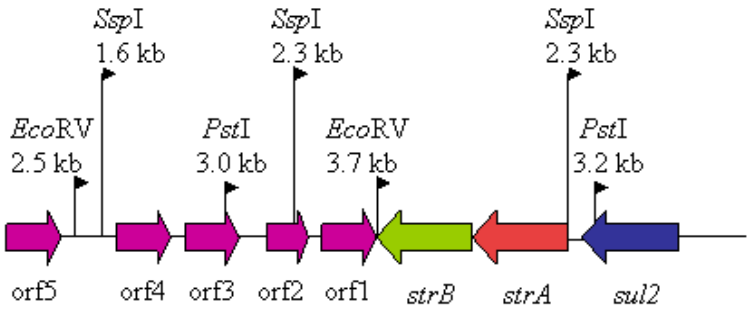


Figure 3.14 The restriction digestion map of p9123 plasmid (accession no. NC_005324) with enzymes *Pst*I, *Ssp*I and *Eco*RV. The genes and the transcriptional directions are indicated by arrows. When digested with enzyme *Pst*I, two fragments of 3.0 kb and 3.2 kb in sizes are obtained. When digested with *Ssp*I, three fragments of 1.6 kb, 2.3 kb and 2.3 kb in sizes are obtained. When digested with *Eco*RV, two fragments of 2.5 kb and 3.7 kb in sizes are obtained. The restriction sites are indicated by black flags with the enzymes and fragment sizes indicated.

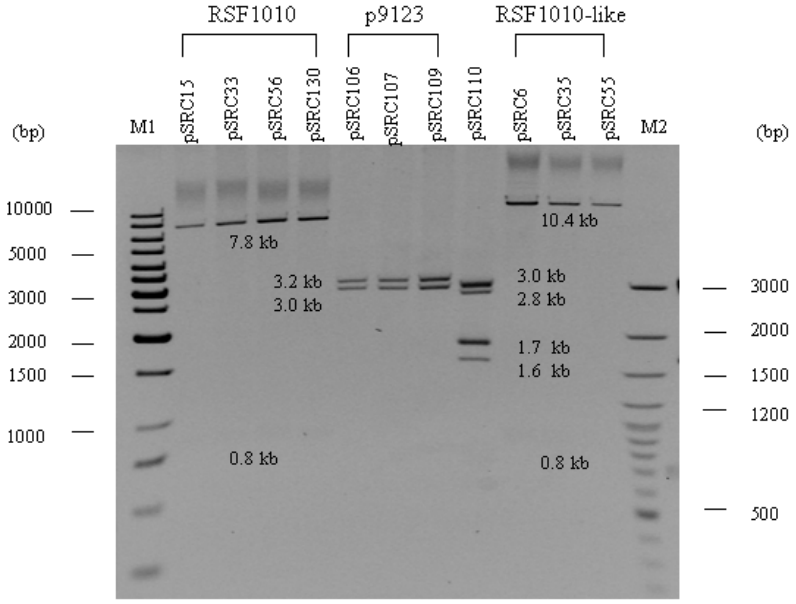
Four strains (SRC15, SRC33, SRC56 and SRC130) that were positive for the RSF1010 PCR, showed further evidence of harbouring RSF1010. When digested with *Pst*I, four plasmids (pSRC15, pSRC33, pSRC56 and pSRC130) displayed a similar digestion profile where a fragment of approximately 7.8 kb was obtained. The 0.8 kb fragment could not be visualised from the gel clearly (Figure 3.15). When digested with *Ssp*I, three fragments of 1.1 kb, 1.7 kb and 5.8 kb were obtained as expected for RSF1010 (Figure 3.16). These 4 plasmids also had the same restriction profile predicted for RSF1010 when digested with enzyme *Eco*RV with two fragments of approximately 2.7 kb and 5.9 kb being observed (Figure 3.17) (Table 3.5). In summary, four *Salmonella* strains including SRC15, SRC33, SRC56 and SRC130 were confirmed to contain RSF1010 based on restriction enzyme digestion profiles.

At approximately 6.2 kb, the plasmids pSRC106, pSRC107 and pSRC109 all have the same size, which is not as large as the RSF1010 plasmid. These plasmids were cut into two fragments of 3.0 kb and 3.2 kb by *Pst*I (Figure 3.15), three fragments measuring 1.6 kb, 2.3 kb and 2.3 kb by *Ssp*I (Figure 3.16), and two fragments of 2.5 kb, 3.7 kb by *Eco*RV (Figure 3.17) (Table 3.5). The enzyme digestion profiles of *Pst*I, *Ssp*I and *Eco*RV of this 6.2 kb plasmid were compared to other small plasmids including p9123 (accession no. NC_005324), pMS260 (accession no. AB10985), and pIE1115 (accession no. AJ293027) digested with the same enzymes using the Gene Construction Kit. The result suggested that the 6.2 kb plasmid was identical or closely related to plasmid p9123. Thus, three *Salmonella* strains SRC106, SRC107 and SRC109 may contain plasmid p9123.

The transformant obtained using the plasmid extracted from SRC110 DNA appeared to contain two small plasmids, one of which is approximately 10 kb and the other estimated to be only 1.7 kb. The 1.7 kb small plasmid did not appear to be digested by *Pst*I, *Ssp*I or *Eco*RV. However, three *Pst*I fragments of 1.6 kb, 2.8 kb and 3.0 kb were obtained for the 10 kb plasmid with the 3.0 kb fragment potentially being a doublet (Figure 3.15). When digested with enzyme *Ssp*I, three fragments of 1.1 kb, 2.3 kb and 4.0 kb were obtained with the 2.3 kb band potentially being a doublet (Figure 3.16). The enzyme *Eco*RV cut this plasmid into three fragments of 2.5 kb, 3.0 kb and 3.7 kb (Figure 3.17) (Table 3.5). The restriction enzyme data suggested the 10 kb plasmid was different from both plasmid p9123 and RSF1010 but shared some similarities.

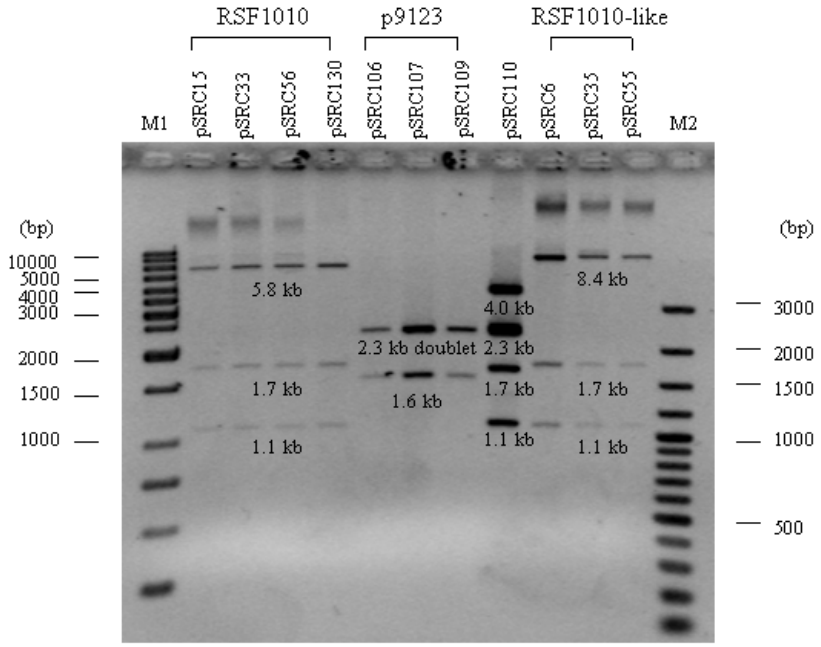
While plasmids pSRC6, pSRC35 and pSRC55 were positive for RSF1010 specific PCR, each plasmid was several kilobases larger than RSF1010. These plasmids were

designated as RSF1010-like plasmids. These 3 plasmids were not only resistant to streptomycin and sulfonamide similar to RSF1010, but were also resistant to tetracycline. These RSF1010-like plasmids were digested with *Pst*I generating a 10.4 kb fragment. A 0.8 kb fragment could not be visualised on the gel (Figure 3.15). When digested with *Ssp*I, the RSF1010-like plasmids were cut into three fragments of 1.1 kb, 1.7 kb and 8.4 kb (Figure 3.16). The RSF1010-like plasmids were digested into 1.8 kb, 3.5 kb and 5.9 kb fragments with *Eco*RV (Figure 3.17) (Table 3.5). The restriction endonuclease analysis results suggest that strains SRC6, SRC35 and SRC55 contain plasmids of approximately 11.2 kb in size, which are similar to RSF1010 plasmid with the addition of a tetracycline resistance gene.



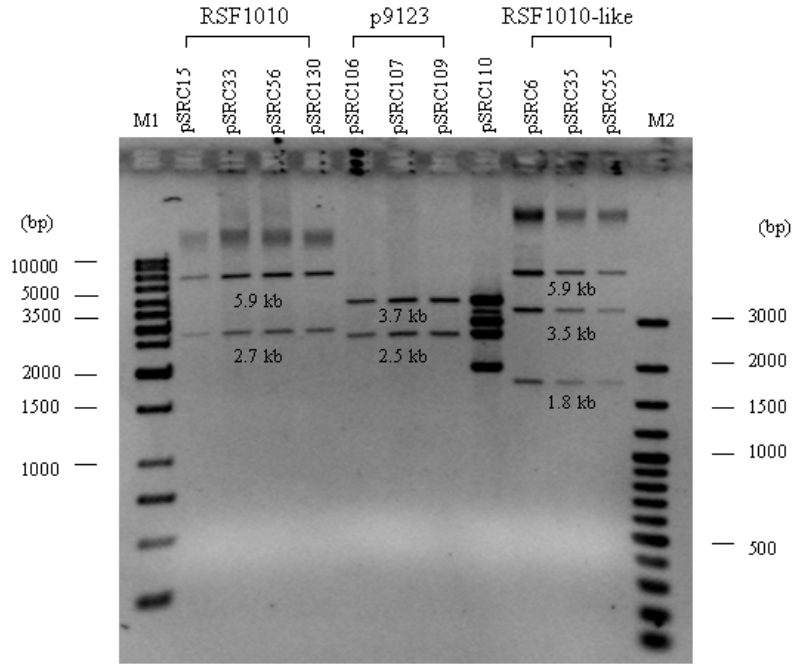
*Pst*I

Figure 3.15 Gel electrophoresis of 11 plasmid extracts digested with enzyme *Pst*I. The numbers above the gel represent plasmid numbers. The size of each digested fragment is labeled in the gel. 1 kb DNA ladders (Fermentas) is labeled M1 and the corresponding sizes are given on the left. 100 bp plus DNA ladders (Fermentas) is labeled M2 and the corresponding sizes are given on the right.



*Ssp*I

Figure 3.16 Gel electrophoresis of 11 plasmid extracts digested with enzyme *Ssp*I. The numbers above the gel represent plasmid numbers. The size of each digested fragment is labeled in the gel. 1 kb and 100 bp plus DNA ladders (Fermentas) are labeled M1 and M2 and the corresponding sizes are given on the left and right of the figure.



*Eco*RV

Figure 3.17 Gel electrophoresis of 11 plasmid extracts digested with enzyme *Eco*RV. The numbers above the gel represent plasmid numbers. The size of each digested fragment is labeled in the gel. M1, 1 kb DNA ladders (Fermentas). M2, 100 bp plus DNA ladders (Fermentas).

Table 3.5 The resistance phenotype, plasmid type and restriction digestion results with enzymes *Ssp*I, *Pst*I and *Eco*RV for 11 small plasmids.

Plasmid number	Resistance phenotype	Plasmid type	Enzyme restriction digestion (kb)		
			*Pst*I	*Ssp*I	*Eco*RV
pSRC15, pSRC33, pSRC56, pSRC130	SmSu	RSF1010	0.8, 7.8	1.1, 1.7, 5.8	2.7, 5.9
pSRC106, pSRC107 pSRC109	SmSu	p9123	3.0, 3.2	1.6, 2.3, 2.3	2.5, 3.7
pSRC110	ApSmSu	not identified	1.6, 2.8, 3.0, 1.7	1.1, 2.3, 4.0, 1.7	2.5, 3.0, 3.7, 1.9
pSRC6, pSRC35, pSRC55	SmSuTc	RSF1010-like	0.8, 10.4	1.1, 1.7, 8.4	1.8, 3.5, 5.9

3.3.7 Detections of *tetA*(A), *tetA*(B), *tetA*(G) genes and transposon Tn*1721*

In this *Salmonella* collection, many strains are resistant to tetracycline. In order to test the collection for the presence of *tetA*(A), *tetA*(B) and *tetA*(G) genes which are known to be prevalent in *Salmonella* strains, a multiplex PCR of *tetA*(A), *tetA*(B) and *tetA*(G) was performed for 136 strains. Three PCR amplicons of 210 bp for the *tetA*(A) gene, 659 bp for *tetA*(B) gene and 844 bp for *tetA*(G) gene were obtained (Figure 3.18). DNA sequence analysis of the *tetA*(A) PCR amplicon derived from strain SRC1 revealed that it was identical to the *tetA*(A) gene from the Gram-negative bacterial transposon Tn*1721* (accession no. X61367). DNA sequence analysis of the *tetA*(B) amplicon derived from strain SRC26 revealed that it was identical to the *tetA*(B) gene from *S. enterica* serovar Typhi (accession no. AY150213). The *tetA*(G) gene is typically associated with SGI1, and this observation was followed up by another PhD student and hence is not presented as part of this study (Levings et al., 2005). The multiplex analysis showed that 50 strains contained the *tetA*(A) gene (36.8%), and 34 strains contained *tetA*(B) (25.0%). One strain harboured both the *tetA*(A) and *tetA*(B) genes. Fifty-one strains contained neither *tetA*(A) nor *tetA*(B) genes (37.5%).

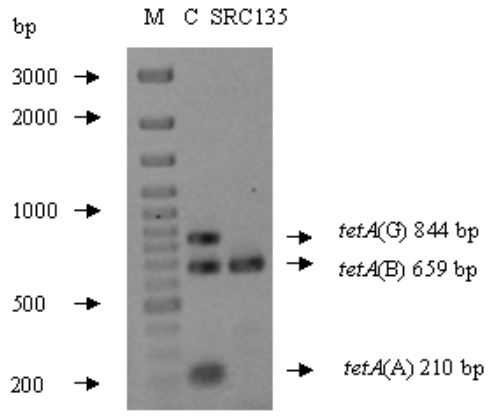


Figure 3.18 Gel electrophoresis of multiplex PCR for *tetA*(A), *tetA*(B) and *tetA*(G) amplicons. +C is a positive control and shows the multiplex PCR amplicons of *tetA*(A), *tetA*(B) and *tetA*(G). SRC135 is used as a representative to show the multiplex PCR amplicon *tetA*(B). 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

In order to identify whether the *tetA*(A) gene was located on the transposon Tn*1721*, a pair of primers TAF/TetAR3 (see Figure 3.6) was used to amplify the *tetA*(A) region of Tn*1721* transposon. A PCR amplicon of 1199 bp was obtained (Figure 3.19). DNA sequence analysis of this PCR amplicon from strain SRC1 confirmed that it was identical to the corresponding fragment on the Gram-negative bacteria transposon Tn*1721* (accession no. X61367). The screening results for *tetA*(A) and transposon Tn*1721* revealed that the *tetA*(A) gene of 47 strains comprised part of the right arm of Tn*1721*. Four strains including SRC6, SRC35, SRC55 and SRC73 contained the *tetA*(A) gene, but no PCR amplicon of the right arm of Tn*1721* was obtained. Further research needs to be undertaken for these four strains. The *tetA*(B) gene is usually located on Tn*10*, which has not screened for in this project.

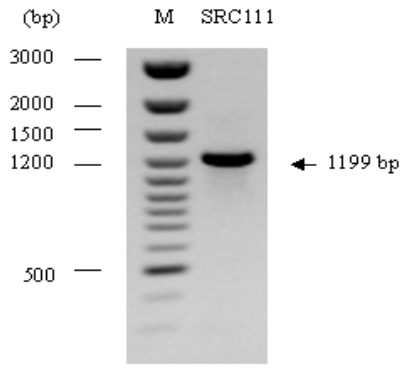


Figure 3.19 Gel electrophoresis of the PCR amplicon of the right arm of transposon Tn*1721*. SRC111 was chosen as a representative for this 1199 bp PCR amplicon (1.0% agarose gel). M, 100 bp plus DNA ladders (Fermentas).

In conclusion, among 136 *Salmonella* strains, 18 strains contained *sul2-strA-strB* and *tetA*(A) genes, 15 strains contained *sul2-strA-strB* and *tetA*(B) genes, 14 strains contained *sul2-strA-strB*. Additionally, 15 strains contained *strA-strB* and *tetA*(A) genes, 11 of which are *S. enterica* serovar Hadar. In total, 25 strains did not contain any of *strA*, *strB*, *sul2*, *tetA*(A) or *tetA*(B) gene (Table 3.6). The PCR screening results of *strA*, *strB*, *sul2*, *strA-strB*, *sul2-strA*, *tetA*(A) and *tetA*(B) for each strain were shown in Appendix II Table A.

Table 3.6 The screening results of antibiotic resistance genes *strA*, *strB*, *sul2*, *strA-strB*, *sul2-strA*, *tetA*(A) and *tetA*(B) among 136 *Salmonella* strains.

Antibiotic resistance cluster type	The strain numbers of each type
sul2-strA-strB, *tetA*(A)*	18
sul2-strA-strB, *tetA*(B)*	15
sul2-strA-strB, no *tetA*(A), no *tetA*(B)*	14
sul2, *strA-strB*, *tetA*(A)	1
strA-strB, *tetA*(A)	15
strA-strB, *tetA*(B)	2
strA-strB, no *tetA*(A), no *tetA*(B)	2
strA, *sul2*, *tetA*(A)	1
strB, *tetA*(A)	3
sul2, *tetA*(A)	1
sul2, no *tetA*(A), no *tetA*(B)	9
tetA(A) only	12
tetA(B) only	17
tetA(A) and *tetA*(B)	1
no *strA*, *strB*, *sul2*, *tetA*(A), *tetA*(B)	25

Asterisk indicates 47 strains containing linked *sul2-strA-strB* genes.

DNA sequence analysis for a representative of each PCR amplicon including *strA-strB*, *sul2*, *sul2-strA*, Tn*5393* (IS*1133*), RSF1010, *tetA*(A), the right arm of Tn*1721* and *tetA*(B), is shown in Table 3.7.

Table 3.7 BlastN sequence search results for sequences of representatives of each PCR amplification and % identity to previously published GenBank sequences. The program used was BLASTN 2.2.18+.

Sequenced strain	Resistance profile	Source	PCR amplicon	Bacteria source (Accession No.)	Identity
SRC13	ApSmSuTcTp	human (F)	*strA-strB*	*S. enterica* serovar Choleraesuis (AB366440)	100%
				S. enterica serovar Paratyphi A (AM412236)	100%
				E. coli (DQ464881)	100%
				S. enterica serovar Typhimurium (AY333434)	100%
				S. enterica serovar Typhi (AL513383)	100%
				uncultured bacteria (AJ851089)	100%
				RSF1010 plasmid (M28829)	99%
SRC136	ApKmSmSu Tp	porcine	*sul2*	*S. enterica* serovar Choleraesuis (AB366440)	100%
				S. enterica serovar Paratyphi A (AM412236)	100%
				E. coli (DQ464881)	100%
				S. enterica serovar Typhimurium (AY333434)	100%
				S. enterica serovar Typhi (AL513383)	100%
				uncultured bacteria (AJ851089)	100%
SRC126	ApSmSuTp	bovine	*sul2-strA*	*S. enterica* serovar Typhimurium (AY333434)	100%
				S. enterica serovar Typhi (AL513383)	100%
				Salmonella plasmid (AJ628353)	100%
				uncultured bacteria (AJ851089)	100%
				S. enterica serovar Paratyphi A (AM412236)	100%
				RSF1010 plasmid (M28829)	99%
SRC39	SmTc(NaCp')	human (M) Indo	IS*1133*	*S. enterica* serovar Heidelberg SL476 (CP001120)	100%
				S. enterica serovar Kentucky plasmid pCVM29188_146 (CP001122)	100%
				Pseudomonas aeruginosa PA7 (CP000744)	100%
				S. enterica serovar Paratyphi A plasmid IncH1 (AM412236)	100%
				Erwinia amylovora plasmid pEa34, transposon Tn*5393* (M95402)	100%
SRC15	SmSu	human (F)	RSF1010	Expression vector pQLICE (EF189157)	100%
				Plasmid RSF1010 (M28829)	100%
				Cloning vector pFL122 (AY785149)	100%
SRC1	CmSmSpSu TcTp (Na)	human (F)	*tetA*(A)	*S. enterica* serovar Schwarzengrund (CP001125)	100%
				E. coli (CP000971)	100%
				S. enterica serovar Dublin (AB366441)	100%
				S. enterica serovar Typhimurium (AY333434)	100%
				Gram-negative bacteria transposon Tn*1721* (X61367)	100%
SRC1	CmSmSpSu TcTp (Na)	human (F)	Tn*1721*	*S. enterica* serovar Schwarzengrund (CP001125)	100%
				E. coli (CP000971)	100%
				S. enterica serovar Dublin (AB366441)	100%
				S. enterica serovar Typhimurium (AY333434)	100%
				Gram-negative bacteria transposon Tn*1721* (X61367)	100%
SRC26	ApKmSmSu TcTp	bovine	*tetA*(B)	*Yersinia ruckeri* (CP000602)	100%
				E. coli (DQ364638)	100%
				S. enterica serovar Typhi (AY150213)	100%
				Shigella flexneri (AF162223)	100%
				Actinobacillus pleuropneumoniae (DQ176855)	100%

3.4 Discussion

The routine use of antibiotics in medicine and agriculture circles has resulted in widespread antibiotic resistance (Suh and Odeh, 2008). Increases in the occurrence of resistance in non-typhoid *Salmonella* spp. in developed and developing countries has been reported (Threlfall, 2002). Resistance to streptomycin, sulfonamide and tetracycline is very common in *Salmonella* strains (Agustin et al., 2005).

Sulfonamides were the first drugs found to suppress bacterial infections. However resistance soon emerged (Radstrom et al., 1991). The main reason for the development of this resistance in Gram-negative bacteria was the establishment of sulfonamide resistance determinants on R plasmids (Radstrom et al., 1991). The first plasmids containing *sul2* were recognized in a collection of *S. enterica* serovar Typhimurium strains in the 1960's (Lawn et al., 1967). Thus, the intensive use of sulfonamides since their introduction in the 1930's probably drove the evolution of R plasmids carrying this resistance (Radstrom et al., 1991). The common region IS*CR2* was found closely associated with the sulfonamide resistance gene *sul2* on the non-conjugative plasmid RSF1010 (Toleman et al., 2006). Common regions were first discovered and reported in the early 1990's as a DNA sequence found in two complex class I integrons, In6 and In7. Because the same sequence was commonly found, it was named as the common region (CR) (Stokes et al., 1993). The original CR sequence in In6 and In7 was designated as IS*CR1*. So far, more than a dozen CRs have been discovered and designated as IS*CR1*, IS*CR2*, IS*CR3* and so on (Toleman et al., 2006). IS*CRs* use a rolling circle (RC) transposition mechanism, which includes RC replicaton and homologous recombination to achieve transposition (Tavakoli et al., 2000). Apart from the *sul2* gene, the *sul1* gene confers sulfonamide resistance and is normally found

associated with class 1 integrons. Clonal expansion and horizontal gene transfer have contributed to the spread of antibiotic resistance class 1 integrons among bacterial species (Krauland et al., 2009). The *sul3* gene has been reported in recent years and also confers sulfonamide resistance (Perreten and Boerlin, 2003).

In this study, 62.5% (85/136) of strains were resistant to tetracycline because these strains contained *tetA*(A) or *tetA*(B) genes. These findings may not be surprising, as these antimicrobials have been widely used in modern animal production systems in many countries (Antunes et al., 2006; Gebreyes et al., 2004; Mazel et al., 2000). Resistant bacteria from food animals and other sources may be passed through the food chain to humans resulting in antibiotic resistant infections (Anderson et al., 2003).

In this study, 11 strains from *S. enterica* serovar Hadar were found to contain transposon Tn*5393*, six strains being isolated from humans, with the other 5 strains from ducks. Transposon Tn*5393* was first identified in the plant pathogen *Erwinia amylovora* (Sundin and Bender, 1996). Subsequently, Tn*5393* transposons have been found in some isolates of different *S. enterica* serovars, such as Hadar and Agona from animal sources (Pezzella et al., 2004). The investigation conducted in this research has provided the first report of Tn*5393* to be found in *S. enterica* serovar Hadar strains sourced from humans. The identification of Tn*5393* in *Salmonella* suggests novel scenarios of resistance transmission among zoonotic and plant pathogens (Miriagou et al., 2006). It may be hypothesised that *Salmonella* has imported this genetic element from plant pathogens possibly via the contamination of animal feeds. As tetracycline and streptomycin are common antibiotics administered in veterinary medicine, their frequent use may have contributed to the spread of these genetic determinants in zoonotic

pathogens (Miriagou et al., 2006). Through handling or consuming contaminated food products, this genetic element was then passed to humans.

Plasmid RSF1010 is very common and has been detected in 14 genera of human- and animal-associated bacteria (Palmer et al., 1997). Four *Salmonella* strains from human and animals were identified to contain this plasmid based on restriction enzyme analysis. DNA sequence analysis of the *strA-strB* PCR amplicon from strain SRC13 was 99% identical to that of RSF1010 plasmid (accession no. M28829), but it was identical to the *strA-strB* region on plasmids from *S. enterica* serovar Paratyphi A (accession no. AM412236), *S. enterica* serovar Typhimurium (accession no. AY333434), *S. enterica* serovar Typhi (accession no. AL513383) and an uncultured bacterium (accession no. AJ851089) (Table 3.7).

The primer pair RSF1010-F/RSF1010-R used in this research is specific for plasmid RSF1010 from *E. coli* (accession no. M28829). Recently, the sequence of another plasmid, pCCK1900 from *Pasteurella multocida* has been deposited into GenBank and the region targeted by these primers is present in this plasmid as well (Kehrenberg et al., 2008). Plasmid pCCK1900, which was isolated from a swine in German, is 10.2 kb in size. Like RSF1010, this plasmid contains the linked *sul2-strA-strB* genes, the replication genes *repA*, *repB* and *repC*, and the mobilization genes *mobA*, *mobB* and *mobC*. Different from RSF1010, a 2325 bp region was detected downstream of the *strB* gene, which included a chloramphenicol-florfenicol resistance gene *floR* and a putative transcriptional regulator gene (Figure 3.20) (Kehrenberg et al., 2008). This region has also been identified on the multi-resistance plasmid pAB5S9 from *Aeromonas bestiarum*, the plasmids from *E. coli* contain genes conferring three amino acids

differences in the FloR proteins (Gordon et al., 2008; Cloeckaert et al., 2000; Blickwede and Schwarz, 2004). It has been assumed that the truncated IS*CR2* element downstream of the *strB* gene has served for the integration of the *floR* gene area into the RSF1010-related plasmid pCCK1900 (Kehrenberg et al., 2008).

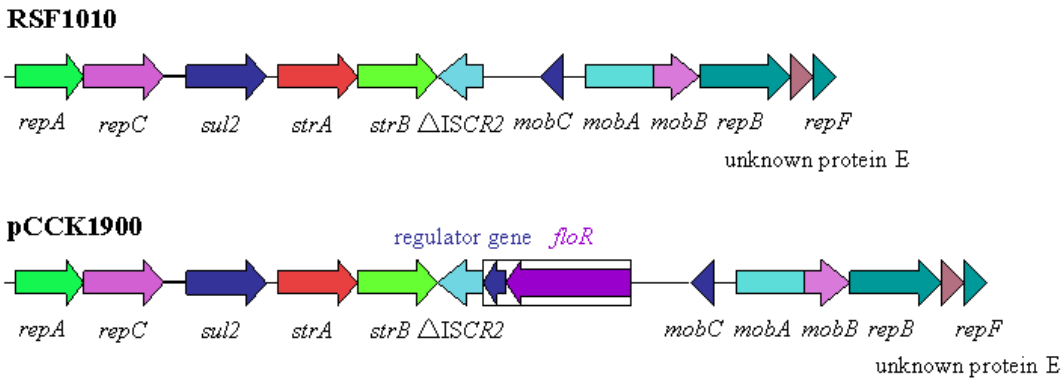


Figure 3.20 The structures of RSF1010 and pCCK1900. Genes and their transcriptional directions are indicated by arrows. The black box on plasmid pCCK1900 highlights the difference between two plasmids (adapted from Kehrenberg et al., 2008).

In this study, three RSF1010-like plasmids were identified by the RSF1010-F/RSF1010-R primer pair as well. The RSF1010-like plasmids are similar to RSF1010 since they shared common restriction fragments but harbour an extra tetracycline resistance gene. Comparing the *Eco*RV restriction profiles of RSF1010 and the RSF1010-like plasmids, the results suggest that the RSF1010-like plasmids may have evolved from RSF1010 via the insertion of a tetracycline resistance gene *tetA*(A) upstream or downstream of the *mobC* gene. Because the region between *mobC* and *strB* is a hot spot for other insertion and deletion events (Kehrenberg et al., 2008), it is possible that *tetA*(A) genes were inserted between the *mobC* and *strB* genes. IS*CR2* element might have served for the integration of the *tetA*(A) gene area into the RSF1010-like plasmid, possibly by homologous recombination.

Three strains were identified to contain plasmid p9123 or a close derivative of it, which was first isolated by Enne et al. (2004) from *E. coli* strains in East London. Plasmid p9123 has a close relationship to pBP1, a plasmid prevalent among *E. coli* which was described in the 1970's and 1980's (Enne et al., 2004). Three strains harbouring p9123 were isolated from chickens in Australia in 2000. The presence of plasmid p9123 may improve the inherent fitness of the host strain via an increase in growth rate conferred by carriage of this plasmid (Enne et al., 2004). This character may explain why this plasmid can exist and spread in enteric bacteria (Enne et al., 2004). There were two small plasmids found in the SRC110 strain. Additional investigations are needed to fully describe the plasmid contents of this strain.

Electroporation, transformation and conjugation methods were used to transfer small plasmids from these 11 *Salmonella* strains to *E. coli* cells. Because RSF1010 and p9123 plasmids are non-conjugative small plasmids, electroporation was the method used to transfer these plasmids to *E. coli* strains. In this project, small plasmids from 8 strains including 4 strains containing RSF1010, 3 strains containing p9123 and 1 strain containing a RSF1010-like plasmid were transferred to *E. coli* DH5α cells. The plasmids of another three strains could not be transferred by electroporation. Thus, a chemical transformation method was attempted for three plasmids and two were transformed into *E. coli* JM109 cells (Promega). The last plasmid was transferred into *E. coli* 294 Rif^r cells by conjugation.

It was suggested by Sundin (2002) that Tn*5393* is the ancestor of plasmid RSF1010. The possible relationship between Tn*5393* and RSF1010 is described as the following (Figure 3.21): (1) evolution of the Tn*5393* transposon. The *strA* and *strB* genes are

usually found within transposon Tn*5393* and its IS-free parent Tn*5393c* (Chiou and Jones, 1993; Sundin and Bender, 1996; L'Abee-Lund and Sorum, 2000). (2) transposition of Tn*5393* into the CR2-*sul2* configuration. As noted previously, RSF1010 contains a continuous 1778 bp segment of Tn*5393* that includes *strA-strB* and the adjacent inverted repeat (IRR) (L'Abee-Lund and Sorum, 2000). However, the *sul2* gene is not in its original context. When gene *sul2* occurs alone, it is found adjacent to the *ori* end of the small mobile element CR2 (Partridge and Hall, 2003). In RSF1010, the Tn*5393* fragment separates the CR2 remnant from the *sul2* gene and 1.6 kb from the centre of the CR2-*sul2* region is missing. This configuration suggests that the resistance gene region in RSF1010 arose via transposition of Tn*5393* into CR2 in the CR2-*sul2* configuration followed by a second event that removed part of Tn*5393* and part of CR2 or via a one-ended transposition event initiated at IRR of Tn*5393* and resolved by another event (Yau et al., 2009). (3) evolution of the non-conjugative lines such as RSF1010 via sequence divergence of the *strA* gene and other genetic rearrangements; (4) widespread transfer of Tn*5393* among environmental bacteria, acquisition of IS elements as necessary to express the *strA-strB* genes, upstream genetic rearrangements of the *strA* gene and further sequence divergence (Sundin, 2002; Radstrom and Swedberg, 1988). The wide distributions of Tn*5393* and non-conjugative broad-host-range plasmids such as RSF1010 have contributed to the prevalence of *strA-strB* genes (Sundin, 2002; Toleman et al., 2006).

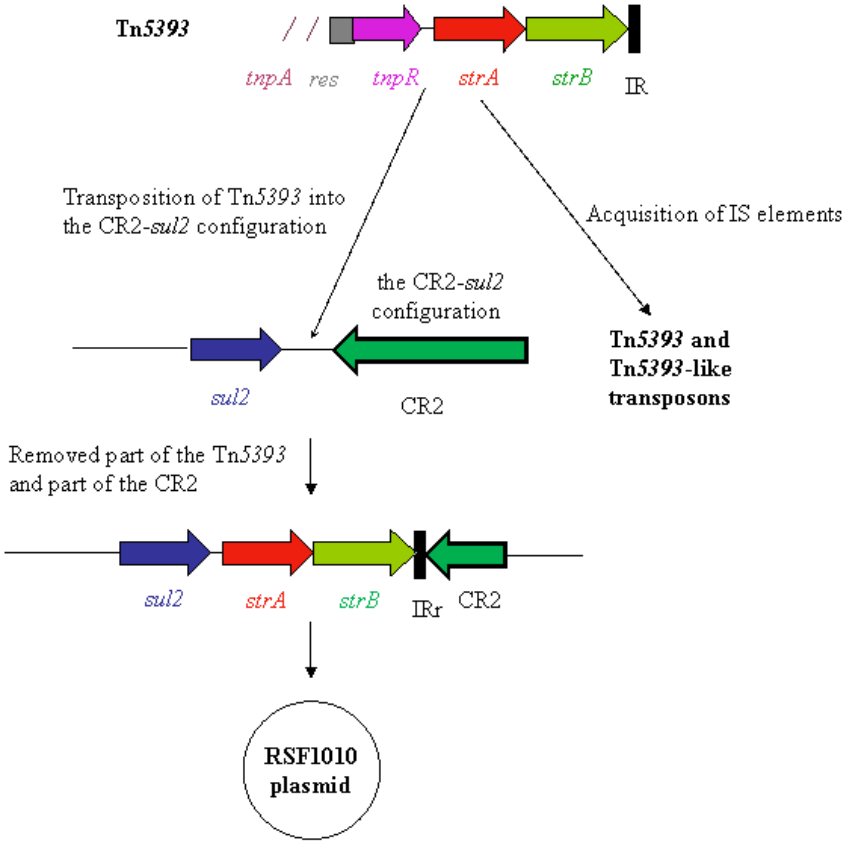


Figure 3.21 The possible relationship between transposon Tn*5393* and plasmid RSF1010. Genes and their transcriptional directions are indicated by arrows. IRt, inverted repeat of Tn*5393* transposon. IRr, inverted repeat of RSF1010 (modified from Sundin, 2002; Yau et al., 2009).

More recently, the assembly of repertoires of resistance genes as distinct clusters or antimicrobial resistance islands is now regarded as an efficient mean by which the dissemination of these markers can occur *en bloc*. Mobile genetic elements together with various genetic recombination mechanisms facilitate this process (Miriagou et al., 2006). Various antibiotic resistance gene clusters have been reported in *Salmonella* (Miriagou et al., 2006). The best known is the *Salmonella* Genomic Island 1 (SGI1) which maps to the chromosome of *S. enterica* serovar Typhimurium DT104 (Randall et al., 2004). Recently Daly et al. (2005) described two 10 kb regions on the chromosome of *S. enterica* serovar Typhimurium phage type DT193 and on the IncI plasmid of *S. enterica* serovar Enteritidis, which conferred resistance to multiple antibiotics. Due to

the formation of these antibiotic resistance clusters, selection for one antibiotic generally means selection of all the genes in the cluster. IS*26* is widely found among plasmids and may be implicated in the acquisition of resistance genes (Doublet et al., 2008).

In summary, 47 strains contained the linked *sul2-strA-strB* genes with 11 strains confirmed as harbouring plasmids including RSF1010, p9123 and RSF1010-like. The remaining 36 strains (strains marked with an asterisk in Table 3.3) were chosen for further investigation which is described in Chapter 4.

Chapter 4: Characterisation of the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* cluster among 36 *Salmonella* strains

4.1 Introduction

In Ireland, the *sul2*-*strA*-*strB* antibiotic resistance region was identified on the chromosome of an *S. enterica* serovar Typhimurium phage type DT193 strain. It was also identified in Italy on an IncI plasmid of *S. enterica* serovar Enteritidis cultured in 1997 (Daly et al., 2005). Both strains harboured a similar 10 kb antibiotic resistance region containing *strB*-*strA*-*sul2*-*repC*-*repA* resembling that of RSF1010 plasmid (accession no. M28829). On the chromosome of *S. enterica* Typhimurium DT193, this region was located downstream of an IP-type 2 class 1 integron, which carries *dfrA1* and *aadA1* gene cassettes and confers antibiotic resistance to streptomycin and spectinomycin. At the other end, an IS*26* element was present downstream of the *repA* gene preceded by a *tnpB* and a $bla_{\text{TEM-1}}$ gene, which was part of the Tn*1* transposon and conferred resistance to β-lactam (Figure 4.1) (Daly et al., 2005). On the 80 kb conjugative IncI plasmid of *S. enterica* serovar Enteritidis, the *strB*-*strA*-*sul2*-*repC*-*repA* region was located downstream of an inverted IP-type 2 class 1 integron. Between the antibiotic resistance region and the inverted class 1 integron, an IS*26* element was inserted, which possibly deleted the 3′-CS of the inverted IP-type 2 class 1 integron. Downstream of the antibiotic resistance gene region, an IS*26* element was also present, preceded by a putative recombinase *rec*-like and tetracycline resistance genes *tetR* and *tetA*(A). The *repA* gene was possibly truncated by the insertion of the IS*26* element downstream. The two IS*26* elements on the IncI plasmid were in the inverted orientation (Figure 4.1) (Daly et al., 2005). The *strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* antibiotic resistance gene region on the *S. enterica* serovar Enteritidis IncI plasmid and

on the chromosome of *S. enterica* serovar Typhimurium DT193 showed a high degree of similarity, which suggested that these strains acquired this region via a common route (Daly et al., 2005). The aim of this chapter was to identify whether the antibiotic resistance gene cluster IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* was present in the *Salmonella* collection under investigation.

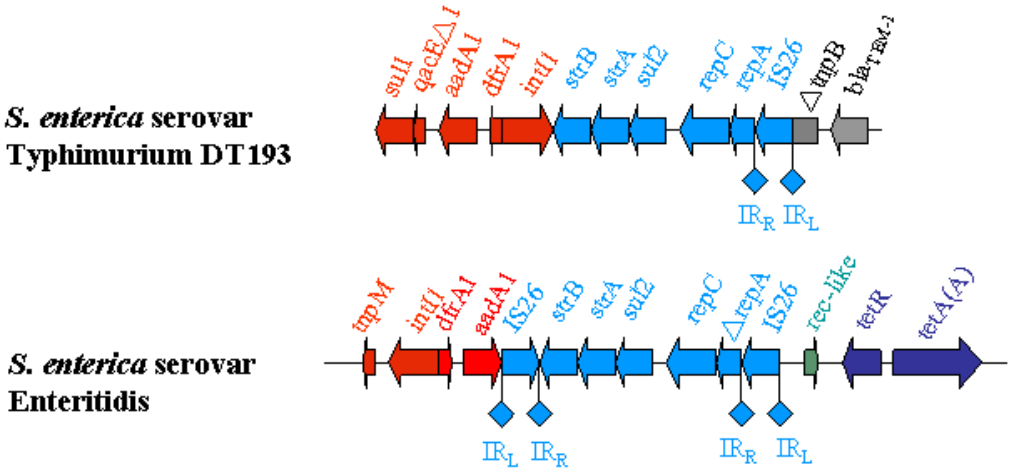


Figure 4.1 The consensus map of the *strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* antibiotic resistance region (blue color) on the chromosome of *S. enterica* serovar Typhimurium DT193 (accession no. AY524415) and on the *S. enterica* serovar Enteritidis IncI plasmid (accession no. AJ628353). Genes and their transcriptional directions are indicated by arrows. IR_L and IR_R, indicate the left and right inverted repeats of IS*26* (adapted from Daly et al., 2005).

4.2 Materials and Methods

4.2.1 PCR amplification of IS*26*-*strB*, *sul2*-*repC* and *repC*-IS*26* regions

The primers used to amplify the IS*26*-*strB* region (JL-D2/strB-F), the *sul2*-*repC* region (sulII-R/JL-D3) and the *repC*-IS*26* region (JL-D1/JL-D2) are indicated in Figure 4.2 and all the primer sequences are listed in Table 4.1. The program used for the amplification was: 94°C for 5 min, followed by 35 cycles 94°C for 30 sec, variable annealing temperature for 1 min, 72°C for 1 min, and finally 72°C for 10 min. The annealing temperature for the IS*26*-*strB* region was 56°C, and for the *sul2*-*repC* and *repC*-IS*26* regions was 57°C. The amplification of the IS*26*-*strB* region was performed

using a thermocycler FTS-960 (Corbett Research), while the amplifications of the *sul2-repC* and *repC*-IS*26* regions were performed using a thermocycler PC-960 (Corbett Research).

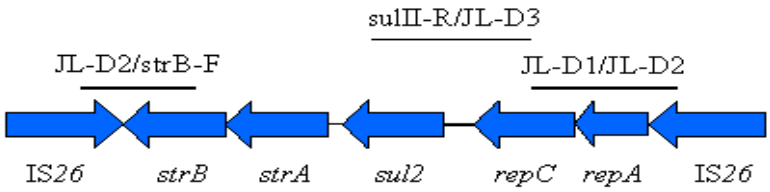


Figure 4.2 The primer positions used to amplify the IS*26-strB* region (JL-D2/strB-F), *sul2-repC* region (sulII-R/JL-D3) and *repC*-IS*26* region (JL-D1/JL-D2). The map is drawn according to the *S. enterica* serovar Enteritidis IncI plasmid (accession no. AJ628353) with a complete *repA* gene. Genes and transcriptional directions are indicated by arrows. The PCR products are shown as lines with the primers indicated.

Table 4.1 The primers used for the IS*26-strB* region (JL-D2/strB-F), *sul2-repC* region (sulII-R/JL-D3) and *repC*-IS*26* region (JL-D1/JL-D2) PCR amplifications.

Primer	Primer sequence (5′-3′)	Location	Nucleotide positions	Accession no.	Reference
strB-F	ATCGTCAAGGGATTGAAACC	*strB*	5877-5896	M95402	Gebreyes and Altier, 2002
sulII-R	ATG CCG GGA TCA AGG ACA AG	*sul2*	8401-8382	M28829	Leverstein-van Hall et al., 2002
JL-D1	AGT CCT GTA TGT GCT TGA GCG	*repC*	40802-40822	AJ851089	this study
JL-D2	AAG GTA TTG AGG TGA TGC G	IS*26*	35512-35530	AJ851089	this study
JL-D3	CGC TCA AGC ACA TAC AGG ACT	*repC*	40822-40802	AJ851089	this study

4.2.2 Restriction endonuclease analysis of the three linkage PCR amplicons

Enzymes *Not*I, *Ssp*I and *Rsa*I were used to digest the three linkage PCR amplicons of IS*26-strB*, *sul2-repC* and *repC*-IS*26*. The digested DNA fragments were analysed by a 2.0% (w/v) agarose gel as described in Chapter 2 (section 2.5).

4.2.3 DNA sequence analysis of the three linkage PCR amplicons

Three linkage PCR amplicons of IS*26-strB*, *sul2-repC* and *repC*-IS*26* were sequenced on both strands according to the method described in Chapter 2 (section 2.8).

4.3 Results

4.3.1 The IS*26-strB* linkage PCR amplification

According to the gene map described by Daly et al. (2005), three pairs of primers including JL-D2/strB-F for the IS*26-strB* region, sulII-R/JL-D3 for *sul2-repC* region, and JL-D1/JL-D2 for *repC*-IS*26* region were designed for 36 *Salmonella* strains, which contained linked *sul2-strA-strB* genes (see Chapter 3). For the IS*26-strB* linkage PCR, an amplicon was obtained with an expected size of 890 bp (Figure 4.3A). The amplicon from strain SRC13 was sequenced as a representative. DNA sequence analysis showed that it was identical to the fragment described for *S. enterica* serovar Choleraesuis (accession no. AB366440), *S. enterica* serovar Paratyphi A (accession no. AM412236), *E. coli* (accession no. DQ464881), *S. enterica* serovar Typhimurium (accession no. AY333434), *S. enterica* serovar Typhi (accession no. AL513383) and an uncultured bacterium (accession no. AJ851089).

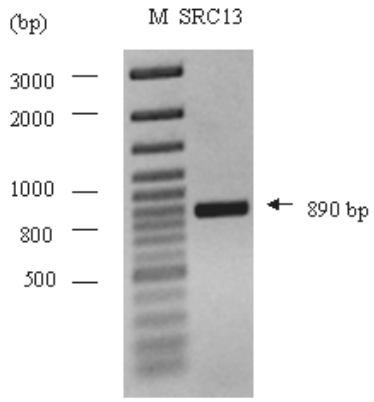


Figure 4.3A Gel electrophoresis of the IS*26-strB* linkage PCR amplicon. Strain SRC13 was chosen as a representative for this 890 bp PCR amplicon (1.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

In total, 33 of the 36 strains within the collection were positive for this PCR (Table 4.2). All the PCR amplicons were further characterised by digestion with restriction enzyme *Not*I. Two fragments were obtained with expected sizes of 690 bp and 200 bp and all the

PCR amplicons had the same RFLP profile (Figure 4.3B). Three strains including SRC1, SRC51 and SRC116 were negative for this PCR amplification.

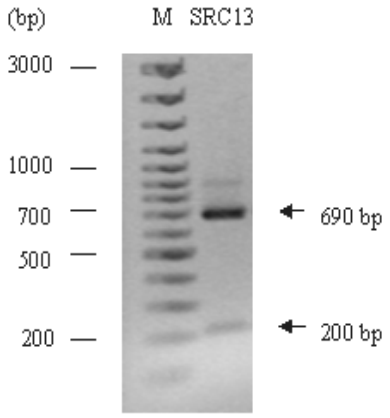


Figure 4.3B Gel electrophoresis of the IS*26-strB* PCR amplicon digested with *Not*I. Two fragments of 690 bp and 200 bp were obtained. Strain SRC13 is displayed as a representative on a 2.0% agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

4.3.2 The *sul2-repC* linkage PCR amplification

For the *sul2-repC* linkage PCR, an amplicon with a predicted size of 1298 bp was obtained (Figure 4.4A). Among the 36 *Salmonella* strains, 34 strains were positive for this linkage PCR (Table 4.2). The PCR amplicon from strain SRC26 was sequenced as a representative. DNA sequence analysis revealed that this fragment was identical to the *sul2-repC* region from *S. enterica* serovar Paratyphi A (accession no. AM412236), *S. enterica* serovar Typhimurium (accession no. AY333434), *S. enterica* serovar Typhi (accession no. AL513383), *S. enterica* serovar Enteritidis IncI plasmid (accession no. AJ628353), *E. coli* (accession no. DQ464881) and an uncultured bacterium (accession no. AJ851089).

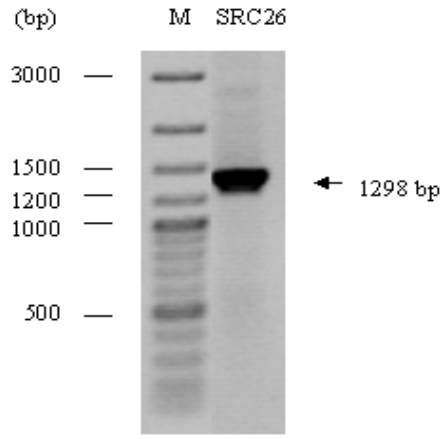


Figure 4.4A Gel electrophoresis of the *sul2-repC* linkage PCR amplicon. SRC26 was chosen as a representative for this PCR amplicon which was 1298 bp in size on a 1.0% agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

All PCR amplicons were characterised by *Ssp*I restriction enzyme digestion. Two fragments were obtained with the predicted sizes of 724 bp and 574 bp and all the PCR amplicons gave the same RFLP profile (Figure 4.4B). Among the 36 strains, 33 were positive for both the IS*26-strB* and *sul2-repC* linkage PCR, while two strains SRC1 and SRC51 were negative for both. Strain SRC116 was positive for *sul2-repC*, but negative for the IS*26-strB* linkage PCR amplification.

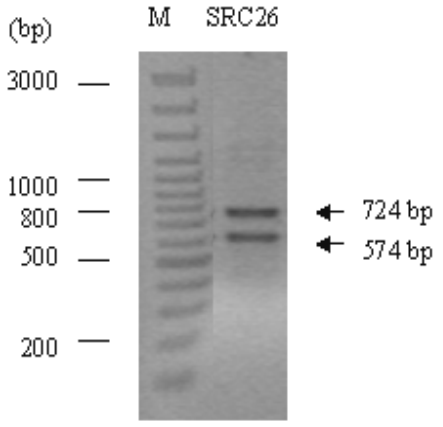


Figure 4.4B Restriction endonuclease analysis of the *sul2-repC* linkage PCR amplicon digested with *Ssp*I. SRC26 was chosen as a representative to show the digested fragments of 724 bp and 574 bp visualized on a 2.0% agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

4.3.3 The *repC*-IS*26* linkage PCR amplification

Three variable sized PCR amplicons were obtained for the *repC*-IS*26* linkage PCR with sizes of 882 bp, 1123 bp and 1274 bp respectively (Figure 4.5A). For the 36 *Salmonella* strains, 34 strains produced an amplicon for this PCR. Of these, 27 gave the larger 1274 bp product, 6 were positive for the smallest 882 bp amplicon and only one was positive for the 1123 bp product. Two strains SRC1 and SRC51 were negative for this PCR (Table 4.2).

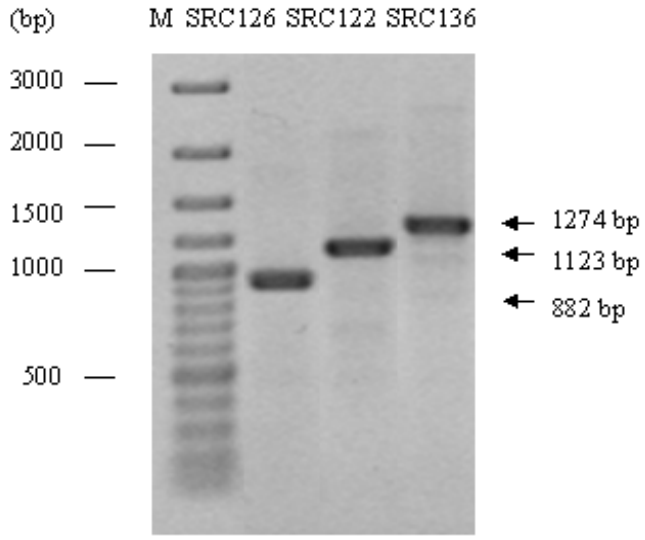


Figure 4.5A Gel electrophoresis of the three different sized amplicons for the *repC*-IS*26* linkage PCR. Strains SRC126, SRC122 and SRC136 were chosen as representatives for each amplicon on a 1.0% agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

All the PCR amplicons were further confirmed and characterised by digestion with restriction enzyme *Rsa*I. As expected, three different RFLP profiles were obtained. The enzyme *Rsa*I was used for the 1274 bp product generating two fragments of 636 bp and 638 bp. The sizes of these two fragments were too close to be differentiated upon agarose gel electrophoresis. The 882 bp amplicon produced two fragments of 636 bp and 246 bp when digested with *Rsa*I. The 1123 bp amplicon was digested into two fragments of 636 bp and 487 bp (Figure 4.5B). The digested fragment of 636 bp was

common for three different sized PCR amplicons, which suggested that they shared a common region.

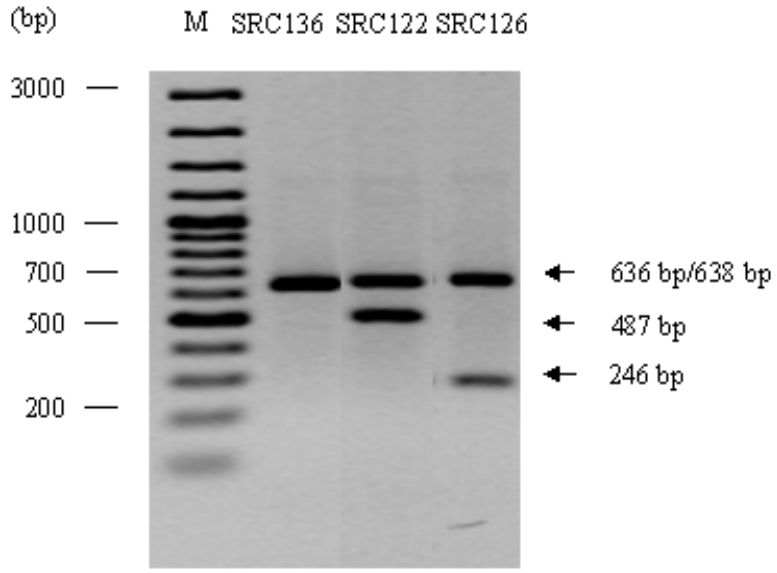


Figure 4.5B Gel electrophoresis of *Rsa*I digested *repC*-IS*26* linkage PCR amplicons. Strain SRC136 was chosen as a representative to show the 636 bp and 638 bp digested fragments for the 1274 bp amplicon, strain SRC122 was used to show the 636 bp and 487 bp digested fragments for the 1123 bp amplicon, and SRC126 was chosen as a representative to show the 636 bp and 246 bp digested fragments for the 882 bp amplicon (2.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

The variable sized *repC*-IS*26* PCR amplicons from three strains were sequenced as representatives. DNA sequence analysis of the amplicon from strain SRC28 revealed that the *repC*-IS*26* region of 1274 bp was identical to the corresponding region from *S. enterica* serovar Paratyphi A (accession no. AM412236), *S. enterica* serovar Typhimurium (accession no. AY333434), *S. enterica* serovar Typhi (accession no. AL513383) and an uncultured bacterium (accession no. AJ851089). When compared to the *repC*-IS*26* fragment from *S. enterica* serovar Typhimurium DT193 (accession no. AY524415), the identity was 99%.

The three different structures all contained the same *repC* and IS*26* genes, the only

difference was in the length of the *repA* gene. When compared to the sequence of *S. enterica* serovar Typhimurium DT193 (accession no. AY524415), the *repC*-IS*26* region of 1123 bp lacks 151 bp of the *repA* gene, while the *repC*-IS*26* region of 882 bp lacks 392 bp of the *repA* gene. Figure 4.6 displays the three different structures of *repC*-IS*26* identified in this study, which were digested with restriction enzyme *Rsa*I. Figure 4.7 shows the alignment of *repC*-IS*26* fragments from *S. enterica* serovar Typhimurium DT193 (accession no. AY524415), 1274 bp PCR amplicon from strain SRC28, *S. enterica* serovar Enteritidis IncI plasmid (accession no. AJ628353), which contains a different deletion on *repA* gene, 1123 bp PCR amplicon from strain SRC122 and 882 bp PCR amplicon from strain SRC126.

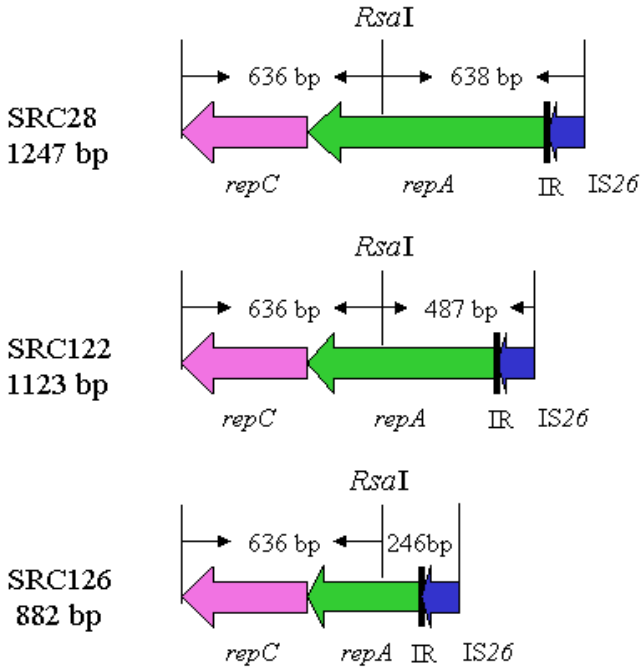


Figure 4.6 The structures of three *repC*-IS*26* linkage PCR amplicons digested with restriction enzyme *Rsa*I. The amplicon of 1274 bp was represented by strain SRC28, the 1123 bp amplicon was shown by strain SRC122, and the 882 bp amplicon was represented by strain SRC126. Genes and their transcription directions are indicated by arrows. The black bar indicates the inverted repeat of the IS*26* element.

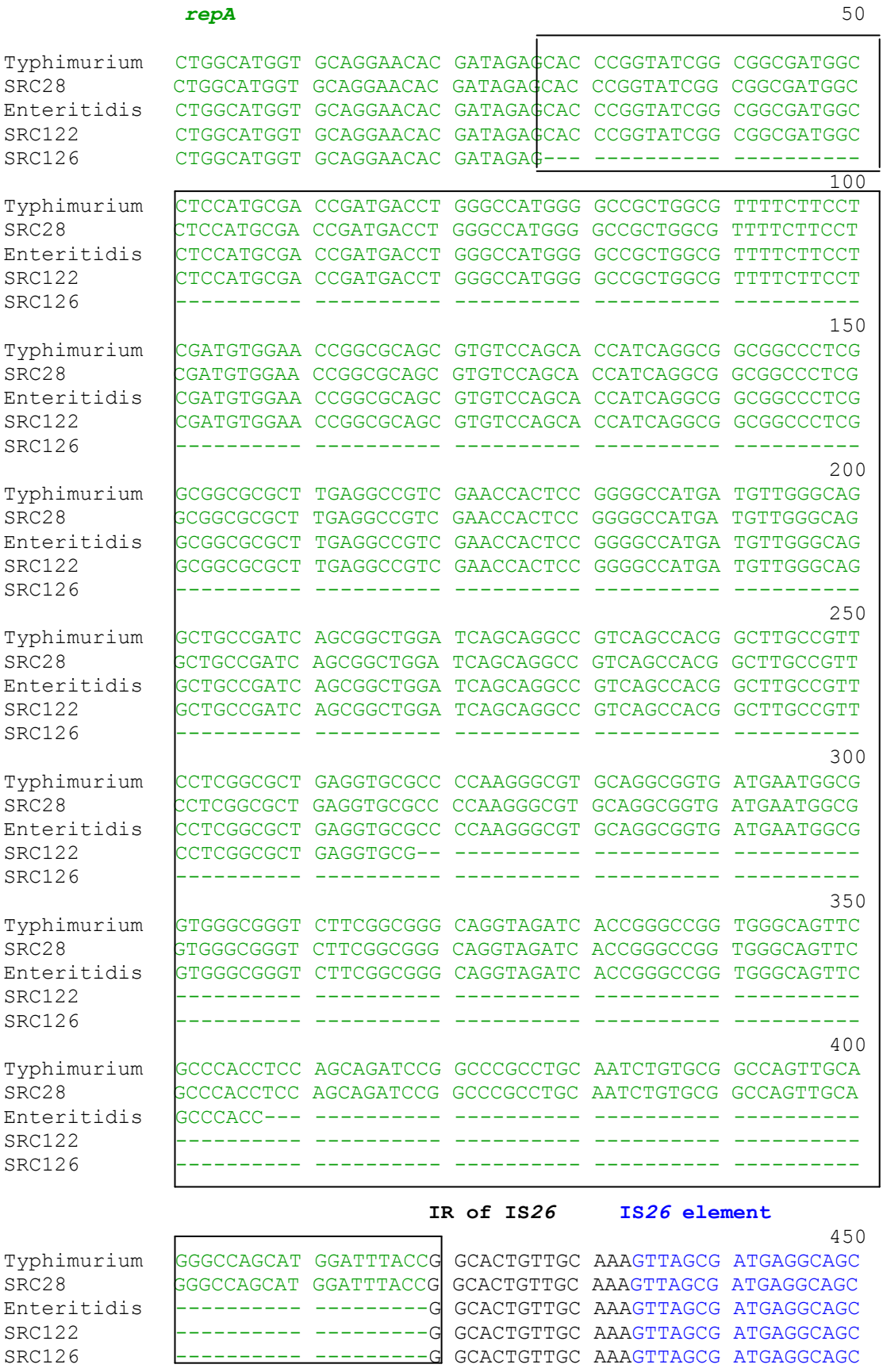


Figure 4.7 DNA sequence alignment of the *repC*-IS*26* fragments from *S. enterica* serovar Typhimurium DT193 (accession no. AY524415), strain SRC28, *S. enterica*

serovar Enteritidis IncI plasmid (accession no. AJ628353), strains SRC122 and SRC126 respectively. The dashed lines indicate spaces used to facilitate alignment.

A summary of PCR screening data for IS*26*-*strB*, *strB*, *strA*-*strB*, *strA*, *sul2*, *strA*-*sul2*, *sul2*-*repC* and *repC*-IS*26* is shown in Table 4.2. The results indicate that among 36 strains containing the linked *strB*-*strA*-*sul2* genes, 33 strains were positive for all three linkage PCRs and harboured the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* gene cluster, though the sizes of the truncated *repA* gene were different. Strains SRC1 and SRC51 were negative for IS*26*-*strB*, *sul2*-*repC* and *repC*-IS*26* linkage PCRs and did not contain the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* region. In order to determine whether the *sul2*-*strA*-*strB* genes were located on a small plasmid, a QIAprep Spin Miniprep Kit (Qiagen) was used to extract plasmid DNA from these two strains, but a plasmid was not detected. A modified Kado and Liu method (Kado and Liu, 1981) was also used to extract large plasmids for these two strains, however no plasmid DNA was detected. It would be nonetheless interesting to uncover where the *sul2*-*strA*-*strB* genes were located for these two strains. Additional research will be required to further this analysis.

Strain SRC116 was positive for the *sul2*-*repC* and *repC*-IS*26* linkage PCR amplifications, but negative for the IS*26*-*strB* linkage PCR. This strain does contain the *strB*-*strA*-*sul2*-*repC*-IS*26* region, similar to the antibiotic resistance gene region found in *S. enterica* serovar Typhimurium DT193 as noted in Daly et al. (2005).

Table 4.2 PCR screening results of IS*26-strB*, *strB*, *strA-strB*, *strA*, *strA-sul2*, *sul2*, *sul2-repC* and *repC*-IS*26* for 36 *Salmonella* strains.

Strain no.	Serovar	Source	Resistance profile	PCR amplifications							
				IS*26-strB*	*strB*	*strA-strB*	*strA*	*strA-sul2*	*sul2*	*sul2-repC*	*repC*-IS*26* (bp)
SRC1	Singapore	human	CmSmSpSuTcTp	-	+	+	+	+	+	-	-
SRC51	Singapore	human	CmSmSpSuTcTp	-	+	+	+	+	+	-	-
SRC126*	Typhimurium PT44	bovine	ApSmSuTp	+	+	+	+	+	+	+	882
SRC13*	Typhimurium PT135	human	ApSmSuTcTp	+	+	+	+	+	+	+	882
SRC14*	Typhimurium PT44	human	ApSmSuTcTp	+	+	+	+	+	+	+	882
SRC31*	Typhimurium PT44	bovine	ApSmSuTcTp	+	+	+	+	+	+	+	882
SRC124*	Typhimurium PT44	bovine	ApSmSuTcTp	+	+	+	+	+	+	+	882
SRC128*	Typhimurium PT44	bovine	ApSmSuTcTp	+	+	+	+	+	+	+	882
SRC132*	Typhimurium PT9	bovine	ApSmSu	+	+	+	+	+	+	+	1274
SRC133*	Typhimurium PT9	bovine	ApKmSmSu	+	+	+	+	+	+	+	1274
SRC131*	Typhimurium PT9	bovine	ApSmSuTp	+	+	+	+	+	+	+	1274
SRC82	Bovismorbificans 32	bovine	ApSmSuTp	+	+	+	+	+	+	+	1274
SRC116	Typhimurium PT135	bovine	ApSmSuTcTp	-	+	+	+	+	+	+	1274
SRC120*	Typhimurium PT29	porcine	ApKmSmSuTp	+	+	+	+	+	+	+	1274
SRC122*	Typhimurium PT29	porcine	ApKmSmSuTp	+	+	+	+	+	+	+	1123
SRC136*	Typhimurium RDNC	porcine	ApKmSmSuTp	+	+	+	+	+	+	+	1274
SRC17	Bovismorbificans	bovine	ApCmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC26*	Typhimurium PT1var2	bovine	ApKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC32*	Typhimurium PT68	bovine	ApKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC123*	Typhimurium PT29	porcine	ApKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC129*	Typhimurium PT44	bovine	ApKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC135*	Typhimurium RDNC	porcine	ApKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC36	Bovismorbificans PT13	human	ApKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC61	Bovismorbificans PT11	bovine	ApKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC117*	Typhimurium PT141	porcine	ApGmKmSmSuTp	+	+	+	+	+	+	+	1274
SRC121	N/D	porcine	ApGmKmSmSuTp	+	+	+	+	+	+	+	1274
SRC23	Seftenberg	porcine	ApCmSmSpSuTcTp	+	+	+	+	+	+	+	1274
SRC29*	Typhimurium PT141	porcine	ApGmKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC30*	TyphimuriumPT141var4	porcine	ApGmKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC118*	Typhimurium PT141	porcine	ApGmKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC60	Bovismorbificans PT13	bovine	ApCmKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC62	Bovismorbificans PT32	bovine	ApCmKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC79	Bovismorbificans 14	bovine	ApCmKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC80	Bovismorbificans 24	bovine	ApCmKmSmSuTcTp	+	+	+	+	+	+	+	1274
SRC27*	Typhimurium PT135	equine	ApCmGmKmSmSpSuTcTp	+	+	+	+	+	+	+	1274
SRC28*	Typhimurium PT135	equine	ApCmGmKmSmSpSuTcTp	+	+	+	+	+	+	+	1274

4.4 Discussion

IS*26* is 820 bp long and carries 14 bp perfect terminal inverted repeats. Upon integration, IS*26* generates an 8 bp duplication of its target sequence (Mollet et al., 1983). IS*26* belongs to the IS*6* family of insertion sequences and is distributed widely among plasmids (Wrighton and Strikes, 1987; Lee et al., 1990; Naas et al., 2001). IS*15*, IS*15*△ and IS*140* are DNA sequences homologous to IS*26*, which suggested that IS*26* is likely to be a member of a family of transposable elements with a wide host range and of considerable importance for horizontal gene transfer between microorganisms (Mollet et al., 1983). Through transposition and cointegration, IS*26* is widely spread among plasmids and implicated in the dissemination of resistance genes in several ways (Doroshenko and Livshits, 2004; Lee et al., 1990; Naas et al., 2001; Wrighton and Strike, 1987). As a transposable element, two IS*26* elements in direct repeat formation can move the intervening DNA segment (Mollet et al., 1983). For example, two IS*26* elements in the same orientation are found at each end of the kanamycin resistance transposon Tn*4352B* (Figure 4.8) (Szczepanowski et al., 2005). A compound IS*26* transposon carrying nine resistance genes has been described by Naas et al. (2001). IS*26* elements are able to facilitate mobilization of chromosomal sequences containing resistance genes (Lee et al., 1990; Ford and Avison, 2004).

Through the sequence comparison of 14 independent integration sites of IS*26* and its relatives, Mollet et al. (1985) revealed that the distribution of integration sites of IS*26* on small multicopy plasmids is nearly random with no striking rules for target selection

by the element. Miriagou et al. (2005) also suggest that the mobile element of IS*26* does not exhibit any marked target site specificity.

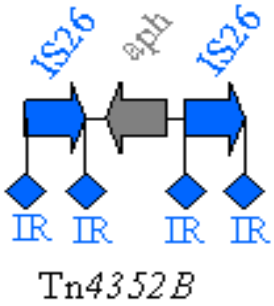


Figure 4.8 The structure of Tn*4352B* transposon. Genes and their transcriptional directions are indicated by arrows. IR, inverted repeat (adapted from Szczepanowski et al., 2005).

A large SGI1-K1 MDR region has been characterised in *Salmonella enteric* serovar Kentucky. This IS*26*-composite transposon contained: (i) an In4-type integron with only one cassette array, *aac(3)-Id–aadA7*, and an IS*6100* element, (ii) part of Tn*21* comprising the mercury resistance operon, (iii) part of Tn*1721* comprising the tetracycline resistance genes *tetR-tetA*(A), (iv) part of Tn*5393* comprising the streptomycin resistance genes *strA-strB*, and an IS*1133* element, (v) part of Tn*3* comprising the $bla_{\text{TEM-1}}$ gene, and (vi) two IS*26* elements (Doublet et al., 2008). Similar IS*26*-composite transposons were also identified in *Salmonella enteric* serovar Newport, which contained an In4-type integron, a mercury resistance operon and parts of Tn*1721* and Tn*5393* (Doublet et al., 2009). Transposition and/or homologous recombination of IS*26* elements associated with integrons and transposons are probably implicated in the building of the SGI1 MDR regions and other plasmids (Doublet et al., 2009). Recently, parts of Tn*21*, Tn*1721*, Tn*5393*, In4-type integron structures, and IS*26* elements were also found in an 86 kb chromosomal resistant island recently identified in

multidrug-resistant *Acinetobacter baumannii* strain AYE (Doublet et al., 2008).

For the linkage PCR amplification of the *repC*-IS*26* region, three deviatives were found in this collection. Six strains contained the same size *repC*-IS*26* region (882 bp) and all belonged to *S. enterica* serovar Typhimurium isolated from human and bovines. Among them, 5 strains had the same antibiotic resistance profiles. Twenty-seven strains contained the same *repC*-IS*26* region of 1274 bp in size and were isolated from different serovars including Typhimurium, Bovismorbificans and Senftenberg. These strains had the same or similar resistance profiles. Only one strain contained the *repC*-IS*26* region of 1123 bp in size and this deletion was unique in this collection. In GenBank, another deviative of *repC*-IS*26* was reported for *S. enterica* serovar Enteritidis IncI plasmid (accession no. AJ628353) which lacks 62 bp of the *repA* gene when compared to *S. enterica* serovar Typhimurium DT193. This particular deviative was not found in this investigation. The occurrence of the *repC*-IS*26* structure might be a useful marker to identify whether they were clonal.

Three different deviatives identified in this research were possibly caused by the IS*26* element, which deleted different amount of the upstream *repA* gene. It is very possible that the deletion is characteristic and this structure was transferred between different bacteria by horizontal transfer. The findings of this study suggest that the association of IS*26* with the *strB-strA-sul2-repC-repA* gene cluster was probably a critical step in the evolution of the antibioitc resistance gene cluster of

IS*26-strB-strA-sul2-repC-repA*-IS*26*.

The *strB-strA-sul2-repC-repA* antibiotic resistance region is very common and has been previously described in different small plasmids such as the broad-host-range non-conjugative plasmids RSF1010 and pBP1, and the IncQ-like plasmid pMS260 (Scholz et al., 1989; Ito et al., 2004; Korfmann et al., 1983). The formation of the IS*26-strB-strA-sul2-repC-repA*-IS*26* antibiotic resistance gene cluster may be derived from this type of small plasmid mediated by IS*26* elements (Daly et al., 2005). It can be hypothesized that the formation of the IS*26-strB-strA-sul2-repC-repA*-IS*26* region in this research was similar to the mechanism described by Daly et al. (2005). It may have occurred through the acquisition of conserved genetic traits *strB-strA-sul2-repC-repA*, by independent rounds of insertion and recombination (Daly et al., 2005). One IS*26* element was inserted upstream of the *strB* gene and created an 8 bp duplication of its target sequence. The other IS*26* element was inserted downstream of the *repA* gene and truncated the 5′ end of this gene into three different sizes as mentioned above. Having flanking IS*26* elements, this antibiotic resistance region can transpose to a recipient replicon. Although a DNA segment can be moved by direct IS*26* copies, but not by inverted IS*26* copies, the presence of these insertion sequences may have contributed to the intrinsic instability of the resistance region, resulting in deletions and inversions (Miriagou et al., 2006).

In our collection, 33 strains contain this IS*26-strB-strA-sul2-repC-repA*-IS*26* antibiotic

resistance gene region. Among them, 23 of these belong to *S. enterica* serovar Typhimurium, 8 belong to *S. enterica* serovar Bovismorbificans, one belongs to *S. enterica* serovar Senftenberg and one strain has no serovar information. These strains were isolated from human, bovine, equine and porcine sources respectively. Thus, this IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* structure is not a rare structure in this collection and horizontal gene transfer mediated by IS*26* elements may have contributed to its spread.

This antibiotic resistance structure of IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* has been identified on different plasmids recently, including plasmid pHCM1 from *S. enterica* serovar Typhi CT18, pU302L from *S. enterica* serovar Typhimurium, pRSB107 from uncultured bacterium without source information, and pAKU_1 from *S. enterica* serovar Paratyphi A (Parkhill et al., 2001; Chen et al., 2007; Szczepanowski et al., 2005; Holt et al., 2007).

Based on the screening results for all the PCR amplifications, the group of 23 *S. enterica* serovar Typhimurium strains (strains marked with an asterisk in Table 4.3), which contain the linked IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* antibiotic resistance gene cluster were chosen for further investigation.

Chapter 5: Characterisation of the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* gene cluster from 23 *S. enterica* serovar Typhimurium strains

5.1 Introduction

5.1.1 The IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* gene cluster

For strains isolated from human and animals, streptomycin resistance linked to sulfonamide resistance is usually found on large self-transmissible R plasmids. Plasmid pHCM1 is a 218 kb conjugative plasmid isolated from *S. enterica* serovar Typhi CT18 belonging to the incompatibility group IncHI1 (Parkhill et al., 2001). An antibiotic resistance gene cluster carrying *bla*$_{TEM-1}$, *sul2*, *strA*-*strB* genes and IS*26* elements was identified on this plasmid. Two IS*26* elements flanking the *strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$ cluster were in a direct orientation, while the third IS*26* element, between the *sul2* and *bla*$_{TEM-1}$ genes, was oppositely oriented. A Tn*21* mercury resistance (*mer*) operon is located upstream of the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* gene cluster. Downstream of this cluster, a fourth IS*26* element was detected after a class 1 integron on this plasmid (Figure 5.1) (Parkhill et al., 2001). The presence of multiple IS*26* copies suggested that IS*26*-mediated transposition could play an important role in the assembly and mobilisation of this antibiotic resistance region (Miriagou et al., 2006).

As noted by Chen et al. (2007), an 84.5 kb IncF-like plasmid pU302L, which was isolated from *S. enterica* serovar Typhimurium phage type U302, contained a similar antibiotic resistance gene cluster. Plasmid pU302L carried 14 complete IS elements and multiple resistance genes including *aac3*, *aph(3')-1, sul2*, *tetA*(A)*/tetR*, *strA/strB*, $bla_{\text{TEM-1}}$, *mph* and a Tn*21 mer* operon. DNA sequence analysis of pU302L revealed that it harboured an IS*26-strB-strA-sul2-repC-repA*-IS*26-*$bla_{\text{TEM-1}}$-IS*1294* gene cluster. This cluster was located between nucleotide 21,660 and 29,080 of plasmid pU302L (accession no. AY333434). Except for the presence of an IS*1294* instead of the IS*26* element downstream of the $bla_{\text{TEM-1}}$ gene, the sequence of the gene cluster is identical to that found on plasmid pHCM1 (Figure 5.1).

The same gene cluster is also evident on plasmid pRSB107, which was isolated from an unknown bacterial host from a wastewater treatment plant (Szczepanowski et al., 2005). Plasmid pRSB107 is approximately 120 kb in size and belongs to the IncF plasmid group. This plasmid conferred antibiotic and mercury resistance and other beneficial properties upon the host bacterium (Szczepanowski et al., 2005). In addition to the same IS*26-strB-strA-sul2-repC-repA*-IS*26-*$bla_{\text{TEM-1}}$-IS*26* antibiotic resistance gene cluster, there were two additional resistance gene insertions within the Tn*21* composite transposon. The Tn*4352B* kanamycin/neomycin resistance transposon was inserted upstream of the IS*26-strB-strA-sul2-repC-repA*-IS*26-*$bla_{\text{TEM-1}}$-IS*26* gene cluster. This transposon is comprised of the *aphA1* gene flanked by two IS*26* elements in direct orientation. A macrolide-resistance operon *mph*(A)-*mrx-mphR*(A) was inserted

downstream of the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster, followed by an IS*6100* element (Szczepanowski et al., 2005). A class 1 integron carrying a trimethoprim resistance gene cassette (*dfr*) with a truncated 3′-CS was located downstream of the IS*6100* element (Szczepanowski et al., 2005). The Tn*21* on plasmid pRSB107 was terminated by a Tn*21*-specific transposition module (*tnpM-tnpR-tnpA*), which is responsible for transposition of this transposon. Upstream of the Tn*4352B* transposon, a Tn*21 mer* operon was found (Figure 5.1) (Szczepanowski et al., 2005). Plasmid pRSB107 exhibited a mosaic structure consisting of modules which have previously been identified on resistance and virulence plasmids or in the chromosomes of human and plant pathogens (Szczepanowski et al., 2005).

Recently, another IncHI1 plasmid pAKU_1 has been isolated from *S. enterica* serovar Paratyphi A (Holt et al., 2007). This 212 kb plasmid was isolated from a Pakistani patient in Karachi in 2002. A 9 kb DNA fragment, which incorporated *bla*$_{TEM-1}$, *sul2*, *strA* and *strB* resistance genes flanked by IS*26* elements, was present on pAKU_1 (Holt et al., 2007). This IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* fragment is identical to the clusters from plasmid pHCM1 and pRSB107 (Parkhill et al., 2001; Szczepanowski et al., 2005). It is also similar to the antibiotic resistance gene cluster on plasmid pU302L (Chen et al., 2007; Holt et al., 2007). A Tn*21 mer* operon was located upstream of the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster. Downstream of this cluster, a truncated Tn*21*-specific transposition module (*tnpR-tnpM*) in an opposite orientation was found. A class 1 integron containing a *dfrA*7 gene

cassette and a 3′-CS containing *qacE△1* and *sul1* was located downstream of the *tnpR-tnpM* genes. A *tra2* conjugal transfer region was also identified (Figure 5.1) (Holt et al., 2007).

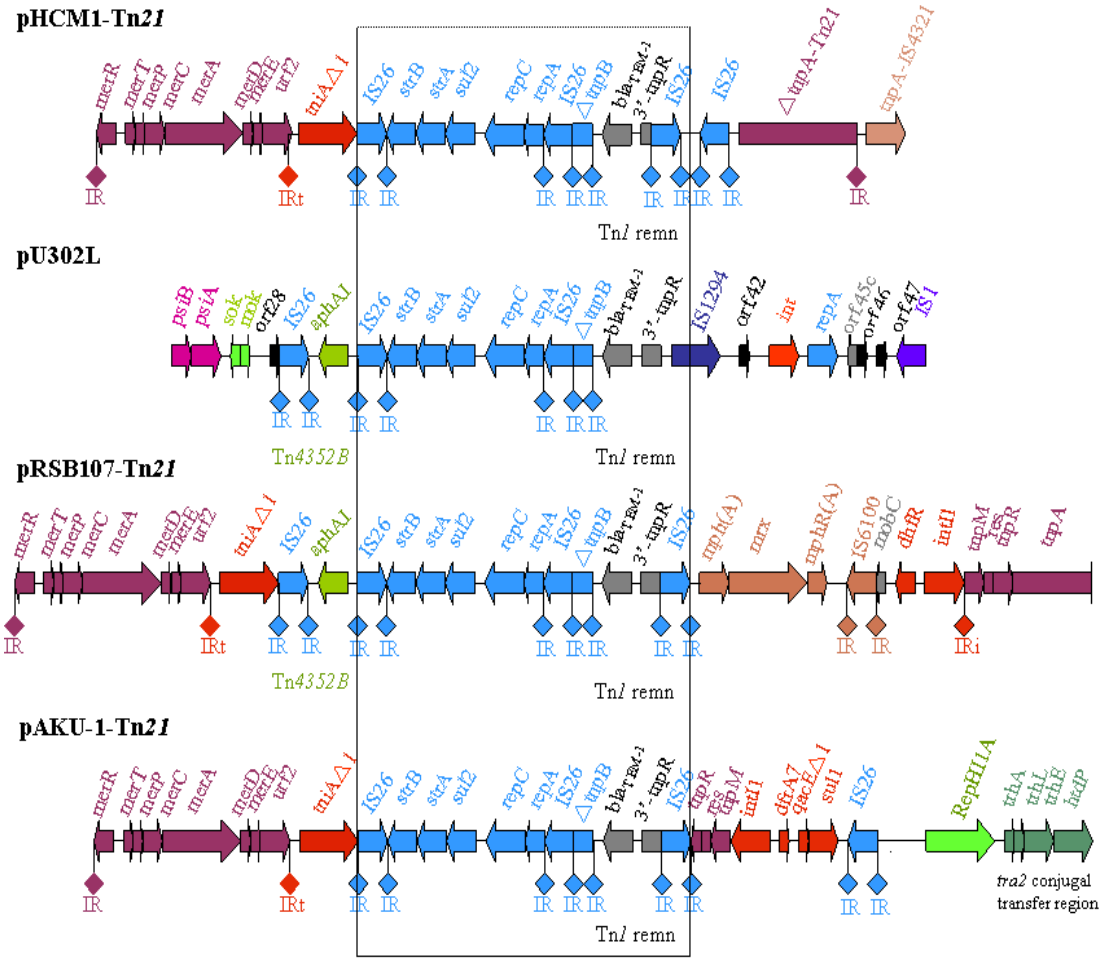


Figure 5.1 The IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$-IS*26* antibiotic resistance gene clusters (blue fill within the box) on plasmid pHCM1 (accession no. AL513383), pRSB107 (accession no. AJ851089) and pAKU_1 (accession no. AM412236). On plasmid pU302L (accession no. AY333434), the IS*26* element downstream of the *bla*$_{\text{TEM-1}}$ was replaced by an IS*1294*. Genes and their transcriptional directions are indicated by arrows (modified from Szczepanowski et al., 2005; Holt et al., 2007; Chen et al., 2007).

The IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$-IS*26* gene cluster is flanked by two IS*26* elements in direct orientation and may be mobile (Chen et al., 2007). Holt et al. (2007) found that the sequences across the boundaries, which included the insertions of

Tn*21* into Tn*9* and the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* antibiotic resistance gene cluster into Tn*21*, were identical for plasmids pAKU_1, pHCM1 and pRSB107. This suggests that there was a possible composite transposon, which was formed by the insertion of IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* into Tn*21*-derived transposons, followed by the insertion of these Tn*21*-derived transposons into a Tn*9* transposon (Holt et al., 2007). This composite transposon is able to insert into large plasmids and move between distinct plasmid backbones as a single unit using the IS*1* ends of Tn*9* (Figure 5.2) (Holt et al., 2007). This hypothesis is supported by observations that the insertion sites of the composite transposon in pAKU_1 and pHCM1 plasmids are different (Holt et al., 2007; Parkhill et al., 2001).

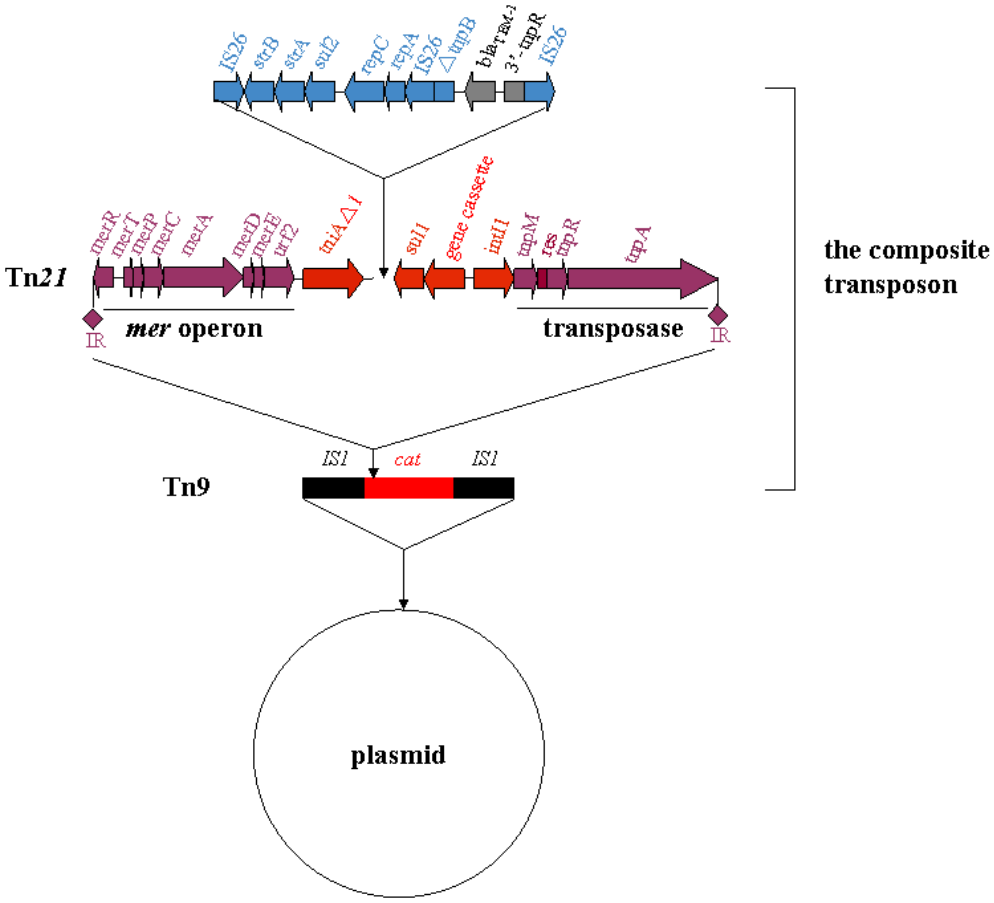


Figure 5.2 Hypothesis for various insertion events that occurred for the multiple

antibiotic resistance plasmid pAKU_1. The IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster (blue fill) was inserted into a Tn*21*-derived transposon, which was then inserted into a Tn*9* to form a composite transposon. The composite transposon has inserted into a large plasmid to form pAKU_1. The genes and their transcriptional directions are indicated by arrows. The Tn*21* transposon contains a *mer* operon, truncated *tniA* gene, a class 1 integron and a Tn*21* transposition system (modified from Holt et al., 2007).

5.1.2 IS*200* elements and epidemiological research

For epidemiological research undertaken during the past decade, plasmid analysis, chromosomal fingerprinting and pulse-field gel electrophoresis (PFGE) have been widely used to tract *S. enterica* isolates (Rubino et al., 1998). IS*200* is a mobile element found in a variety of eubacterial genera including *Salmonella, Escherichia, Shigella, Vibrio*, *Enterococcus*, *Clostridium*, *Helicobacter* and *Actinobacillus* (Beuzon et al., 2004). IS*200* elements are small (707–711 bp) and contain a single gene. Unlike typical mobile elements, IS*200* transposes rarely and is distributed widely. These characteristics make IS*200* a suitable molecular marker for epidemiological and ecological studies, especially when the number of IS*200* chromosomal copies is high (Beuzon et al., 2004). IS*200* has been found in many isolates of *S. enterica* with the exception of serovars Agona, Bovismorbificans, Dar-es-salam, Panama and Choleraesuis and some strains of serovar Arizonae, (Gibert et al., 1990; Olsen and Skov, 1994; Rubino et al., 1998). IS*200* fingerprinting is extensively used for *S. enterica* strain discrimination (Beuzon et al., 2004). IS*200* fingerprints can predict evolutionary relatedness among *Salmonella* isolates. Evolutionary distance can be reflected by the divergence in the copy number and distribution of IS*200* elements (Rubino et al., 1998). Significant observations have been made by the use of IS*200* epidemiologically and

phylogenetically in several *S. enterica* serovars such as Enteritidis, Abortusovis, Heidelberg, Dublin, Typhimurium, Brandeburg, Typhi and Paratyphi BdT+ (Olsen et al., 1994; Schiaffino et al., 1996; Stanley et al., 1992; Chowdry et al., 1993; Stanley et al., 1993; Baquar et al., 1994; Threlfall et al., 1994; Weill et al., 2005; Levings et al., 2006). For *S. enterica* serovars in which the number of IS*200* copies is low, IS*200* fingerprinting often needs to be combined with other methods to discriminate among *Salmonella* serotypes or strains (Beuzon et al., 2004).

In this chapter, gene probes comprising bla_{TEM}, the class 1 integron specific integrase gene *intI1*, the mercury resistance gene *merA* and their relationship with the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* gene cluster will be characterised for 23 *S. enterica* serovar Typhimurium.

5.2 Materials and methods

5.2.1 Southern hybridisations using bla_{TEM}, *intI1*, *merA*, *dfrA5* and IS*200* gene probes

5.2.1.1 Preparing bla_{TEM}, *intI1*, *merA, dfrA5* and IS*200* gene probes

Primer pairs Tem-F/Tem-R, L2/L3, CV-D1/CV-D2, JL-D6/JL-D4 and IS200-F/IS200-R were used to amplify bla_{TEM}, *intI1*, *merA*, *dfrA5* and IS*200* gene probes. The exact primer sequences are listed in Table 5.1.

Table 5.1 Primers used in each PCR amplification.

Primer ID	Primer sequence (5′ – 3′)	Gene	Position	Accession no.	Reference
L2	GAC GAT GCG TGG AGA CC	*intI1*	27266-27246	AF261825.2	Sallen et al., 1995
L3	CTT GCT GCT TGG ATG CC	5′-CS	27908-27892	AF261825.2	Maguire et al., 2001
L1	GGCATCCAAGCAGCAAGC	5′-CS	27892-27909, 37164-37181	AF261825.2	Levings et al., 2005
Tem-F	TTC TTG AAG ACG AAA GGG C	bla_{TEM}	6707-6689	L27758	Brinas et al., 2002
Tem-R	ACG CTC AGT GGA ACG AAA AC	bla_{TEM}	5500-5519	L27758	Brinas et al., 2002
CV-D1*	CAG CCG CAG TTC GTC TAT G	Tn*501merA*	2548-2566	Z00027	Levings et al., 2007
CV-D2*	TCG TCA GGT AGG GGA ACA AC	Tn*501merA*	2941-2922	Z00027	Levings et al., 2007
JL-D6	TTAAAGCCTTGACGTACAAC	*dfrA5*	210-229	DQ133160	this study
JL-D4	TTGATAGCAATAGTTAATG	*dfrA5*	571-553	DQ133160	this study
JL-D1	AGT CCT GTA TGT GCT TGA GCG	*repC*	40802-40822	AJ851089	this study
JL-D2	AAG GTA TTG AGG TGA TGC G	IS*26*	35512-35530	AJ851089	this study
JL-D3	CGC TCA AGC ACA TAC AGG ACT	*repC*	40822-40802	AJ851089	this study
JL-D5	TGT TTG AGC TAA AGT GTT GC	*dfrA5*	554-535	DQ133160	this study
JL-D7	CGC ATC ACC TCA ATA CCT T	IS*26*	35530-35512	AJ851089	this study
JL-D13	AAG CGT CGC GGT TTC TG	*tnpB*	42980-42964	AJ851089	this study
JL-D14	CAG AAA CCG CGA CGC TT	*tnpB*	42964-42980	AJ851089	this study
JL-D43	TGA ACC ACC GAT CAG CGA AC	*repA*	41283-41302	AJ851089	this study
JL-D44	TGA GCG GAT ACA TAT TTG AA	Tn*1*	44182-44201	AJ851089	this study
DB-T1	TGC CAC GCT CAA TAC CGAC	IS*6100*	41120-41138	AF261825.2	Boyd et al., 2002
IS6100-Rv2	AAT GGT GGT TGA GCA TGC C	IS*6100*	41475-41457	AF261825.2	Levings et al., 2005
TemSFW	GTATGGATCCTCAACATTTCCGTG	bla_{TEM}	37-60	AF506748	Daly et al., 2005
TemSRV	ACC AAT GCT TAA TCA GTG AGG CA	bla_{TEM}	897-875	AF506748	Daly et al., 2005
sulI-F(C)*	CGA TCA GAT GCA CCG TGT TT	*sul1*	29512-29531	AF261825.2	unpublished data
sulI-R	TTT ACA GGA AGG CCA ACG GT	*sul1*	39424-39405	AF261825.2	Leverstein-van Hall et al., 2002
strB-out	AGA GGA GCA ACG CGA TCT AGC	*strB*	1639-1659	M28829	Palmer et al., 1997
IS200-F	CCT AAC AGG CGC ATA CGA TC	IS*200*	66-85	X56834	Gibert et al., 1990; Burnens et al., 1997
IS200-R	ACA TCT TGC GGT CTG GCA AC	IS*200*	603-622	X56834	Gibert et al., 1990; Burnens et al., 1997
aphAI-IAB(R)	CAA ACC GTT ATT CAT TCG TGA	*aphAI*	42069-42089	AF024666	Randall et al., 2004
aphAI-IAB(F)	AAA CGT CTT GCT CGA GGC	*aphAI*	42529-42511	AF024666	Randall et al., 2004

* Primer designed by C. Venturini.

In the amplification reaction, DIG-labeled dNTPs (Roche) replaced the standard dNTPs. The programs used for the PCR amplifications were as follows: 1. For *bla*$_{TEM,}$ 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 1.5 min, and finally 72°C for 10 min; 2. For *intI1*, *merA* and *dfrA5*, 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 50°C-59°C for 45 sec, 72°C for 45 sec, and finally 72°C for 10 min. The annealing temperature for the *intI1* probe was 58°C , for the *merA* probe it was 59°C, and for *dfrA5* it was 50°C; 3. For IS*200*, 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. The amplifications were all performed using the FTS-960 thermocycler (Corbett Research).

5.2.1.2 Southern hybridisations using *bla*$_{TEM}$, *intI1*, *merA*, *dfrA5* and IS*200* gene probes

In order to identify whether these *S. enterica* serovar Typhimurium strains contain the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* antibiotic resistance gene cluster on Tn*21*-like transposons, Southern hybridisations with the *bla*$_{TEM}$, *intI1* and *merA* gene probes were performed on these strains. Because the *Bgl*II restriction enzyme does not cut either the *strA*, *strB*, *sul2*, *bla*$_{TEM-1}$, *intI1* nor *merA* genes, it was used to digest chromosomal DNA for each *Salmonella* strain for the *bla*$_{TEM}$, *intI1* and *merA* Southern hybridisations. Enzyme *Ban*II was used to digest the chromosomal DNA for the *dfrA5* Southern hybridisation because it can cut *intI1*, *qacE*△*1*, *sul1*, orf5 and IS*6100*, but does not cut the *dfrA5* gene cassette. Enzyme *Pst*I was chosen to digest the chromosomal DNA for the IS*200* Southern hybridisation since it does not cut within IS*200* in *Salmonella* (Casin et al., 1999). Southern hybridisations of the *bla*$_{TEM}$, *intI1*, *merA*, *dfrA5* and IS*200* gene probes were performed according to the method described in Chapter 2 (section 2.9).

5.2.2 Linkage PCR amplifications

The primer sequences used for linkage PCR amplifications are listed in Table 5.1. The primers for IS*26*-*aphAI*, *aphAI*-*strB*, IS*26*-*tnpB*, *tnpB*-bla_{TEM-1} and bla_{TEM-1}-IS*26* PCR amplifications, are given in Figure 5.3. These assays and those shown in Figure 5.4 were designed to determine if the arrangement of genes found in pRSB107-Tn*21* are presented in our collection of *S. enterica* serovar Typhimurium strains.

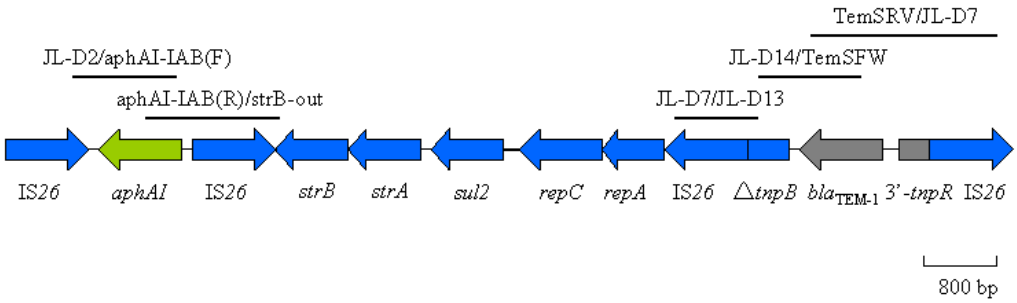


Figure 5.3 The primer positions for the IS*26*-*aphAI* region [JL-D2/aphAI-IAB(F)], *aphAI*-*strB* [aphAI-IAB(R)/strB-out], IS*26*-*tnpB* (JL-D7/JL-D13), *tnpB*-bla_{TEM-1} (JL-D14/TemSFW) and bla_{TEM-1}-IS*26* (TemSRV/JL-D7) linkage PCR amplifications. The gene and their transcriptional directions are indicated by arrows. The fragments amplified by PCR are shown as lines with the primers indicated above.

The primers used for PCR amplification of the Tn*1*-IS*6100* region, the IS*6100*-*sul1* region and the *sul1*-*intI1* region, are indicated in Figure 5.4.

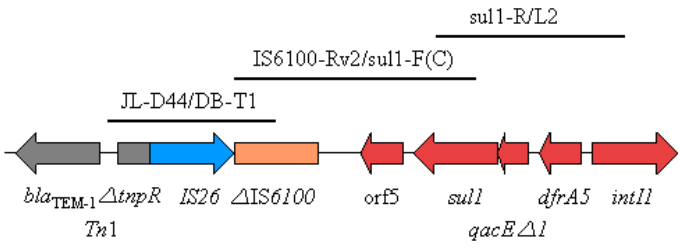


Figure 5.4 The primer positions of Tn*1*-IS*6100* (JL-D44/DB-T1), IS*6100*-*sul1* [IS6100-Rv2/sul1-F(C)] and *sul1*-*intI1* (sul1-R/L2) linkage PCR amplifications. The gene and their transcriptional directions are indicated by arrows. The fragments amplified by PCR are shown as lines with the primer pairs indicated.

The primers used for PCR amplification of Tn*1*-*dfrA5* and *dfrA5*-*intI1*, are indicated in Figure 5.5.

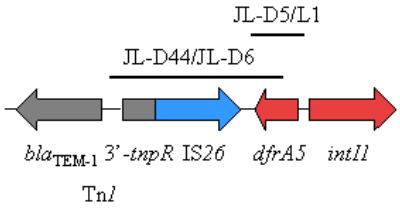


Figure 5.5 The primer positions of Tn*1*-*dfrA5* (JL-D44/JL-D6) and *dfrA5*-*intI1* (JL-D5/L1) linkage PCR amplifications. The genes and their transcriptional directions are indicated by arrows. The fragments amplified by PCR amplifications are shown as lines with the primers indicated above.

The programs used for each linkage PCR are listed in Table 5.2. The amplifications were performed either using the thermocycler FTS-960 or PC-960 (Corbett Research).

Table 5.2 Programs and thermocyclers used for linkage PCR amplifications.

PCR linkage	First cycle	Followed by 35 cycles	Last cycle	Thermocycler
IS*26*-*aphAI*	94°C, 5 min	94°C, 30 sec; 59°C, 1 min; 72°C, 1 min	72°C, 6 min	FTS-960
aphAI-*strB*	94°C, 5 min	94°C, 30 sec; 58°C, 1 min; 72°C, 1.5min	72°C, 6 min	FTS-960
IS*26*-*tnpB*	94°C, 5 min	94°C, 30 sec; 57°C, 1 min; 72°C, 1 min	72°C, 10min	FTS-960
tnpB-bla_{TEM-1}	94°C, 5 min	94°C, 30 sec; 60°C, 1 min; 72°C, 1.5 min	72°C, 10min	FTS-960
bla_{TEM-1}-IS*26*	94°C, 5 min	94°C, 30 sec; 60°C, 1 min; 72°C, 2 min	72°C, 10min	FTS-960
Tn*1*-*dfrA5*	94°C, 5 min	94°C, 30 sec; 53°C, 1 min; 72°C, 1.5 min	72°C, 10min	FTS-960
dfrA5-*intI1*	94°C, 5 min	94°C, 30 sec; 62°C, 1 min; 72°C, 1 min	72°C, 10min	PC-960
Tn*1*-IS*6100*	94°C, 5 min	94°C, 30 sec; 58°C, 1 min; 72°C, 1.5 min	72°C, 10min	PC-960
IS*6100*-*sul1*	94°C, 5 min	94°C, 30 sec; 58°C, 1 min; 72°C, 1.5 min	72°C, 10min	PC-960
sul1-*intI1*	95°C, 5 min	95°C, 30 sec; 62°C, 1 min; 72°C, 2 min	72°C, 10min	PC-960

5.2.3 DNA sequence analysis of each linkage PCR amplicon

Representatives of each linkage PCR amplicon including the IS*26*-*aphAI*, *aphAI*-*strB*, IS*26*-*tnpB*, *tnpB*-bla_{TEM-1}, bla_{TEM-1}-IS*26*, Tn*1*-*dfrA5*, *dfrA5*-*intI1*, Tn*1*-IS*6100*, IS*6100*-*sul1* and *sul1*-*intI1* regions were fully sequenced and analysed according to the method described in Chapter 2 (section 2.8).

5.2.4 Restriction endonuclease analysis of each linkage PCR amplicon

The method for this analysis is that described in Chapter 2 (section 2.6). The following is a list of the enzymes used for each PCR amplicon: enzyme *Hind*III was used to digest IS*26*-*aphAI*; *Dra*I was used to digest *aphAI*-*strB*; *Sal*I was used to digest IS*26*-*tnpB*;

*Sca*I was used to digest *tnpB-bla*$_{TEM-1}$; *Bam*HI was used to digest *bla*$_{TEM-1}$-IS*26*; *Pst*I was used to digest Tn*1*-*dfrA5*; and *Ase*I was used to digest *dfrA5*-*intI1*.

5.3 Results

5.3.1 Characterisation of the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* gene cluster using Southern hybridizations and PCR amplifications

5.3.1.1 Southern hybridisations using *bla*$_{TEM}$, *intI1* and *merA* gene probes

Twenty-three *S. enterica* serovar Typhimurium strains were found to contain the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* gene cluster (see Chapter 4), which is similar to the antibiotic resistance gene cluster appearing on plasmids pRSB107, pHCM1, pU302L and pAKU_1. Southern hybridisations using *bla*$_{TEM}$, *intI1* and *merA* gene probes revealed that all strains contained the *bla*$_{TEM}$ gene, 21 strains contained the *intI1* gene and 16 strains carried the *merA* gene. Sixteen of the 23 strains contained all three (*bla*$_{TEM}$, *intI1* and *merA*) genes. Except for strain SRC26, 15 of these were located on the same sized fragments of approximately 23 kb (Figure 5.6). Strain SRC26 contained these three genes, but the *intI1* gene was located on a different restriction fragment (Figure 5.6). Because all 23 strains were positive for the *bla*$_{TEM}$ gene by Southern hybridisation, a screening PCR for the *bla*$_{TEM-1}$ gene was performed for each of the 23 strains and this result confirmed that each strain contained the *bla*$_{TEM-1}$ gene (data not shown). It is very possible that a Tn*21*-like transposon derivate, similar to that carried by the pRSB107 plasmid, may be harboured among the 23 *S. enterica* serovar Typhimurium strains.

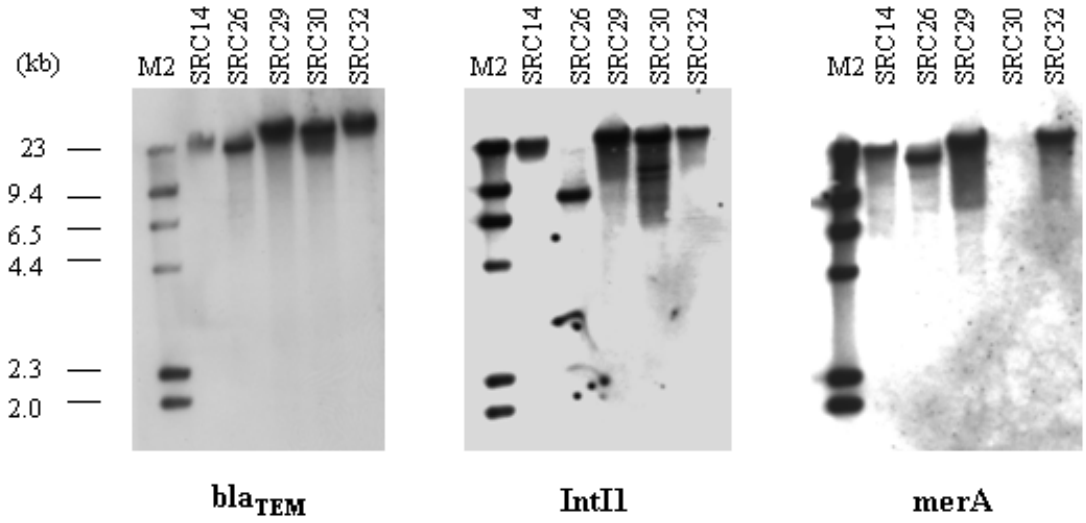


Figure 5.6 Southern hybridisation results of *Bgl*II digested DNA probed using *bla*$_{TEM}$, *intI1* and *merA* gene probes. The designations above the gel indicate strain numbers. Strain SRC14 contained all three genes on a fragment approximately 23 kb in size. Strains SRC29 and SRC32 contained all three genes as well, which were located on a fragment much larger than 23 kb. SRC26 also contained the three genes, but the *intI1* gene was located on a different sized restriction fragment. SRC30 contained only the *bla*$_{TEM}$ and *intI1* genes, which were located on a fragment larger than 23 kb. DIG-labeled marker II (Roche) is labeled M2 and the corresponding sizes are given on the left.

5.3.1.2 The IS*26-tnpB* linkage PCR amplification

In order to investigate the antibiotic resistance gene cluster IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* among 23 *S. enterica* serovar Typhimurium strains, several pairs of primers were designed to link the *bla*$_{TEM-1}$ gene to the identified IS*26-strB-strA-sul2-repC-repA*-IS*26* gene cluster. The first linkage PCR was designed to link the IS*26* downstream of the *sul2* gene to the *tnpB* gene upstream of the *bla*$_{TEM-1}$ gene (Figure 5.3). A PCR amplicon was obtained with the expected size of 924 bp (Figure 5.7A). All 23 strains were positive for this PCR. DNA sequence analysis of the amplicon from strain SRC26 revealed that it was identical to the IS*26-tnpB* fragment from *S. enterica* serovar Paratyphi A (accession no. AM412236), *S. enterica* serovar Typhimurium (accession no. AY333434), *S. enterica* serovar Typhi (accession no. AL523383) and an uncultured bacterium (accession no. AJ851089). All the PCR

amplicons were further characterised by restriction enzyme digestion using *Sal*I. Two fragments of 590 bp and 334 bp were obtained as expected. The enzyme digestion result confirmed that each of the PCR amplicons had the same RFLP profile (Figure 5.7 B).

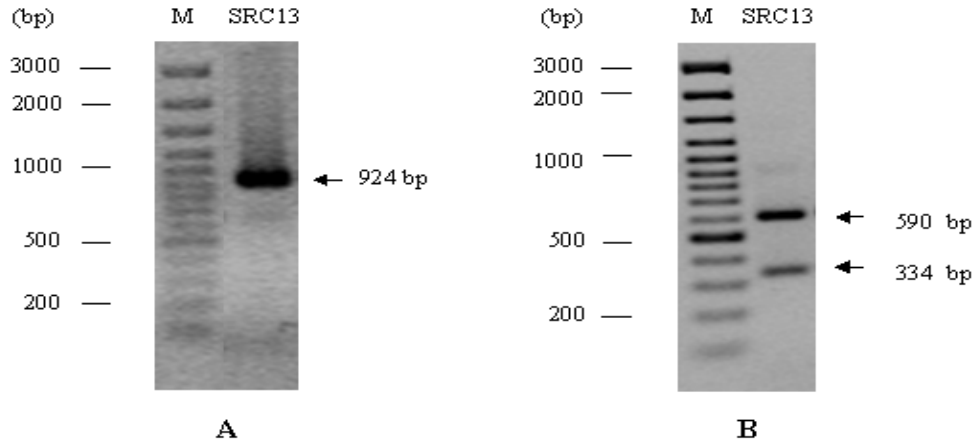


Figure 5.7A Gel electrophoresis of the IS*26*-*tnpB* linkage PCR amplicon. Strain SRC13 was chosen as a representative for this 924 bp PCR product (1.0% agarose gel). **B** Gel electrophoresis of the IS*26*-*tnpB* linkage PCR amplicon digested using restriction enzyme *Sal*I. Strain SRC13 was used as a representative to show the digested fragments of 590 bp and 334 bp (2.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left

Two PCR of *repC*-IS*26* (Chapter 4 Figure 4.5A and B) and IS*26*-*tnpB* (Figure 5.7A and B) were performed and used to link the *repC*-*repA*-IS*26*-*tnpB* region. Because there are usually more than one copy of IS*26* in *S. enterica* and other *Enterobacteria* spp., a PCR linking *repA*-*tnpB* was also performed. The expected sizes of PCR amplicons were obtained and all the PCR products which were further characterised by restriction digestion using enzyme *Pst*I (data not shown). These analyses confirmed that the *tnpB* gene was linked to the *repC*-*repA*-IS*26* region.

5.3.1.3 The *tnpB*-*bla*$_{\text{TEM-1}}$ linkage PCR amplification

In order to determine whether the *bla*$_{\text{TEM-1}}$ is located downstream of the *tnpB* gene in the same location as that for the antibiotic resistance gene cluster of plasmid pRSB107, a linkage PCR was designed to link the *tnpB* gene to *bla*$_{\text{TEM-1}}$ gene (Figure 5.3). A 1170

bp PCR amplicon was obtained for all the strains and the size was the same as that expected from plasmid pRSB107 (Figure 5.8A). DNA sequence analysis of the amplicon from strain SRC26 confirmed that it was identical to the *tnpB*-*bla*$_{TEM-1}$ region from an uncultured bacterium (accession no. AJ851089), *S. enterica* serovar Paratyphi A (accession no. AM412236) and *S. enterica* serovar Typhimurium (accession no. AY333434). All remaining PCR amplicons were further characterised by restriction digestion using *Sca*I. Two fragments, at the expected sizes of 861 bp and 309 bp, were obtained for all the 23 strains (Figure 5.8B).

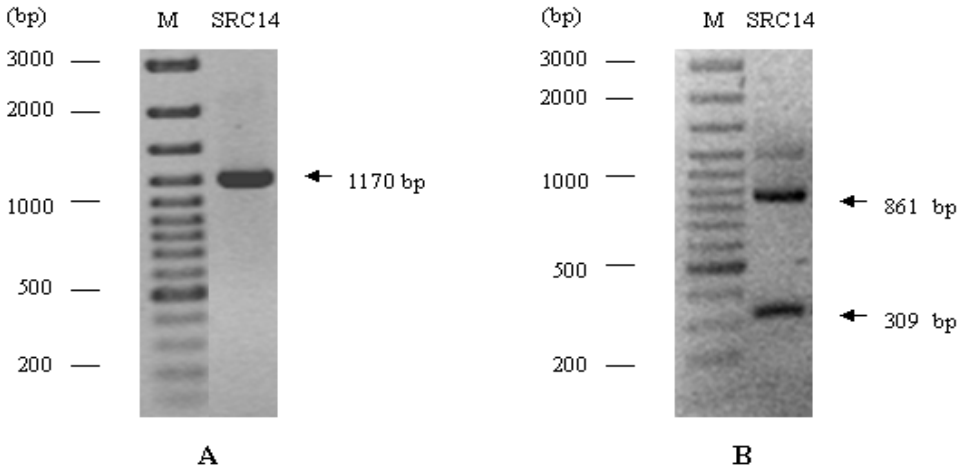


Figure 5.8A Gel electrophoresis of the *tnpB*-*bla*$_{TEM-1}$ linkage PCR amplicon. SRC14 was chosen as a representative for this 1170 bp PCR product (1.0 % agarose gel). **B** Gel electrophoresis of *ScaI* restriction digestion of the *tnpB*-*bla*$_{TEM-1}$ linkage PCR amplicon. SRC14 was chosen as a representative to show the digested fragments of 861 bp and 309 bp (2.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

5.3.1.4 The *bla*$_{TEM-1}$-IS*26* linkage PCR amplification

A linkage PCR was designed to link the *bla*$_{TEM-1}$-IS*26* region (Figure 5.3). The PCR product is predicted to be 2060 bp according to the sequence on plasmid pRSB107, however the result of this amplification showed that 10 strains were negative. For the positive strains, three different sized PCR amplicons were obtained. The PCR amplicons of 10 strains were of the expected 2060 bp. Two PCR amplicons were obtained for both

strains SRC27 and SRC28, the sizes of which were approximately 2060 bp and 1000 bp. The PCR amplicon from SRC31 was smaller than the others, being 1964 bp in size (Figure 5.9A). DNA sequence analysis of the 2060 bp amplicon from strain SRC26 confirmed that it was identical to the *bla*$_{TEM-1}$-IS*26* fragment from an uncultured bacterium pRSB107 plasmid (accession no. AJ851089), *S. enterica* serovar Paratyphi A (accession no. AM412236) and *S. enterica* serovar Choleraesuis (accession no. AB366440).

All the PCR amplicons were further characterised by restriction enzyme digestion using *Bam*HI. The 2060 bp PCR amplicons were digested into two fragments with expected sizes of 1233 bp and 827 bp. The 2060 bp PCR amplicons from strains SRC27 and SRC28 were also digested into two fragments of 1233 bp and 827 bp, but the 1000 bp PCR amplicons were not digested (fragment indicated by arrowhead; Figure 5.9B). Further analysis needs to be undertaken for strains SRC27 and SRC28. The 1964 bp PCR amplicon from strain SRC31 was digested into two fragments of approximately 1233 bp and 731 bp (Figure 5.9B).

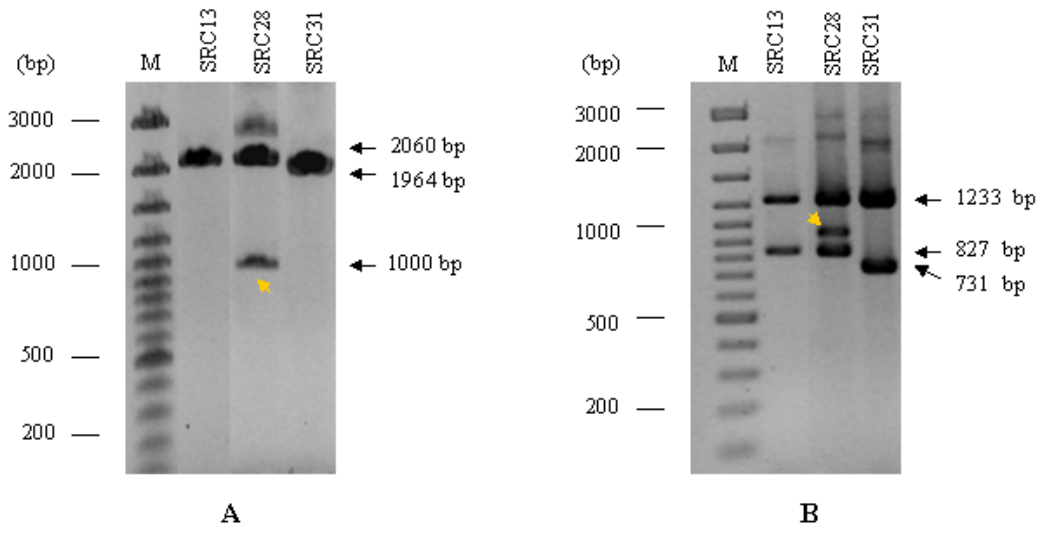


Figure 5.9A Gel electrophoresis of the *bla*$_{TEM-1}$-IS*26* linkage PCR amplicons. Strain SRC13 is a representative for the 2060 bp PCR amplicon, SRC28 is a representative for

the 2060 bp and 1000 bp amplicons, while SRC31 produces a 1964 bp amplicon (1.0% agarose gel). **B** Gel electrophoresis of *Bam*HI restriction digestion of the bla_{TEM-1}-IS*26* linkage PCR amplicons. SRC13 shows the digested fragments of 1233 bp and 827 bp. SRC27 is used to show the digested fragments of 1233 bp and 827 bp, and also the undigested amplicon of 1000 bp. Strain SRC31 shows the digested fragments of 1233 bp and 731 bp (2.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

5.3.1.5 Southern hybridisation using a bla_{TEM} gene probe to *Pst*I-digested whole cell chromosomal DNA derived from 23 *S. enterica* serovar Typhimurium strains.

Southern hybridisation using a bla_{TEM} gene probe was performed for 23 strains in order to confirm that the IS*26* element was downstream of the bla_{TEM-1} gene and whether it was the only bla_{TEM-1} gene carried by these *S. enterica* serovar Typhimurium strains. *Pst*I was used to digest the chromosomal DNA of the 23 *Salmonella* strains. *Pst*I cuts IS*26* and bla_{TEM-1} and cleaves the IS*26*-△*tnpB*-bla_{TEM-1}-IS*26* region into two fragments of 1007 bp and 1221 bp (Figure 5.10A).

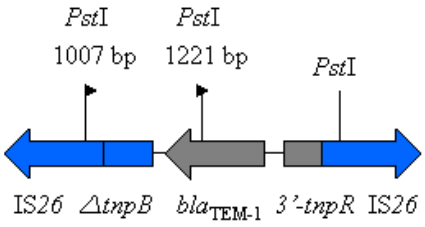


Figure 5.10A The predicted restriction digestion map of the IS*26*-△*tnpB*-bla_{TEM-1}-3′-*tnpR*-IS*26* region using *Pst*I (accession no. AJ851089). This region is digested into two fragments of 1007 bp and 1221 bp.

The Southern hybridisation result revealed that there were three different bla_{TEM} hybridisation profiles among the 23 *S. enterica* serovar Typhimurium strains. One profile produced two fragments of approximately 1.0 kb and 1.2 kb in sizes as expected, and these 11 strains contained the IS*26*-△*tnpB*-bla_{TEM-1}-3′-*tnpR*-IS*26* gene cluster. The second profile found in 10 strains had two fragments of approximately 1.0 kb and 3.5 kb in size. These 10 strains only contained the IS*26*-△*tnpB*-bla_{TEM-1} region without the IS*26* element downstream of the bla_{TEM-1} gene. The third profile found on strains

SRC27 and SRC28 gave three fragments of approximately 1.0 kb, 1.2 kb and 10 kb, which suggested that these two strains may contain two or more bla_{TEM-1} genes in their genome (Figure 5.10B). Southern hybridisations of *Pst*I-digested DNA using the bla_{TEM} gene probe further confirmed the results of the IS*26-tnpB*, *tnpB*-bla_{TEM-1} and bla_{TEM-1}-IS*26* linkage PCR amplifications. Those strains which were positive for all three PCR amplifications produced hybridisation fragments of 1.0 kb and 1.2 kb. Those strains positive for IS*26-tnpB* and *tnpB*-$bla_{TEM-1,}$ but negative for bla_{TEM-1}-IS*26* linkage PCR, generated bands of 1.0 kb and 3.5 kb.

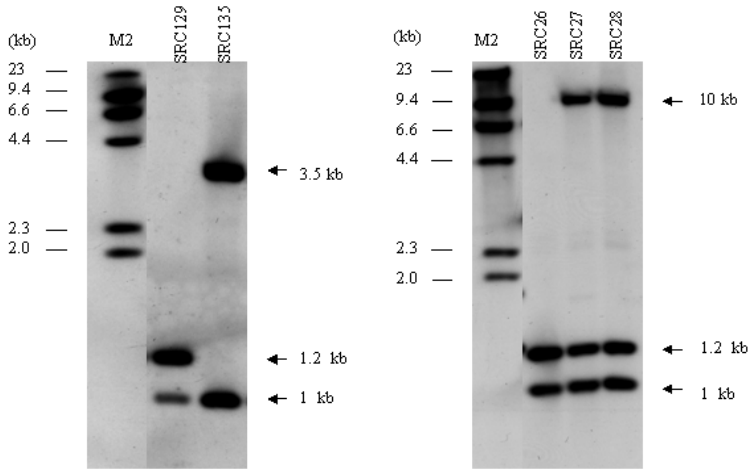


Figure 5.10B Southern hybridisation using the bla_{TEM} gene probe for 23 *Salmonella* strains digested with *Pst*I. SRC129 and SRC26 were chosen as representatives to show the fragments of 1 kb and 1.2 kb. SRC135 was used to show the fragments of 1 kb and 3.5 kb. SRC27 and SRC28 showed the fragments of 1 kb, 1.2 kb and 10 kb. DIG-labeled marker II (Roche) is labeled M2 and the corresponding sizes are given on the left.

5.3.1.6 Linking the kanamycin/neomycin resistance gene *aphAI* to *strB*

Given 15 strains were kanamycin resistant in the group of strains under investigation, a linkage PCR was designed to determine if the *aphAI* is linked to *strB* as it is in the sequence from plasmid pRSB107 (accession no. AJ851089). The primer pair aphAI-IAB(R)/strB-out used to amplify this region is indicated in Figure 5.3. Standard PCR amplification was performed for all 23 *S. enterica* serovar Typhimurium strains and a positive PCR amplicon of the expected size, 1460 bp, was obtained for 14 strains

(Figure 5.11A). All 14 strains displayed resistance to kanamycin. One strain, SRC30, displayed resistance to kanamycin, but did not produce an amplicon with this PCR. DNA sequence analysis of a representative amplicon from strain SRC26 confirmed that it was identical to the region from plasmid pRSB107 (accession no. AJ851089), *S. enterica* serovar Typhimurum (accession no. AY333434) and *E. coli* (accession no. CP000971). All the PCR amplicons were characterised by restriction enzyme digestion using *Dra*I. Two fragments were obtained with the expected sizes of 1196 bp and 264 bp (Figure 5.11B). All the PCR amplicons produced the same RFLP profile.

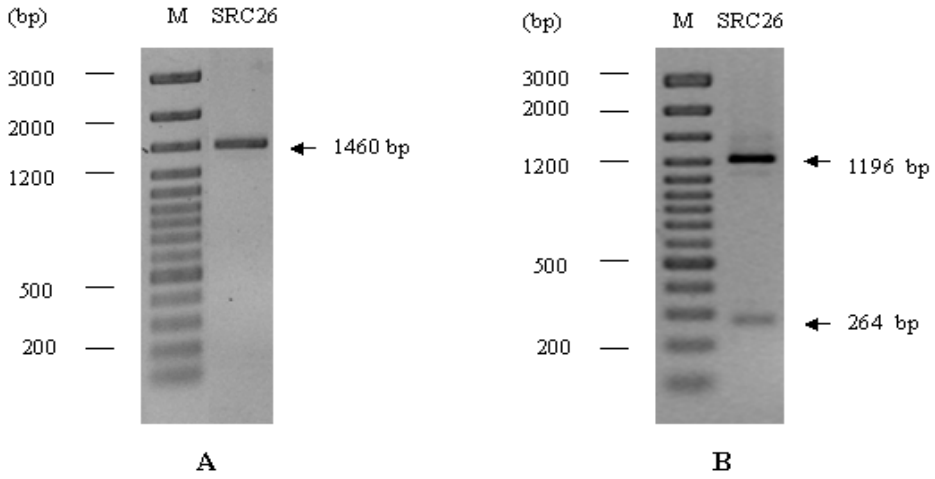


Figure 5.11A Gel electrophoresis of the *aphAI-strB* linkage PCR amplicon. Strain SRC26 was chosen as a representative for the 1460 bp amplicon produced for 14 strains (1.0% agarose gel). **B** Gel electrophoresis of *Dra*I restriction digestion of the *aphAI-strB* linkage PCR amplicon. The PCR product digested with *Dra*I for strain SRC26 (producing fragments of 1196 bp and 264 bp) is shown as a representative (2.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

5.3.1.7 The IS*26-aphAI* linkage PCR

A further linkage PCR from IS*26* to *aphAI* was designed according to the sequence of plasmid pRSB107 (accession no. AJ851089). The primer pair JL-D2/aphAI-IAB(F) was used to amplify the IS*26-aphAI* region (see Figure 5.3). Standard PCR amplification was performed for the 23 *S. enterica* serovar Typhimurium strains and a positive PCR amplicon was obtained for 14 of these, which are kanamycin resistant. The PCR product

size was 1093 bp as predicted from the plasmid pRSB107 sequence (Figure 5.12A). DNA sequence analysis of the amplicon from strain SRC26 revealed that it was identical to the corresponding fragment from plasmid pRSB107 (accession no. AJ851089), *S. enterica* serovar Choleraesuis (accession no. EU219534) and *S. enterica* serovar Typhimurum (accession no. AY333434). All the PCR amplicons were further characterised by digestion with *Hind*III. Two restriction fragments were obtained with the predicted sizes of 533 bp and 560 bp. These two fragments could not be discriminated by gel electrophoresis (Figure 5.12 B). Strain SRC30, which is resistant to kanamycin, was negative for both *aphAI-strB* and IS*26-aphAI* linkage PCR amplifications.

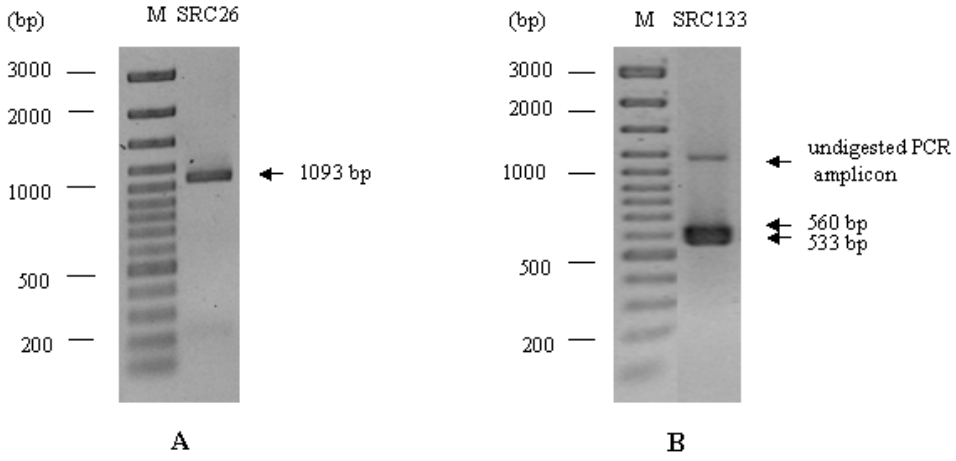


Figure 5.12A Gel electrophoresis of the IS*26-aphAI* linkage PCR amplicon. The 1093 bp amplicon from strain SRC26 was chosen as a representative (1.0% agarose gel). **B** Gel electrophoresis of *Hind*III restriction enzyme digestion of the IS*26-aphAI* linkage PCR amplicon. *Hind*III fragments of 560 bp and 533 bp derived by digestion of the 1093 bp amplicon from SRC133 were used as a representative (2.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

5.3.1.8 The *dfrA5-intI1* linkage PCR

Because 21 *S. enterica* serovar Typhimurium strains contained the class 1 integron specific integrase gene *intI1*, an analysis of the gene cassette in the integron was undertaken. Cassette associated trimethoprim resistance was found to be associated with

the *dfrA5* gene cassette, based on previous studies on the 136 isolates within the complete strain collection (R. Levings, personal communication). Normally, the *dfrA5* gene cassette is linked to the class 1 integron specific integrase gene *intI1*, and thus a primer pair JL-D5/L1 was designed (see Figure 5.5). PCR amplification was performed for 23 *S. enterica* serovar Typhimurium strains and a positive PCR amplicon of 555 bp was obtained for 19 strains (Figure 5.13A). DNA sequence analysis of the PCR product from strain SRC26 confirmed that it was identical to the *dfrA5-intI1* fragment from *E. coli* (accession no. AB188269), plasmid pLM020 (accession no. X12868) and an uncultured bacterium (accession no. AY139589).

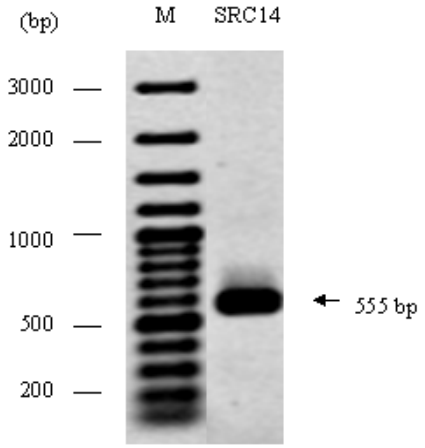


Figure 5.13A Gel electrophoresis of the *dfrA5-intI1* linkage PCR amplicon. Strain SRC14 was chosen as a representative for the 555 bp PCR amplicon (1.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given to the left of the figure.

All the PCR amplicons were characterised by restriction enzyme digestion using *Ase*I. Two fragments of 131 bp and 424 bp in sizes were produced as expected. All 19 strains gave the same RFLP profile (Figure 5.13B).

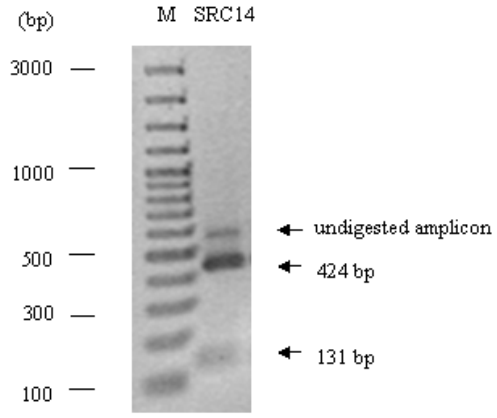


Figure 5.13B Gel electrophoresis of *Ase*I restriction enzyme digestion of the *dfrA5-intI1* linkage PCR amplicon. Strain SRC14 was used as a representative to show the digested fragments of 131 bp and 424 bp (2.0% agarose gel). M, 100 bp plus DNA ladders (Fermentas).

5.3.1.9 Summary

The results of Southern hybridisations of the bla_{TEM}, *intI1* and *merA* gene probes were collated with all the linkage PCR amplification data, which classified the 23 *S. enterica* serovar Typhimurium strains into 3 groups (Table 5.3). One group includes 10 strains containing the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$ antibiotic resistance gene cluster. All of these strains contained the integrase gene *intI1* with *dfrA5* gene cassette and 7 of these also contained the *merA* gene. Nine of these strains also contained the *aphAI* gene located upstream of this antibiotic resistance gene cluster (Table 5.3).

The second group accounted for 11 strains, which contained the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster. Nine of these contained the integrase gene *intI1* with *dfrA5* gene cassette and *merA* gene. The other two strains from this group were found to contain the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster, but did not harbour *intI1*, *dfrA5* cassette or *merA* genes. Only 3 strains from this group were found to contain the *aphAI* gene (Table 5.3).

The third group accounted for two strains, SRC27 and SRC28, containing either the antibiotic resistance gene cluster IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*26* or IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$. These strains both contained the *intI1* gene but not the *merA* gene or *dfrA5* gene cassette. Both strains harboured the *aphAI* gene which was located upstream of the antibiotic resistance gene cluster (Table 5.3). Additional investigations are needed to further characterise these two strains. All the linkage PCR amplifications for each of the 23 *Salmonella* strains are listed in Appendix II (Table B).

Table 5.3 The results of the antibiotic resistance gene clusters and Southern hybridisations (*bla*$_{TEM}$, *intI1* and *merA*) for 23 *S. enterica* serovar Typhimurium strains.

Strain no.	Serovar	Source	Resistance phenotype	Antibiotic resistance gene cluster	*bla*$_{TEM}$ (S)	*intI1* (S)	*merA* (S)
Group 1							
SRC30	Typhimurium PT141var4	P	ApGmKmSmSuTcTp	IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	-
SRC29	Typhimurium PT141	P	ApGmKmSmSuTcTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	+
SRC32	Typhimurium PT68	B	ApKmSmSuTcTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	+
SRC117	Typhimurium PT141	P	ApGmKmSmSuTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	+
SRC118	Typhimurium PT141	P	ApGmKmSmSuTcTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	+
SRC120	Typhimurium PT29	P	ApKmSmSuTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	+
SRC122	Typhimurium PT29	P	ApKmSmSuTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	-
SRC123	Typhimurium PT29	P	ApKmSmSuTcTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	-
SRC135	Typhimurium RDNC	P	ApKmSmSuTcTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	+
SRC136	Typhimurium RDNC	P	ApKmSmSuTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	+
Group 2							
SRC13	Typhimurium PT135	H	ApSmSuTcTp	IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	+	+
SRC14	Typhimurium PT44	H	ApSmSuTcTp	IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	+	+
SRC31	Typhimurium PT44	B	ApSmSuTcTp	IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	+	+
SRC124	Typhimurium PT44	B	ApSmSuTcTp	IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	+	+
SRC126	Typhimurium PT44	B	ApSmSuTp	IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	+	+
SRC128	Typhimurium PT44	B	ApSmSuTcTp	IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	+	+
SRC131	Typhimurium PT9	B	ApSmSuTp	IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	+	+
SRC132	Typhimurium PT9	B	ApSmSu	IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	-	-
SRC26	Typhimurium PT1var2	B	ApKmSmSuTcTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	+	+
SRC129	Typhimurium PT44	B	ApKmSmSuTcTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	+	+
SRC133	Typhimurium PT9	B	ApKmSmSu	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*	+	-	-
Group 3							
SRC27	Typhimurium PT135	E	ApCmGmKmSmSpSuTcTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	-
SRC28	Typhimurium PT135	E	ApCmGmKmSmSpSuTcTp	IS*26*-*aphIAB*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$	+	+	-

S indicates Southern hybridisation.

5.3.2 Further characterisation for 11 *S. enterica* serovar Typhimurium strains in group 2 (Table 5.3)

5.3.2.1 PCR amplifications of Tn*1*-IS*6100*, IS*6100*-*sul1* and *sul1*-*intI1* regions from strain SRC26

In order to explore genes flanking the $bla_{\text{TEM-1}}$-IS*26* region in strain SRC26, a linkage PCR was undertaken to link the $bla_{\text{TEM-1}}$ gene with the *mph*(A) gene of the macrolide-resistance operon *mph*(A)-*mrx*-*mphR*(A). This structure is found downstream of the $bla_{\text{TEM-1}}$-IS*26* region on plasmid pRSB107, but no PCR amplicon was detected for SRC26. PCR amplifications were also undertaken to link the *mph*(A) gene to the *mrx* gene and *mrx* to the *mphR*(A) gene of the macrolide-resistance operon for strain SRC26, however no PCR amplicon was obtained. Apparently, there is no macrolide-resistance operon *mph*(A)-*mrx*-*mphR*(A) within the genome of strain SRC26. A further linkage PCR was designed to link Tn*1* to IS*6100* because IS*6100* is detected downstream of the macrolide-resistance operon *mph*(A)-*mrx*-*mphR*(A) on plasmid pRSB107. A gradient PCR was performed using the primer pair JL-D44/DB-T1 (Figure 5.4) to link Tn*1* with IS*6100*. A PCR amplicon of approximately 1.7 kb was obtained for strain SRC26 (Figure 5.14A). DNA sequence analysis revealed that the amplicon encompassed an IS*6100* element linked to the IS*26*-$bla_{\text{TEM-1}}$ region.

There is a gene cassette *dhfR* and a class 1 integron specific integrase gene *intI1* located downstream of the IS*6100* element on plasmid pRSB107, so a linkage PCR was designed to link the IS*6100* element with the *intI1* gene for strain SRC26, but no amplicon was generated. Considering strain SRC26 contains the *sul1* gene (data not shown), there is a possibility that the *sul1* gene is a part of the class 1 integron and might be located not far away from the IS*6100* element. To confirm this hypothesis, two primer pairs IS6100-Rv2/sul1-F(C) targeting the IS*6100*-*sul1* region and sul1-R/L2

targeting the *sul1-intI1* region were designed (Figure 5.4). A PCR amplicon approximately 2.5 kb was obtained for the IS*6100-sul1* linkage PCR (Figure 5.14B). DNA sequence analysis of this PCR product revealed that it encompassed orf5 and *sul1* genes downstream of the IS*6100* element. Subsequently, a PCR amplicon of approximately 2.0 kb was obtained for the *sul1-intI1* linkage PCR (Figure 5.14C). DNA sequence analysis revealed that this PCR product contained *qacE△1*, *dfrA5* and *intI1* genes after *sul1*. These data suggest that an In4-like class 1 integron is located downstream of the $bla_{\text{TEM-1}}$-IS*26* region with the 25 bp inverted repeat of the IS*6100* element downstream of the IS*26* element missing. DNA sequence analysis of the three PCR amplicons revealed that SRC26 contained a △IS*6100*-orf5-*sul1-qacE△1-dfrA5-intI1* structure downstream of the IS*26-strB-strA-sul2-repC-repA*-IS*26*-$bla_{\text{TEM-1}}$-IS*26* gene cluster.

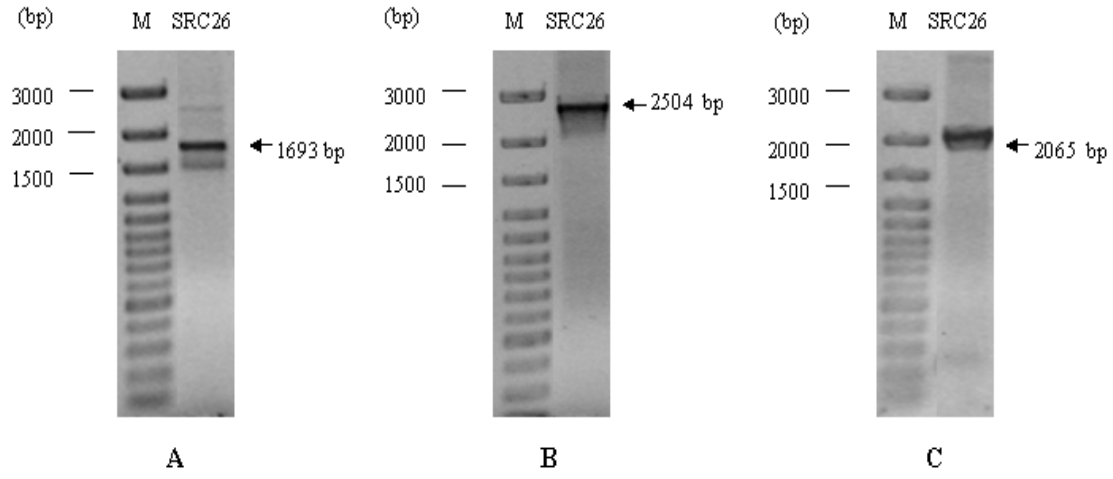


Figure 5.14A Gel electrophoresis of the Tn*1*-IS*6100* linkage PCR amplicon for strain SRC26. **B** Gel electrophoresis of the IS*6100-sul1* linkage PCR amplicon for SRC26. **C** Gel electrophoresis of the *sul1-intI1* PCR product for SRC26 (1.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

5.3.2.2 PCR amplification of Tn*1*-*dfrA5* from the other *S. enterica* serovar Typhimurium strains

5.3.2.2.1 Tn*1*-*dfrA5* linkage PCR

As no IS*6100* element and *sul1* gene were detected in strain SRC14 (data not shown), a primer pair JL-D44/JL-D6 (Figure 5.5) was designed to link Tn*1* to the *dfrA5* cassette directly for strain SRC14. A positive PCR amplicon of approximately 1770 bp was obtained. This PCR was carried out for other strains including SRC13, SRC31, SRC124, SRC126, SRC128, SRC129, SRC131, SRC132 and SRC133 in group 2. In total, three different sizes of PCR amplicons of approximately 2027 bp, 1770 bp and 1674 bp were obtained. Six strains including SRC13, SRC14, SRC124, SRC126, SRC128 and SRC131 contained the amplicon of 1770 bp. SRC31 contained the 1674 bp amplicon and the amplicon size of SRC129 was 2027 bp (Figure 5.15A). Two strains, SRC132 and SRC133, were negative for this PCR which can be readily explained due to the absence of the *dfrA5* cassette in these strains.

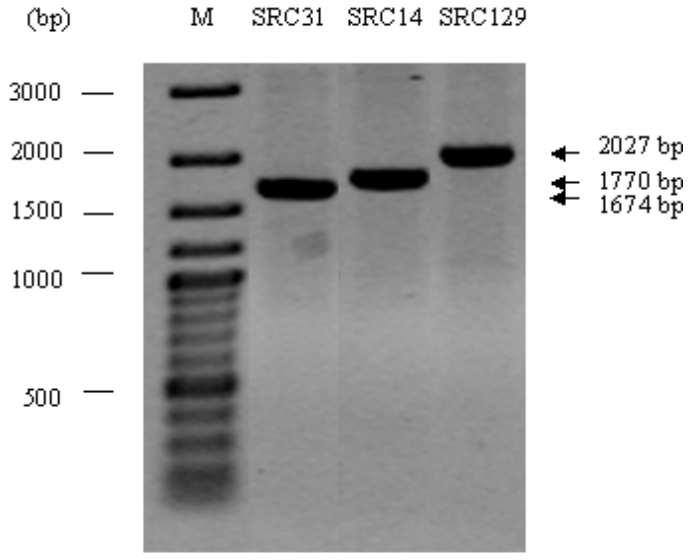


Figure 5.15A Gel electrophoresis of the Tn*1*-*dfrA5* linkage PCR amplicons. SRC31 was used to show the 1674 bp amplicon, SRC14 was chosen as a representative for the 1770 bp amplicon and SRC129 produced the 2027 bp amplicon (1.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

5.3.2.2.2 *Pst*I enzyme digestion of the Tn*1-dfrA5* linkage PCR amplicons

All the PCR amplicons were characterised by digestion with restriction enzyme *Pst*I. The 2027 bp amplicon from strain SRC129 was digested to two fragments of 1402 bp and 625 bp. The 1770 bp amplicons from six strains were digested to 1145 bp and 625 bp fragments. Two digested fragments of 1145 bp and 529 bp were obtained for strain SRC31 (Figure 5.15B).

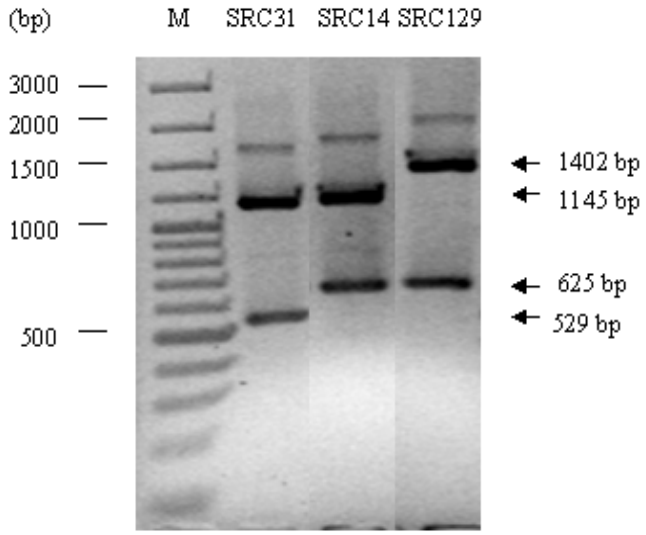


Figure 5.15B *Pst*I enzyme restriction digestion of the Tn*1-dfrA5* linkage PCR amplicons. Strain SRC31 showed the digested fragments of 1145 bp and 529 bp for the 1674 bp PCR amplicon. Strain SRC14 was chosen as a representative for the digested fragments of 1145 bp and 625 bp for the 1770 bp PCR amplicon. SRC129 was chosen to show the restriction digestion result of the 2027 bp amplicon, where 1402 bp and 625 bp fragments were obtained on a 2.0% agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

The DNA sequence alignment of the three different sized Tn*1-dfrA5* linkage PCR amplcons (Figure 5.16) revealed that the 3′-*tnpR* gene in SRC31 was 96 bp shorter than other strains such as SRC14. Compared to those other strains, SRC129 contained an extra 257 bp nucleotides, including 225 bp of the *qacE△1* gene and 32 bp of the 3′-CS of the class 1 integron (Figure 5.16). Six strains including SRC13, SRC14, SRC124, SRC126, SRC128 and SRC131, contained the same $bla_{\text{TEM-1}}$-3′-*tnpR*-IS*26-dfrA5*

structure. The structure of SRC31 was *bla*$_{TEM-1}$-3′-△*tnpR*-IS*26*-*dfrA5* and SRC129 contained a *bla*$_{TEM-1}$-3′-*tnpR*-IS*26*-△*qacE*△*1*-*dfrA5* structure.

```
                                        3′-tnpR                                  50
Paratyphi   CGACAGGATG GTGACCACCA TTTGCCCCAT ATCACCGTCG GTACTGATCC
Typhi       CGACAGGATG GTGACCACCA TTTGCCCCAT AT-------- ----------
SRC31       CGACAGGATG GTGACCACCA TT-------- ---------- ----------
SRC14       CGACAGGATG GTGACCACCA TTTGCCCCAT ATCACCGTCG GTACTGATCC
SRC129      CGACAGGATG GTGACCACCA TTTGCCCCAT ATCACCGTCG GTACTGATCC
                                                                    100
Paratyphi   CGTCATCAAT GAACCGGACT GCCACGCCCT GAGCGTCAAA TTCCTTTATC
Typhi       ---------- ---------- ---------- ---------- ----------
SRC31       ---------- ---------- ---------- ---------- ----------
SRC14       CGTCATCAAT GAACCGGACT GCCACGCCCT GAGCGTCAAA TTCCTTTATC
SRC129      CGTCATCAAT GAACCGGACT GCCACGCCCT GAGCGTCAAA TTCCTTTATC
                                  IR of IS26      IS26                      150
Paratyphi   AGTTGGATCA TATCGGCGGC ACTGTTGCAA ATAGTCGGTG GTGATAAACT
Typhi       ---------- -------GGC ACTGTTGCAA ATAGTCGGTG GTGATAAACT
SRC31       ---------- -------GGC ACTGTTGCAA ATAGTCGGTG GTGATAAACT
SRC14       AGTTGGATCA TATCGGCGGC ACTGTTGCAA ATAGTCGGTG GTGATAAACT
SRC129      AGTTGGATCA TATCGGCGGC ACTGTTGCAA ATAGTCGGTG GTGATAAACT
                                                                    200
Paratyphi   TATCATCCCC TTTTGCTGAT GGAGCTGCAC ATGAACCCAT TCAAAGGCCG
Typhi       TATCATCCCC TTTTGCTGAT GGAGCTGCAC ATGAACCCAT TCAAAGGCCG
SRC31       TATCATCCCC TTTTGCTGAT GGAGCTGCAC ATGAACCCAT TCAAAGGCCG
SRC14       TATCATCCCC TTTTGCTGAT GGAGCTGCAC ATGAACCCAT TCAAAGGCCG
SRC129      TATCATCCCC TTTTGCTGAT GGAGCTGCAC ATGAACCCAT TCAAAGGCCG
                                                                    250
Paratyphi   GCATTTTCAG CGTGACATCA TTCTGTGGGC CGTACGCTGG TACTGCAAAT
Typhi       GCATTTTCAG CGTGACATCA TTCTGTGGGC CGTACGCTGG TACTGCAAAT
SRC31       GCATTTTCAG CGTGACATCA TTCTGTGGGC CGTACGCTGG TACTGCAAAT
SRC14       GCATTTTCAG CGTGACATCA TTCTGTGGGC CGTACGCTGG TACTGCAAAT
SRC129      GCATTTTCAG CGTGACATCA TTCTGTGGGC CGTACGCTGG TACTGCAAAT
                                                                    300
Paratyphi   ACGGCATCAG TTACCGTGAG CTGCAGGAGA TGCTGGCTGA ACGCGGAGTG
Typhi       ACGGCATCAG TTACCGTGAG CTGCAGGAGA TGCTGGCTGA ACGCGGAGTG
SRC31       ACGGCATCAG TTACCGTGAG CTGCAGGAGA TGCTGGCTGA ACGCGGAGTG
SRC14       ACGGCATCAG TTACCGTGAG CTGCAGGAGA TGCTGGCTGA ACGCGGAGTG
SRC129      ACGGCATCAG TTACCGTGAG CTGCAGGAGA TGCTGGCTGA ACGCGGAGTG
                                                                    350
Paratyphi   AATGTCGATC ACTCCACGAT TTACCGCTGG GTTCAGCGTT ATGCGCCTGA
Typhi       AATGTCGATC ACTCCACGAT TTACCGCTGG GTTCAGCGTT ATGCGCCTGA
SRC31       AATGTCGATC ACTCCACGAT TTACCGCTGG GTTCAGCGTT ATGCGCCTGA
SRC14       AATGTCGATC ACTCCACGAT TTACCGCTGG GTTCAGCGTT ATGCGCCTGA
SRC129      AATGTCGATC ACTCCACGAT TTACCGCTGG GTTCAGCGTT ATGCGCCTGA
                                                                    400
Paratyphi   AATGGAAAAA CGGCTGCGCT GGTACTGGCG TAACCCTTCC GATCTTTGCC
Typhi       AATGGAAAAA CGGCTGCGCT GGTACTGGCG TAACCCTTCC GATCTTTGCC
SRC31       AATGGAAAAA CGGCTGCGCT GGTACTGGCG TAACCCTTCC GATCTTTGCC
SRC14       AATGGAAAAA CGGCTGCGCT GGTACTGGCG TAACCCTTCC GATCTTTGCC
SRC129      AATGGAAAAA CGGCTGCGCT GGTACTGGCG TAACCCTTCC GATCTTTGCC
                                                                    450
Paratyphi   CGTGGCACAT GGATGAAACC TACGTGAAGG TCAATGGCCG CTGGGCGTAT
Typhi       CGTGGCACAT GGATGAAACC TACGTGAAGG TCAATGGCCG CTGGGCGTAT
SRC31       CGTGGCACAT GGATGAAACC TACGTGAAGG TCAATGGCCG CTGGGCGTAT
SRC14       CGTGGCACAT GGATGAAACC TACGTGAAGG TCAATGGCCG CTGGGCGTAT
SRC129      CGTGGCACAT GGATGAAACC TACGTGAAGG TCAATGGCCG CTGGGCGTAT
```

```
                                                                      500
Paratyphi    CTGTACCGGG CCGTCGACAG CCGGGGCCGC ACTGTCGATT TTTATCTCTC
Typhi        CTGTACCGGG CCGTTGACAG CCGGGGCCGC ACTGTCGATT TTTATCTCTC
SRC31        CTGTACCGGG CCGTCGACAG CCGGGGCCGC ACTGTCGATT TTTATCTCTC
SRC14        CTGTACCGGG CCGTCGACAG CCGGGGCCGC ACTGTCGATT TTTATCTCTC
SRC129       CTGTACCGGG CCGTCGACAG CCGGGGCCGC ACTGTCGATT TTTATCTCTC
                                                                      550
Paratyphi    CTCCCGTCGT AACAGCAAAG CTGCATACCG GTTTCTGGGT AAAATCCTCA
Typhi        CTCCCGTCGT AACAGCAAAG CTGCATACCG GTTTCTGGGT AAAATCCTCA
SRC31        CTCCCGTCGT AACAGCAAAG CTGCATACCG GTTTCTGGGT AAAATCCTCA
SRC14        CTCCCGTCGT AACAGCAAAG CTGCATACCG GTTTCTGGGT AAAATCCTCA
SRC129       CTCCCGTCGT AACAGCAAAG CTGCATACCG GTTTCTGGGT AAAATCCTCA
                                                                      600
Paratyphi    ACAACGTGAA GAAGTGGCAG ATCCCGCGAT TCATCAACAC GGATAAAGCG
Typhi        ACAACGTGAA GAAGTGGCAG ATCCCGCGAT TCATCAACAC GGATAAAGCG
SRC31        ACAACGTGAA GAAGTGGCAG ATCCCGCGAT TCATCAACAC GGATAAAGCG
SRC14        ACAACGTGAA GAAGTGGCAG ATCCCGCGAT TCATCAACAC GGATAAAGCG
SRC129       ACAACGTGAA GAAGTGGCAG ATCCCGCGAT TCATCAACAC GGATAAAGCG
                                                                      650
Paratyphi    CCCGCCTATG GTCGCGCGCT TGCTCTGCTC AAACGCGAAG GCCGGTGCCC
Typhi        CCCGCCTATG GTCGCGCGCT TGCTCTGCTC AAACGCGAAG GCCGGTGCCC
SRC31        CCCGCCTATG GTCGCGCGCT TGCTCTGCTC AAACGCGAAG GCCGGTGCCC
SRC14        CCCGCCTATG GTCGCGCGCT TGCTCTGCTC AAACGCGAAG GCCGGTGCCC
SRC129       CCCGCCTATG GTCGCGCGCT TGCTCTGCTC AAACGCGAAG GCCGGTGCCC
                                                                      700
Paratyphi    GTCTGACGTT GAACACCGAC AGATTAAGTA CCGGAACAAC GTGATTGAAT
Typhi        GTCTGACGTT GAACACCGAC AGATTAAGTA CCGGAACAAC GTGATTGAAT
SRC31        GTCTGACGTT GAACACCGAC AGATTAAGTA CCGGAACAAC GTGATTGAAT
SRC14        GTCTGACGTT GAACACCGAC AGATTAAGTA CCGGAACAAC GTGATTGAAT
SRC129       GTCTGACGTT GAACACCGAC AGATTAAGTA CCGGAACAAC GTGATTGAAT
                                                                      750
Paratyphi    GCGATCATGG CAAACTGAAA CGGATAATCG GCGCCACGCT GGGATTTAAA
Typhi        GCGATCATGG CAAACTGAAA CGGATAATCG GCGCCACGCT GGGATTTAAA
SRC31        GCGATCATGG CAAACTGAAA CGGATAATCG GCGCCACGCT GGGATTTAAA
SRC14        GCGATCATGG CAAACTGAAA CGGATAATCG GCGCCACGCT GGGATTTAAA
SRC129       GCGATCATGG CAAACTGAAA CGGATAATCG GCGCCACGCT GGGATTTAAA
                                                                      800
Paratyphi    TCCATGAAGA CGGCTTACGC CACCATCAAA GGTATTGAGG TGATGCGTGC
Typhi        TCCATGAAGA CGGCTTACGC CACCATCAAA GGTATTGAGG TGATGCGTGC
SRC31        TCCATGAAGA CGGCTTACGC CACCATCAAA GGTATTGAGG TGATGCGTGC
SRC14        TCCATGAAGA CGGCTTACGC CACCATCAAA GGTATTGAGG TGATGCGTGC
SRC129       TCCATGAAGA CGGCTTACGC CACCATCAAA GGTATTGAGG TGATGCGTGC
                                                                      850
Paratyphi    ACTACGCAAA GGCCAGGCCT CAGCATTTTA TTATGGTGAT CCCCTGGGCG
Typhi        ACTACGCAAA GGCCAGGCCT CAGCATTTTA TTATGGTGAT CCCCTGGGCG
SRC31        ACTACGCAAA GGCCAGGCCT CAGCATTTTA TTATGGTGAT CCCCTGGGCG
SRC14        ACTACGCAAA GGCCAGGCCT CAGCATTTTA TTATGGTGAT CCCCTGGGCG
SRC129       ACTACGCAAA GGCCAGGCCT CAGCATTTTA TTATGGTGAT CCCCTGGGCG
                                                                      900
Paratyphi    AAATGCGCCT GGTAAGCAGA GTTTTTGAAA TGTAAGGCCT TTGAATAAGA
Typhi        AAATGCGCCT GGTAAGCAGA GTTTTTGAAA TGTAAGGCCT TTGAATAAGA
SRC31        AAATGCGCCT GGTAAGCAGA GTTTTTGAAA TGTAAGGCCT TTGAATAAGA
SRC14        AAATGCGCCT GGTAAGCAGA GTTTTTGAAA TGTAAGGCCT TTGAATAAGA
SRC129       AAATGCGCCT GGTAAGCAGA GTTTTTGAAA TGTAAGGCCT TTGAATAAGA
                                    IR of IS26     qacEΔ1            950
Paratyphi    CAAAAGGCTG CCTCATCGCT AACTTTGCAA CAGTGCC--- ----------
Typhi        CAAAAGGCTG CCTCATCGCT AACTTTGCAA CAGTGCC--- ----------
SRC31        CAAAAGGCTG CCTCATCGCT AACTTTGCAA CAGTGCC--- ----------
SRC14        CAAAAGGCTG CCTCATCGCT AACTTTGCAA CAGTGCC--- ----------
SRC129       CAAAAGGCTG CCTCATCGCT AACTTTGCAA CAGTGCCACA CCGACAGGGA
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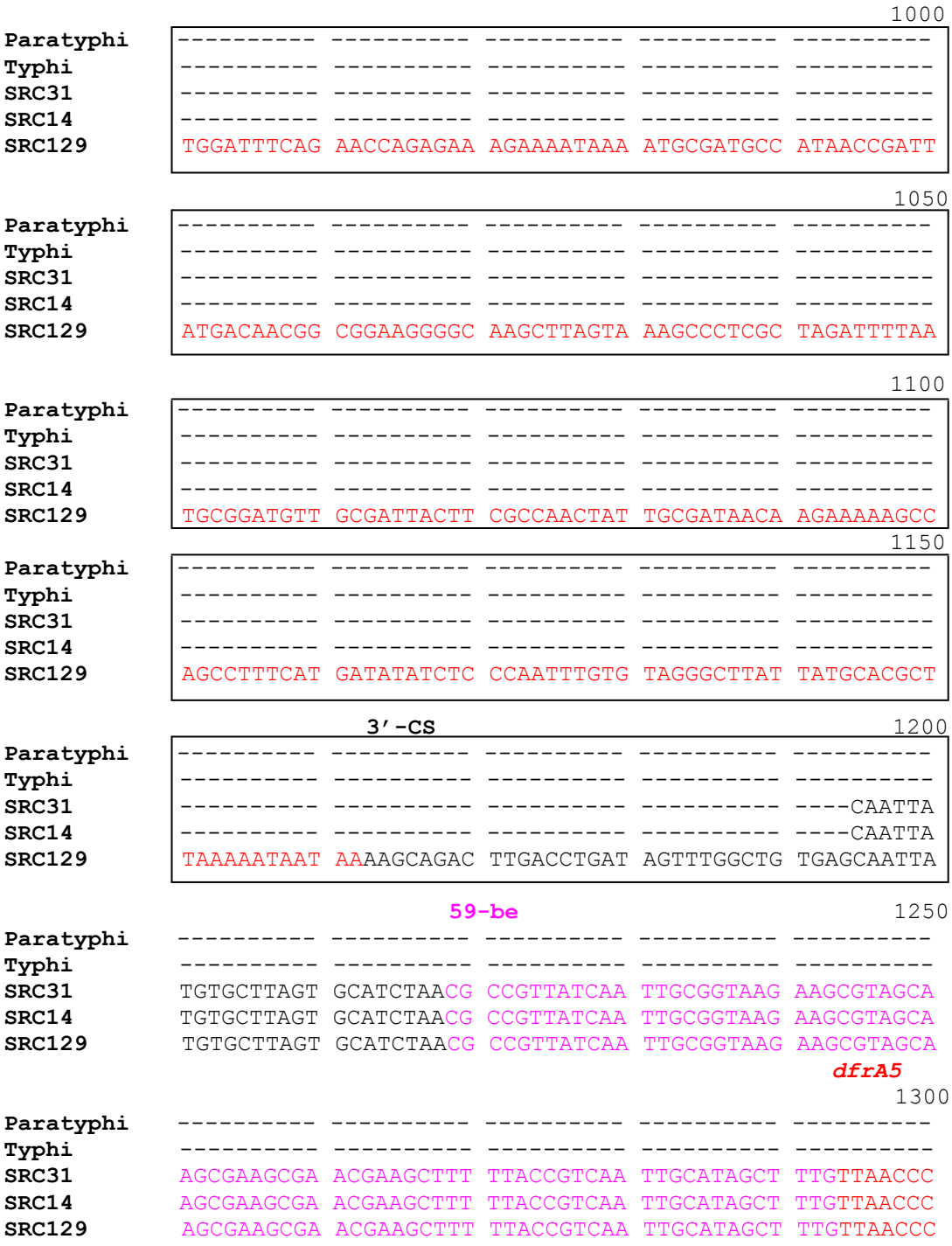


Figure 5.16 DNA sequence alignment of *S. enterica* serovar Paratyphi A (accession no. AM412236), *S. enterica* serovar Typhi CT18 (accession no. AL513383), and the Tn*1*-*dfrA5* linkage PCR amplicons from SRC31, SRC14 and SRC129. The dashed lines indicate spaces used to facilitate allignment.

5.3.2.3 Southern hybridisation using a *dfrA5* gene probe

Southern hybridisation using a *dfrA5* gene probe was carried out against three strains including SRC14, SRC26 and SRC129 to determine how many *dfrA5* cassettes were

present in these strains and also confirm these structures. Enzyme *Ban*II was chosen to digest chromosomal DNA because it does not cut the *dfrA5*. A *Ban*II fragment containing *dfrA5* from SRC14 was approximately 7 kb and it was approximately 1.7 kb for strain SRC26. Strain SRC129 contained two *dfrA5* cassettes and their sizes were approximately 6 kb and 7 kb (Figure 5.17). The sizes of these *Ban*II fragments were consistent with the structures identified in these strains.

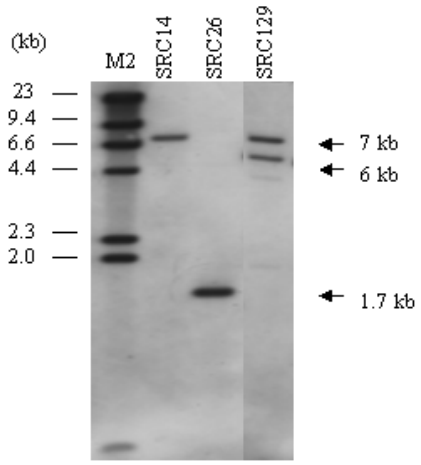


Figure 5.17 Southern hybridisation employing the *dfrA5* gene probe for 3 *S. enterica* serovar Typhimurium strains. SRC14 contained an approximately 7 kb *dfrA5* fragment. SRC26 was used to show the 1.7 kb fragment identified using the *dfrA5* gene probe. Strain SRC129 contained two *dfrA5* cassettes with the sizes of approximately 6 kb and 7 kb. DIG-labeled marker II (Roche) is labeled M2 and the corresponding sizes are given on the left.

5.3.2.4 Summary

In summary, strain SRC26 harboured the multiply antibiotic resistance gene cluster IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$-IS*26-*△IS*6100*-orf5-*sul1-qacE*△*1-dfrA5 -*intI1* (Figure 5.18). The 25 bp inverted repeat of the IS*6100* (on the left side) was missing. SRC129 was found to contain two *dfrA5* gene cassettes by Southern hybridisation. Apart from a *dfrA5* gene cassette within the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$-IS*26-qacE*△*1-dfrA5-intI1* gene cluster, this strain contained another *dfrA5* elsewhere (Figure 5.18). Strain SRC14 contained the

IS*26-strB-strA-sul2-repC-*△*repA*-IS*26-bla*$_{TEM-1}$-IS*26-dfrA5-intI1* antibiotic resistance gene cluster (Figure 5.18). Strain SRC31 contained the IS*26-strB-strA-sul2-repC-*△*repA*-IS*26-bla*$_{TEM-1}$-IS*26-dfrA5-intI1* gene cluster, however the 3′-*tnpR* gene located within the *bla*$_{TEM-1}$-IS*26* region was 96 bp shorter than that contained within other strains (Figure 5.18).

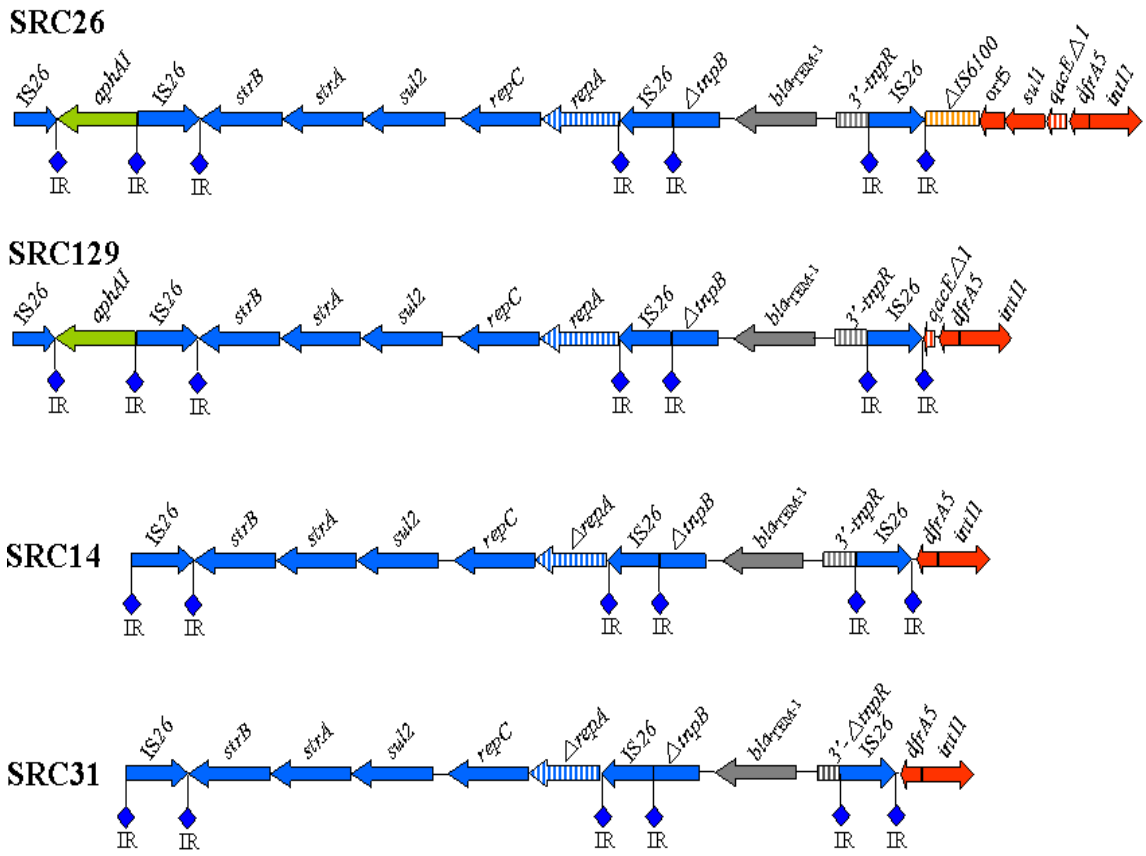


Figure 5.18 Illustration of the antibiotic resistance gene clusters found in different *S. enterica* serovar Typhimurium strains including SRC26, SRC129, SRC14 and SRC31. Differences are marked using stripes. The genes and their transcriptional orientations are indicated by arrows. The inverted repeat of IS*26* is indicated as IR.

5.3.3 IS*200* profiles

In order to investigate the clonal relationship between the 23 *S. enterica* serovar Typhimurium strains, a Southern hybridisation using an IS*200* gene probe was performed. Enzyme *Pst*I which does not cleave IS*200* was used to digest the

chromosomal DNA for all strains. This experiment was repeated one more time and the bands seen were genuine bands. Southern hybridisation with IS*200* probe revealed that 23 strains were divided into two groups. Group I (Figure 5.19A) (Table 5.4) contained 10 strains and possessed an IS*200* profile of six bands: 23 kb; 6.6 kb; 4.8 kb; 3.4 kb; 2.4 kb and 1.9 kb. The Southern hybridisation result of IS*200* suggests that these 10 strains are clonal. Group II contained 13 strains, which were further divided into 4 subgroups: IIa, IIb, IIc and IId (Figure 5.19A, Figure 5.19B) (Table 5.4). The IS*200* profile of group IIa (9 strains) was characterised by five bands of 23 kb, 9.4 kb, 4.8 kb, 2.4 kb and 1.9 kb. Group IIb (1 strain) shared the same five bands with group IIa, and carried an extra 5.0 kb band. Group IIc (2 strains) also shared the same 5 bands with group IIa, but carried an extra 2.3 kb band. Group IId (1 strain) shared the same 4 bands (23 kb, 9.4 kb, 4.8 kb and 1.9 kb) with group IIa, but carried two bands of 6.6 kb and 2.2 kb. Group I shared 4 bands (23 kb, 4.8 kb, 2.4 kb and 1.9 kb) in common with group II.

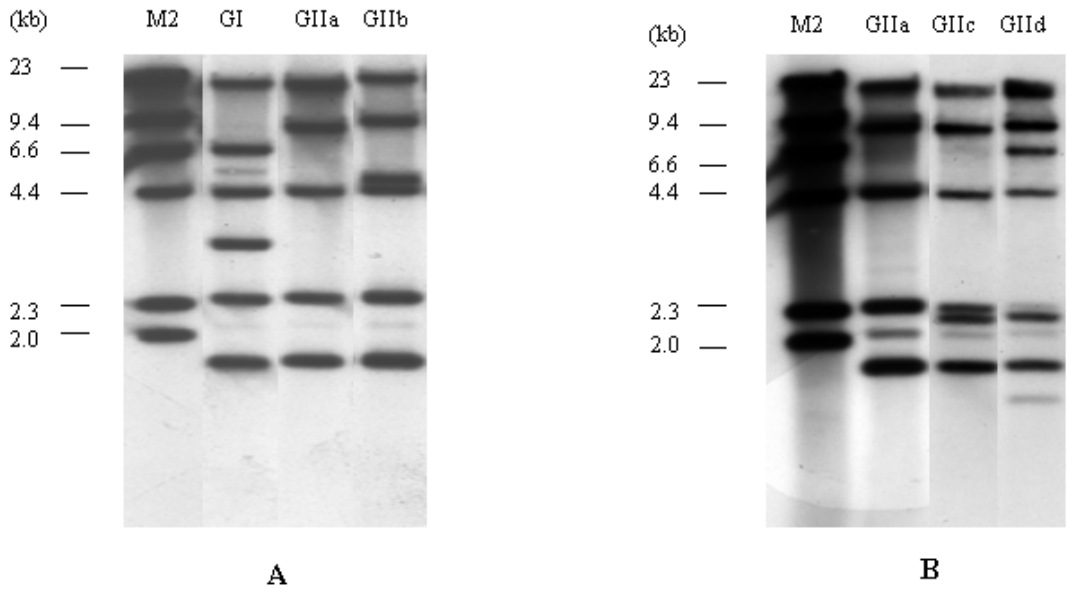


Figure 5.19 A Representatives IS*200* profiles for group I and group IIa and IIb. **B** Representatives IS*200* profiles for group IIa, IIc and IId. Abbreviations: GI, group I; GIIa, group IIa; GIIb, group IIb; GIIc, group IIc; GIId, group IId. DIG-labeled marker II (Roche) is labeled M2 and the corresponding sizes are given on the left.

Table 5.4 IS*200* profiles and their corresponding *S. enterica* serovar Typhimurium strains.

IS*200* profile	Strains
Group I	SRC29, SRC30, SRC32, SRC117, SRC118, SRC120, SRC122, SRC123, SRC135, SRC136
Group IIa	SRC13, SRC14, SRC27, SRC28, SRC31, SRC124, SRC126, SRC128, SRC129
Group IIb	SRC26
Group IIc	SRC132, SRC133
Group IId	SRC131

5.4 Discussion

Over 100 variants of the TEM β-lactamase have been identified and many of them display an extended-spectrum or an inhibitor-resistant phenotype (Bush and Jacoby, 1997; Chaibi et al., 1999; Goussard and Courvalin, 1999). These are all variants of TEM-1 or TEM-2 encoded by Tn*1*, Tn*2* and Tn*3* transposons. The $bla_{\text{TEM-1a}}$ found in Tn*3* and the $bla_{\text{TEM-1b}}$ identified in Tn*2* differs by three nucleotides but encode identical TEM-1 proteins. The $bla_{\text{TEM-2}}$ in Tn*1* differs by five or six nucleotides from the $bla_{\text{TEM-1}}$ types and there is a single amino acid difference between TEM-2 and TEM-1 proteins (Partridge and Hall, 2005). Plasmid-mediated β-lactamase TEM-1 is by far the most prevalent and produced by 80%–100% of ampicillin-resistant *Salmonella* strains. Other β-lactamases, for examples TEM-2, OXA-1, OXA-2 and SHV-1, are reported with frequency (Casin et al., 1999).

In this Chapter, bla_{TEM} Southern hybridisation was performed on 23 *S. enterica* serovar Typhimurium strains which were digested with *Pst*I and all of these were confirmed to contain the $bla_{\text{TEM-1}}$ gene. This Southern hybridisation was integral as it confirmed that IS*26*-△*tnpB*-$bla_{\text{TEM-1}}$-3′-*tnpR*-IS*26* was linked together, based on the sizes of the *Pst*I fragments. There is only one $bla_{\text{TEM-1}}$ gene on plasmid pRSB107 and the fragments

identified by Southern hybridisation should be the same as the structure found in that study. There are two *bla*$_{\text{TEM-1}}$ genes on plasmid pU302L, with the predicted Southern hybridisation profile of *bla*$_{\text{TEM}}$ gene probe differing. There should be 4 fragments including 1007 bp, 3244 bp, 3379bp and 6634 bp in the Southern hybridisation profile of pU302L according to its restriction digestion map of *Pst*I using the GCK 2.5 program. The Southern hybridisation of *bla*$_{\text{TEM}}$ probe is thus a very useful tool to identify the *bla*$_{\text{TEM}}$ gene and discriminate between different structures.

S. enterica serovar Typhimurium displays a great diversity on the basis of IS*200* copy number variations, which is a powerful tool for strain differentiation and estimation of their genomic relatedness (Stanley et al., 1993). From the result of the IS*200* Southern hybridisation, the 23 *S. enterica* serover Typhimurium strains were separated into two related clones. Except for one strain, SRC131, group I shared 4 bands including 23 kb, 4.8 kb, 2.4 kb and 1.9 kb in common with group II and the three bands (4.8 kb, 2.4 kb and 1.9 kb) are the *S. enterica* serovar Typhimurium specific serovar bands (Casin et al., 1999). The common size band of 23 kb suggested that these two groups were closely related. Apart from this similarity, group I contained two extra *Pst*I fragments of 6.6 kb and 3.4 kb in size, while group II contained an additional 9.4 kb fragment. The different bands of the two groups indicated that these strains might have recently diverged from each other and that the difference in the genes around *bla*$_{\text{TEM-1}}$ might, in part, explain these different patterns.

Strain SRC131 lacked the IS*200* element on the 2.4 kb fragment, which has been mapped within the *fliB-fliA* intergenic region of the flagllar gene complex of *S. enterica* serovar Typhimurium (Burnens et al., 1997). This unusual feature was also found in two

rare isolates from Asia, which lacked the 1.9 kb and 2.4 kb fragments and one isolate from France, which lacked the 2.4 kb fragment (Stanley et al., 1993; Casin et al., 1999). Considering the stability of the IS*200* insertion sites in the *S. enterica* serovar Typhimurium genome, strain SRC131 might represent a different clonal line of this serovar.

Ten strains containing the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$ gene cluster were found to possess the group I IS*200* pattern, which suggests that these strains are clonal. Additional studies by others have been taken using one of these strains, SRC32, further verifying the existence of this antibiotic resistance gene cluster. Additionally, this gene cluster of IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$ is located on a Tn*21*-like transposon (Connor O'Meara, unpublished data). The Tn*21* family is widely distributed in both clinical and environmental isolates of Gram-negative bacteria (Liebert et al., 1999). An epidemiological study, conducted in 1999, on genetic elements mediating multidrug resistance in pathogenic avian *E. coli* isolates, concluded that more than half of the one hundred isolates examined carried a Tn*21*-like transposon (Bass et al., 1999).

Thirteen strains were included in the group II IS*200* pattern. Eleven of these strains contained the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$-IS*26* gene cluster. The other two strains, SRC27 and SRC28, contained either the antibiotic resistance gene cluster IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$-IS*26* or IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$. Because these 13 strains were clonal, it is very possible that strain SRC27 and SRC28 contained the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$-IS*26* gene cluster and the other *bla*$_{\text{TEM-1}}$

gene was located elsewhere in these two strains. Plasmid pU302L possesses the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*1294* antibiotic resistance gene cluster and also contains another *bla*$_{\text{TEM-1}}$ gene on the same plasmid (Chen et al., 2007). It is also possible that these two *bla*$_{\text{TEM-1}}$ genes are harboured by two plasmids in one strain. More work need to be undertaken for these two strains to differentiate these possibilities.

Transposons Tn*1* and Tn*3* include the ampicillin resistance gene *bla*$_{\text{TEM-1,}}$ resolvase gene *tnpR* and transposase gene *tnpA* (see Figure 1.5) (Partridge and Hall, 2005). A comparison of the complete sequence of Tn*1* and Tn*3* revealed that they are about 99% identical to each other over most of their 4.95 kb lengths, with most of the differences confined to a region close to the *res* site (Partridge and Hall, 2005). On plasmid pRSB107, Szczepanowski et al. (2005) mentioned that the *bla*$_{\text{TEM-1}}$-3′-*tnpR* was a remnant of transposon Tn*1*. The insertion of IS*26* probably truncated the *tnpR* gene and deleted the *tnpA* gene of Tn*1* to form the *bla*$_{\text{TEM-1}}$-3′-*tnpR*-IS*26* structure (Szczepanowski et al., 2005). This hypothesis was supported by the data in this study. For strain SRC31, there was a further 96 bp nucleotide truncation of the 3′-*tnpR* gene, when compared to the 3′-*tnpR* of other strains such as strain SRC14. A truncation of 3′-*tnpR* also occurred in plasmid pHCM1, which contained 86 bp less than the normal 3′-*tnpR* gene (Parkhill et al., 2001). The truncation of the *tnpR* gene was possibly caused by the IS*26* element downstream, which was inserted within *tnpR* and truncated this gene at the 5′ end. A sequence comparison of the *bla*$_{\text{TEM-1}}$-3′-*tnpR* region of plasmid pRSB107, Tn*1* and Tn*3* suggested that this region was more likely to be a remnant of Tn*3*. The structure of *bla*$_{\text{TEM-1}}$-3′-*tnpR*-IS*26* in this study possibly derived from the insertion of an IS*26* element into the *tnpR* gene of a Tn*3* tranposon. The

IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* gene cluster was possibly developed via the insertion of the *bla*$_{TEM-1}$-3′-*tnpR*-IS*26* region downstream of the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* cluster (Figure 5.20). Chen et al. (2007) also suggested that the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* cluster was inserted into a plasmid next to a *bla* gene to form this antibiotic resistance gene cluster of IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*1294*.

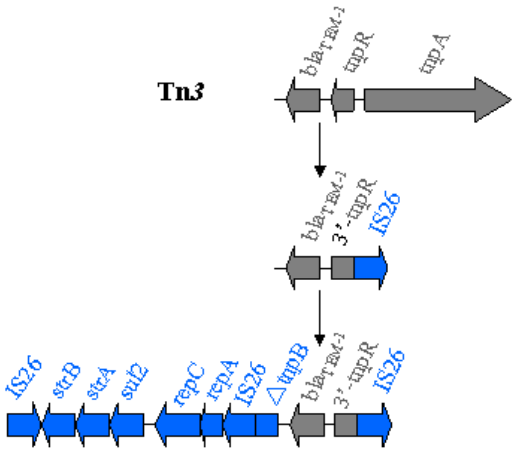


Figure 5.20 The hypothetical derivation of the *bla*$_{TEM-1}$ gene and the structure of IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26*.

The gene cluster of IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* was first described by Parkhill et al. (2001) on plasmid pHCM1. It was suggested by Parkhill et al. (2001) that this IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* cluster was inserted into the *tetA*(C) gene of the R27 tetracycline resistance operon in several sequential IS element-mediated events. The same IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* gene cluster was integrated into a Tn*21*-like transposon on plasmid pRSB107 (Szczepanowski et al., 2005). Holt et al. (2007) suggested that a possible composite transposon, which contained the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* gene cluster, was able to move between distinct plasmid backbones as a single unit.

In this study, it is very possible that the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster moved as one genetic element as suggested by Chen et al. (2007). This cluster might disrupt an existing class 1 integron (In4 type) and truncate the 3′-CS of the class 1 integron. The three deviatives of the Tn*1-dfrA5* region (Figure 5.18) detected among the 23 *S. enterica* serovar Typhimurium strains provide powerful support for this hypothesis. Strain SRC26 contained a nearly complete class 1 integron (In4 type) (IS*6100*-orf5-*sul1-qacE△1-dfrA5-intI1*) downstream of the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* cluster, where only one inverted repeat of the IS*6100* element was missing. Strain SRC129 was truncated more in the 3′-CS than strain SRC26 and has lost the IS*6100*, orf5, *sul1* and most of the q*acE△1* gene. Only part of the *qacE△1*, *dfrA5* cassette and *intI1* gene were located downstream of the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster. Strain SRC14 only harboured the *dfrA5* cassette and *intI1* gene downstream of this cluster. Of 11 strains containing the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster, 2 were isolated from humans and 7 from bovines. In total, 4 phage types, PT44, PT135, PT1var2 and PT9 were involved. These observations suggest that the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster, which might be located on Tn*21*-like transposons, moved as one genetic element rather than as a composite transposon between distinct plasmid backbones or chromosomes. One might speculate that the antibiotic resistance gene cluster was transferred to human via horizontal transfer through contaminated food.

The IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster has been found on different plasmids. On plasmid pHCM1, this antibiotic resistance gene cluster was

located between the *tniA△1* gene and the fourth IS*26* element on a Tn*21*-like transposon (Parkhill et al., 2001). On plasmid pAKU_1, the gene cluster of IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*26* was identified between the *tniA△1* gene and a truncated Tn*21*-specific transposition module (*tnpR*-*tnpM*) (Holt et al., 2007). The IS*26*-*aphAI*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*1294* gene cluster was characterized between the open reading frames orf28 and orf42 on plasmid pU302L (Chen et al., 2007). While on plasmid pRSB107, the IS*26*-*aphA1*-IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*26* gene cluster was inserted between the *tniA△1* gene and a macrolide-resistance operon *mph*(A)-*mrx*-*mphR*(A) located on a Tn*21*-like transposon (Szczepanowski et al., 2005). Plasmid pHCM1, pAKU_1 and pU302L were all isolated from *Salmonella enterica* strains, while plasmid pRSB107 was from sewage water without source information. These antibiotic resistance gene clusters were all located on large plasmids (84.5 kb-212 kb) either belonging to IncHI1 or IncF incompatibility groups (Parkhill et al., 2001; Szczepanowski et al., 2005; Holt et al., 2007; Chen et al., 2007). In this study, 23 *S. enterica* serovar Typhimurium strains containing antibiotic resistance gene clusters of IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*26* or IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$ were chosen for further research to investigate if these elements are harboured on plasmids.

Chapter 6: Plasmid conjugation for 23 *S. enteric* serovar Typhimurium strains

6.1 Introduction

Plasmids play an important role in transferring antibiotic resistance genes among bacteria, which either belong to the same or different genera and species. According to their mobilisation ability, plasmids can be classified as conjugative, mobilisable and non-conjugative (Bennett, 2008).

6.1.1 Conjugative plasmids

Conjugative plasmids can be transferred by conjugation. Conjugative plasmids have been shown to be important mediators of antibiotic resistance gene transfer. Firstly, conjugative plasmids may mediate gene transfer in various environments including soil, rhizosphere, plant surfaces, water and human gut. Secondly, conjugative plasmids in donor cells may be transferred to recipient cells of different genera or even different kingdoms (Dionisio et al., 2002). Specifically, when multidrug resistant gene clusters are located on conjugative plasmids, they are easily spread among genera, species and strains (Sherley et al., 2004). The majority of high molecular weight plasmids found are conjugative plasmids and confer resistance to multiple antibiotics, examples of which include plasmids pRMH760, pRSB107, pHCM1 and pAKU-1 (Partridge and Hall, 2004; Szczepanowski et al., 2005; Parkhill et al., 2001; Holt et al., 2007).

Conjugative plasmids can exhibit a broad or narrow host range. Broad host range plasmids are able to transfer between widely different bacterial species. For example, the resistance plasmid RP1, which was first identified in a clinical strain of *Pseudomonas aeruginosa*, appears to be able to transfer between most Gram-negative

bacteria (Bennett, 2008). Narrow host range plasmids transfer to a restricted number of similar bacterial species (Bennett, 2008). To date, many narrow-host-range plasmids such as pR100, pC15-1a, pRMH760 and pRSB107, which belong to incompatibility group F, and pHCM1, which belongs to IncHI1 group, have been characterised (Novais et al., 2006).

Most serovars of *S. enterica* do not possess plasmids. However serovars which are frequently associated with infections of humans and farm animals, including *S. enterica* serovars Enteritidis, Typhimurium, Dublin, Cholerae-suis, Gallinarum, Pullorum, Brandenburg and Abortus-ovis usually harbour plasmids (Rychlik et al., 2006; Martinez et al., 2007). Strains of these serovars usually contain serovar specific virulence plasmids, typically 50-100 kb in size. Additional to serovar specific virulence plasmids, *Salmonella* can also harbour other high molecular weight plasmids, which can confer resistances to antibiotics (Rychlik et al., 2006). For example, large conjugative plasmids were found in *S. enterica* serovar Brandenburg strains by Martinez et al. (2007). This large conjugative plasmid contained a Tn*21*-related transposon and conferred resistance to multiple antibiotics including streptomycin, spectinomycin, sulfonamide, chloramphenicol and mercury (Martinez et al., 2007). This Tn*21*-related transposon contained four parts including the *mer* opern which contains *merR*, *merT*, *merP*, *merC*, *merA*, *merD*, *merE* and *urf2* genes; a *tni* region containing *tniA* and truncated *tniB* genes; a class 1 integron with *aadA1* and *dfrA1* gene cassettes, and a Tn*21* transposition system including *tnpM*, *tnpR* and *tnpA* genes. A chloramphenicol resistance gene *catA1* was located downstream of the Tn*21*-related transposon. Two IS*1b* elements were located at each end of this antibiotic resistance region (Figure 6.1). Large self-transferable plasmids carrying integrons were also identified within the multidrug-resistant *S.*

enterica serovar Wien, Ohio and Typhimurium isolated from swine (Martinez et al., 2007).

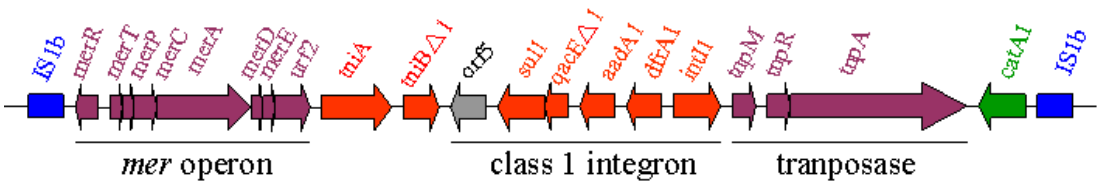


Figure 6.1 The Tn*21*-related transposon identified in isolates of *S. enterica* serovar Brandenburg. Genes and their transcriptional directions are indicated by arrows (adapted from Martinez et al., 2007).

Among *Salmonella* isolates, the most frequently observed plasmids belong to IncH, IncF and IncI incompatibility groups, followed by the IncN, IncP and IncQ groups (Rychlik et al., 2006). Plasmids of the IncH group were first characterised after an outbreak of chloramphenicol resistant typhoid fever in Mexico in 1972 (Anderson and Smith, 1972). IncH plasmids are divided into IncHI and IncHII subgroups. Most of the IncHI plasmids are large genetic elements carrying antibiotic and heavy metal resistance genes and are often associated with human pathogens (Taylor, 1989). The IncHII group plasmids are compatible with IncHI plasmids and transfer at high levels (Bradley et al., 1982). The IncHI group is further subdivided into IncHI1, IncHI2 and IncHI3 subgroups, which share little DNA similarity (Grindley et al., 1973; Roussel and Chabbert, 1978; Smith et al., 1973; Whiteley and Taylor, 1983). The plasmid R27 isolated in England in 1962, the plasmid pHCM1 isolated from Vietnam in 1993, and the plasmid R478 isolated from the Gram-negative opportunistic pathogen *Serratia marcescens*, all belong to the IncHI1 group (Sherburne et al., 2000; Parkhill et al., 2001; Gilmour et al., 2004). The multidrug resistance IncHI1 plasmid pAKU_1 was recently reported by Holt et al. (2007) and this 212 kb plasmid was isolated from a Pakistani patient in Karachi in 2002.

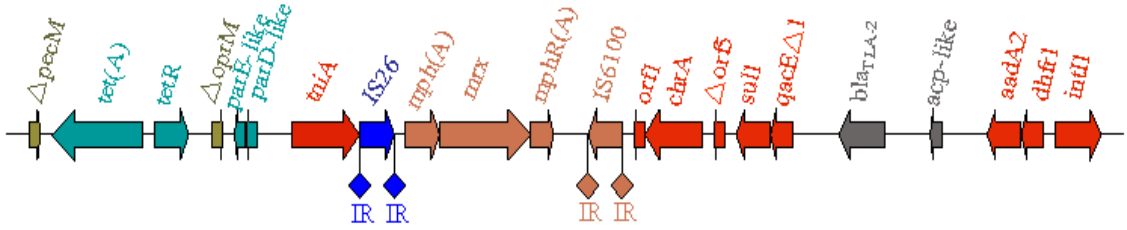
During the early 1970's, the IncFI group plasmids encoding multiple antibiotic resistances were frequently reported in *Salmonella* isolated in Europe, the Middle East and North Africa (Anderson et al., 1977). Twenty-nine multiple antibiotic resistant *S. enterica* serovar Typhimurium strains isolated in 1997 in Albania from sporadic cases of infantile gastroenteritis were found to carry IncFI plasmids (Carattoli, 2003).

6.1.2 Mobilisable plasmids

Naturally occurring mobilisable plasmids carry the genetic information necessary for relaxosome formation and processing, but lack the functions required for mating pair formation (Francia et al., 2004). Mobilisable plasmids can transfer to other strains when helped by a conjugative plasmid co-resident in the cell. Triparental mating can help overcome some of these barriers to efficient plasmid mobilisation.

The mobilisable plasmid designated pRSB101 was reported by Szczepanowski et al. in 2004. This plasmid, isolated from a wastewater treatment plant, was approximately 47 kb in size and conferred resistance to tetracycline, erythromycin, roxythromycin, sulfonamides, cephalosporins, spectinomycin, streptomycin, trimethoprim, nalidixic acid and low concentrations of norfloxacin (Szczepanowski et al., 2004). Plasmid pRSB101 contained a Tn*402*-like transposon, which was approximately 20 kb in size (Figure 6.2). This transposon contained tetracycline resistance genes *tetA*(A)-*tetR*, a macrolide resistance operon *mph(A)-mrx-mph(R)*, and a class 1 integron containing *dhfr1* and *aadA2* gene cassettes (Figure 6.2). Truncated *pecM* and *oprM* genes were located upstream and downstream of the *tetA*(A)-*tetR* genes, followed by the *parE*-like and *parD*-like genes, which were predicted to constitute a plasmid-stabilisation system. A *tniA* gene was located upstream of the macrolide resistance operon, which was

flanked by IS*26* and IS*6100* elements. An open reading frame orf1 and a putative chromate ion transporter gene *chrA* were inserted upstream of the 3′-CS of the class 1 integron and truncated the open reading frame orf5. Between the *qacE△1* and the *aadA2* gene of the class 1 integron, a bla_{TLA-2} gene and a putative acyl-carrier protein gene *acp*-like were inserted (Szczepanowski et al., 2004). Plasmid pRSB101 was mobilisable with the help of the IncP-1α plasmid RP4 (Szczepanowski et al., 2004). Since pRSB101-like plasmids were also identified in the final effluents of a wastewater treatment plant, Szczepanowski et al. (2004) suggested that the antibiotic resistance genes carried by these plasmids will be disseminated widely among environmental bacteria.



Tn*402*-like

Figure 6.2 Map of the Tn*402*-like transposon on plasmid pRSB101 (accession no. AJ698325). Genes and their transcriptional directions are indicated by arrows. IR, inverted repeat (adapted from Szczepanowski et al., 2004).

6.1.3 Non-conjugative plasmids

Non-conjugative plasmids are incapable of initiating conjugation and can only be transferred by transformation, or accidentally and at a low level with the help of conjugative plasmids (Bennett, 2008). Small plasmids such as RSF1010 and pBP1 are non-conjugative broad-host-range plasmids (Scholz et al. 1989; Korfmann et al., 1983).

6.2 Materials and methods

6.2.1 Conjugation

Conjugation experiments were performed on 23 *S. enterica* serovar Typhimurium strains characterised in Chapter 5 according to the protocol described in Chapter 2 (section 2.15). Those strains, for which conjugation could not be detected, were repeated twice more to confirm the result.

6.2.2 Triparental mating

Triparental mating experiments were carried out on 8 *S. enterica* serovar Typhimurium strains, where conjugation could not be detected according to the method described in Chapter 2 (section 2.16). Those strains, where conjugation could not be detected by triparental mating, were repeated twice more to confirm the result.

6.2.3 Chromosomal DNA extraction for 19 *E. coli* transconjugants

Chromosomal DNA was extracted for 19 *E. coli* tranconjugants by the X-tractor Gene™ robot (Corbett Robotics) following the manufacture's protocol as described in Chapter 2 (section 2.3.3).

6.2.4 PCR amplification

6.2.4.1 Multiplex PCR amplification of *strA*, *strB* and *sul2* genes

The multiplex PCR of *strA*, *strB* and *sul2* was performed on 19 transconjugants. The primer positions are indicated in Figure 6.3 and all the primer sequences are listed in Table 6.1. The program used for the amplification was: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2.5 min, and finally 72°C for 10 min. The amplification was performed using a thermocycler PC-960 (Corbett Research).

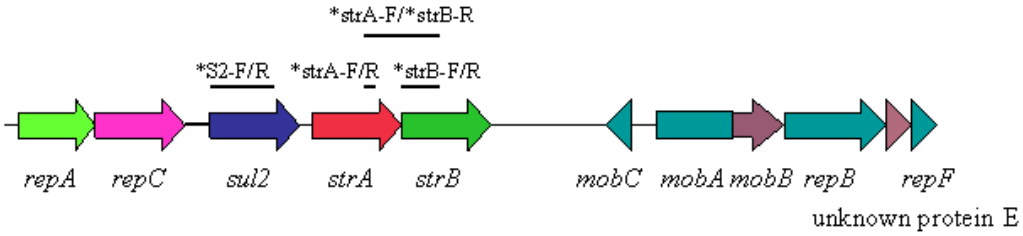


Figure 6.3 The primer positions for *strA* (*strA-F/strA-R), *strB* (*strB-F/strB-R), and *sul2* (*S2-F/S2-R) on RSF1010 plasmid (accession no. M28829). The genes and their transcriptional directions are indicated by arrows. Gene *mobA* is truncated and indicated by a box. Fragments amplified by the multiplex PCR are shown as lines with the primer pairs indicated.

Table 6.1 The primers used for the *strA*, *strB* and *sul2* multiplex PCR, *Salmonella invA* gene and *E. coli uspA* gene amplifications.

Primer	Primer sequence (5′-3′)	Location	Nucleotide positions	Accession no.	Reference
*strA-F	TAC CGG ACG AGG ACA AGA GT	*strA*	502-521	M28829	unpublished data
*strA-R	GAC CCG TGC ATT GAA GAG TT	*strA*	666-647	M28829	unpublished data
*strB-F	GCC TGT TTT TCC TGC TCA TT	*strB*	877-896	M28829	unpublished data
*strB-R	CGC GTG GAC GTA GTC AGT T	*strB*	1321-1303	M28829	unpublished data
*S2-F	TTT TCG GCA TCG TCA ACA TA	*sul2*	7894-7913	M28829	unpublished data
*S2-R	CAA GCT CTG CAG CGA GTG T	*sul2*	8579-8561	M28829	unpublished data
Salm3	GCT GCG CGC GAA CGG CGA AG	*invA*	484-503	U43272	Ferreti et al., 2001
Salm4	TCC CGG CAG AGT TCC CAT T	*invA*	870-852	U43271	Ferreti et al., 2001
FD-uspAF	AAA GTT TCT CTG ATC CAC GTA G	*uspA*	439-460	X67639	unpublished data
FD-uspAR	GTC GTA GAG GGA AGA TTA TTC	*uspA*	792-772	X67639	unpublished data

* indicates primers designed by C. Venturini.
FD indicates primers designed by F. Dawes.

6.2.4.2 PCR amplification of *Salmonella invA* gene and *E. coli uspA* genes

The PCR amplifications of *Salmonella* specific *invA* gene with primer Salm3/Salm4 and *E. coli* specific *uspA* gene with primer FD-uspAF/FD-uspAR were performed on 19 transconjugants respectively. All the primer sequences are listed in Table 6.1. The program used for *invA* and *uspA* PCR amplifications was: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 53°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. The amplifications were performed using a thermocycler FTS-960 (Corbett Research).

6.2.4.3 PCR amplification of gene *tetA*(A), *tetA*(B), the right arm of Tn*1721* transposon, *merA* and the *dfrA5-intI1* region

The primer pairs of tet(A)-F/tet(A)-R and tet(B)-F/tet(B)-R were those used in the multiplex PCR described in Chapter 3 and their sequences are listed in Table 3.1. The program used for the *tetA*(A) and *tetA*(B) PCR amplifications was: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. PCR amplification of the right arm of Tn*1721* transposon was performed on those transconjugants verified to contain the *tetA*(A) gene. The primer pair TAF/TetAR3 (Table 3.1) and the PCR program was described in Chapter 3. PCR amplifications of the *merA* gene and the *dfrA5-intI1* region were performed on 19 transconjugants and the primer sequences are listed in Table 5.1 (Chapter 5). The programs used for *merA* and the *dfrA5-intI1* region were the same as those described in Chapter 5. The amplifications were all performed using a thermocycler FTS-960 (Corbett Research).

6.2.5 Southern hybridisations

6.2.5.1 Southern hybridisation using the bla_{TEM} gene probe

Southern hybridisation using the bla_{TEM} probe was performed on 19 transconjugants. The restriction enzyme used to digest all the chromosomal DNA was *Pst*I. Southern hybridisation using the bla_{TEM} gene probe was also performed on plasmids extracted from 3 transconjugants including pSRC14, pSRC26 and pSRC27. *E. coli* 294 Rif^r strain was used as a negative control. *Salmonella* strain SRC26 was used as a positive control. No enzyme was used to digest the extracted plasmids. The method of Southern hybridisation using the bla_{TEM} probe was the same as described in Chapter 5 (section 5.2.1).

6.2.5.2 Southern hybridisation using an *aphAI* gene probe

6.2.5.2.1 Making the *aphAI* gene probe

The primer pair of aphAI-IAB(F)/aphAI-IAB(R) was used to amplify the *aphAI* gene probe and the primer sequences are listed in Table 5.1. The program used for amplifying the *aphAI* gene probe was: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec, and finally 72°C for 10 min. The amplification was performed using a thermocycler FTS-960 (Corbett Research).

6.2.5.2.2 Southern hybridisation using the *aphAI* probe

Southern hybridization using the *aphAI* probe was performed on 19 transconjugants according to the method described in Chapter 2 (section 2.9). The restriction enzyme used to cut the chromosomal DNA of all the transconjugants was *Pst*I.

6.2.6 Plasmid extraction

Plasmids including pSRC14, pSRC26 and pSRC27 were extracted from three transconjugants according to the method described in Chapter 2 (section 2.11).

6.3 Results

6.3.1 Conjugation of *Salmonella* plasmids

In an attempt to conjugate *Salmonella* strains into *E. coli* 294 Rif^r recipient cells, the conjugation method was performed on 23 *S. enterica* serovar Typhimurium strains selecting for ampicillin. Twelve transconjugants were obtained and the strain numbers are shown in Table 6.2. For the remaining 11 strains, triparental mating was undertaken, which resulted in a further 7 transconjugants upon selection with ampicillin. Four *Salmonella* strains, SRC120, SRC123, SRC128 and SRC136, could not transfer to *E.*

coli by either method with ampicillin selection. Four transconjugant plasmids, pSRC30, pSRC32, pSRC118 and pSRC135 did not harbour tetracycline resistance which was present in the original *Salmonella* strains. Transconjugant plasmid pSRC29 contained neither tetracycline nor trimethoprim resistance. Except for the five strains mentioned, the other transconjugants contained the same antibiotic resistance profiles as their original *Salmonella* strains (Table 6.2).

6.3.2 PCR amplifications

6.3.2.1 *Salmonella invA* gene and *E. coli uspA* gene amplifications

PCR amplification of the *Salmonella* specific *invA* gene was performed on 19 transconjugants and as expected an amplicon was not obtained (data not shown). PCR amplification of the *E. coli* specific *uspA* gene was carried out on 19 transconjugants and as expected an amplicon was obtained for each strain (Figure 6.4).

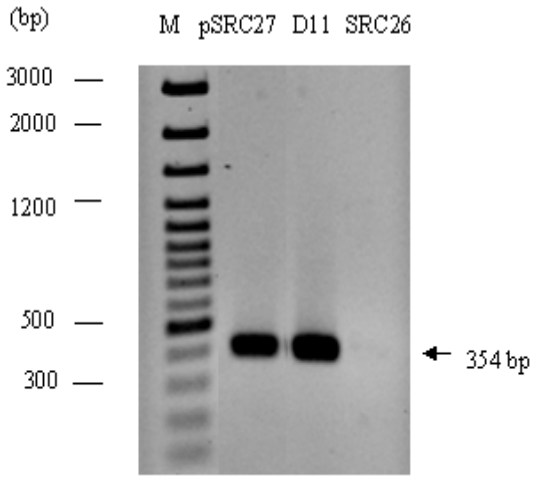


Figure 6.4 Gel electrophoresis of PCR amplicon of the *E. coli uspA* gene. Transconjugant pSRC27 was chosen as a representative for the 354 bp amplicon. *E. coli* strain D11 was a positive control and *Salmonella* strain SRC26 was a negative control for this PCR amplification on a 1.0% agarose gel. The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

Table 6.2 Conjugation and triparental mating results for 23 *S. enterica* serovar Typhimurium strains. The strains without an asterisk were transferred to *E. coli* 294 Rif^r by conjugation. The strains indicated by an asterisk were transferred to *E. coli* 294 Rif^r by triparental mating. Antibiotic resistance not transferred to *E. coli* 294 Rif^r recipients are highlighted in yellow.

Strains	Serovar/phage type	Resistance profile of *Salmonella* donor	Plasmid	Resistance profile of transconjugant
SRC13	Typhimurium PT135	ApSmSuTcTp	pSRC13	ApSmSuTcTp
SRC14	Typhimurium PT44	ApSmSuTcTp	pSRC14	ApSmSuTcTp
SRC26	Typhimurium PT1var2	ApKmSmSuTcTp	pSRC26	ApKmSmSuTcTp
SRC27	Typhimurium PT135	ApCmGmKmSmSpSuTcTp	pSRC27	ApCmGmKmSmSpSuTcTp
SRC28	Typhimurium PT135	ApCmGmKmSmSpSuTcTp	pSRC28	ApCmGmKmSmSpSuTcTp
SRC29*	Typhimurium PT141	ApGmKmSmSuTcTp	pSRC29	ApGmKmSmSu
SRC30	Typhimurium PT141var4	ApGmKmSmSuTcTp	pSRC30	ApGmKmSmSuTp
SRC31	Typhimurium PT44	ApSmSuTcTp	pSRC31	ApSmSuTcTp
SRC32*	Typhimurium PT68	ApKmSmSuTcTp	pSRC32	ApKmSmSuTp
SRC117*	Typhimurium PT141	ApGmKmSmSuTp	pSRC117	ApGmKmSmSuTp
SRC118	Typhimurium PT141	ApGmKmSmSuTcTp	pSRC118	ApGmKmSmSuTp
SRC122*	Typhimurium PT29	ApKmSmSuTp	pSRC122	ApKmSmSuTp
SRC124*	Typhimurium PT44	ApSmSuTcTp	pSRC124	ApSmSuTcTp
SRC126*	Typhimurium PT44	ApSmSuTp	pSRC126	ApSmSuTp
SRC129	Typhimurium PT44	ApKmSmSuTcTp	pSRC129	ApKmSmSuTcTp
SRC131	Typhimurium PT9	ApSmSuTp	pSRC131	ApSmSuTp
SRC132	Typhimurium PT9	ApSmSu	pSRC132	ApSmSu
SRC133	Typhimurium PT9	ApKmSmSu	pSRC133	ApKmSmSu
SRC135*	Typhimurium RDNC	ApKmSmSuTcTp	pSRC135	ApKmSmSuTp

6.3.2.2 Multiplex PCR amplification of *strA*, *strB* and *sul2* genes

Multiplex PCR of *strA*, *strB* and *sul2* was developed by another PhD student (C. Venturini) for *E. coli* strains. This multiplex PCR was used for *strA*, *strB* and *sul2* screening of the 19 *E. coli* transconjugants. Four amplicons were obtained with sizes of 165 bp (*strA*), 450 bp (*strB*), 700 bp (*sul2*) and 800 bp (*strA-strB* region) (Figure 6.5). The result (Table 6.3) revealed that all the transconjugants were positive for *strA*, *strB*, *strA-strB* and *sul2* genes, identical to the original *Salmonella* strains.

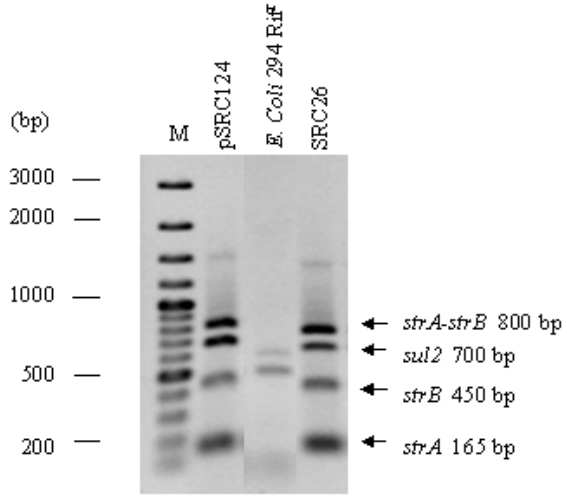


Figure 6.5 Gel electrophoresis of the multiplex PCR amplicons for the *strA*, *strB*, *strA-strB* and *sul2* genes for 19 *E. coli* transconjugants. The transconjugant containing pSRC124 was chosen as a representative to show the four amplicons of 165 bp, 450 bp, 700 bp and 800 bp. Strain *E. coli* 294 Rif^r was used as a negative control. *Salmonella* strain SRC26 was used as a positive control (1.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled and the corresponding sizes are given on the left.

6.3.2.3 PCR amplifications of *tetA*(A), the right arm of transposon Tn*1721* and *tetA*(B)

The *tetA*(A) and *tetA*(B) genes were PCR screened separately for each of the 19 *E. coli* transconjugants. The screening result for the *tetA*(A) gene revealed that five transconjugants contained this gene (Figure 6.6A) (Table 6.3).

The *tetA*(A) gene was located on the right arm of transposon Tn*1721* in the *Salmonella* strains, and thus that arm was screened for those *E. coli* transconjugants containing the *tetA*(A) gene. The screening result confirmed that all the *tetA*(A) genes for these 5 transconjugants were located on the right arm of transposon Tn*1721* (Figure 6.6B) (Table 6.3).

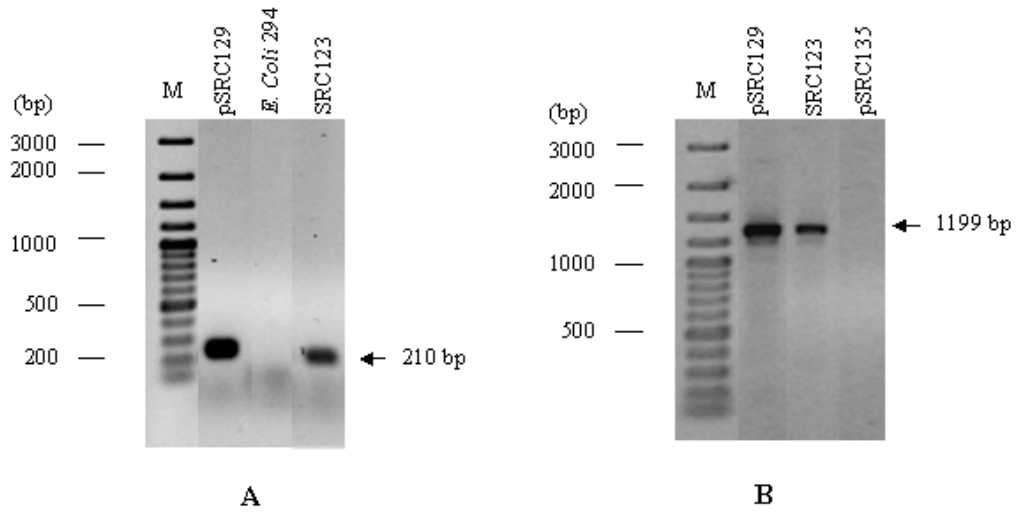


Figure 6.6 A Gel electrophoresis of the *tetA*(A) PCR amplicon for 19 *E. coli* transconjugants. The *E. coli* transconjugant containing pSRC129 was chosen as a representative for the 210 bp PCR amplicon. *E. coli* 294 Rif^r was used as a negative control and *Salmonella* strain SRC123 was used as a positive control (1.0% agarose gel). **B** Gel electrophoresis of the PCR amplicon of the right arm of transposon Tn*1721*. The *E. coli* transconjugant containing pSRC129 was used to show the 1199 bp PCR amplicon. *Salmonella* strain SRC123 was used as a positive control. The *E. coli* transconjugant containing pSRC135 was used as a negative control (1.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

For screening using the *tetA*(B) gene primer, a positive PCR amplicon was obtained with a size of approximately 659 bp as expected (Figure 6.7). The PCR screening revealed that the *tetA*(B) genes of 3 *Salmonella* strains, SRC26, SRC27 and SRC28 were transferred to tranconjugants, while the *tetA*(B) gene of another 5 strains, SRC29, SRC30, SRC32, SRC118 and SRC135 did not transfer (Table 6.3).

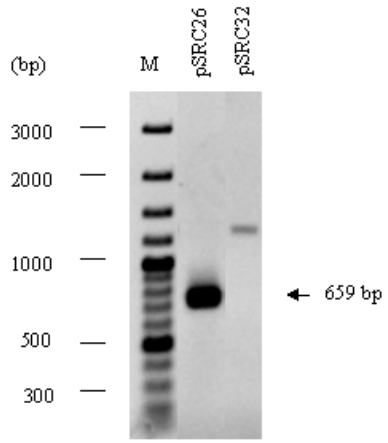


Figure 6.7 Gel electrophoresis of the PCR amplicon for the *tetA*(B) gene. The *E. coli* transconjugant containing pSRC26 was used as a representative for this 659 bp PCR product. The *E. coli* transconjugant containing pSRC32 was chosen as a representative for those strains whose *tetA*(B) gene was not transferred from *Salmonella* to *E. coli* (1.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

6.3.2.4 PCR amplification of the *merA* gene and the *dfrA5-intI1* region

The *merA* gene was screened for detection in the 19 transconjugants and an amplicon of 394 bp was obtained as expected (Figure 6.8). The PCR screening result confirmed that the *merA* gene present in 13 of these *Salmonella* strains was transferred to *E. coli* recipient cells.

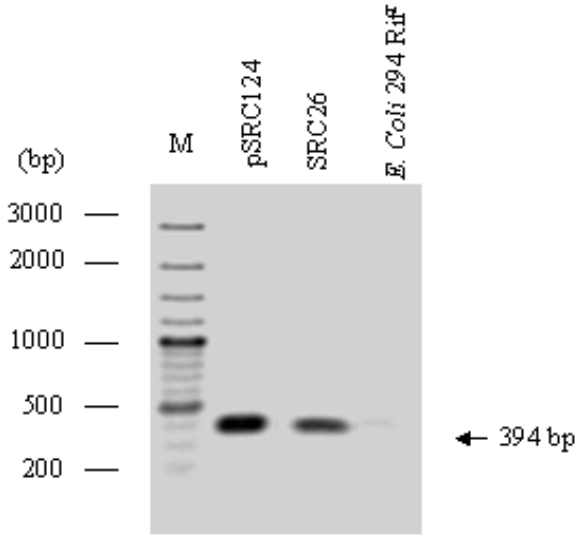


Figure 6.8 Gel electrophoresis of the PCR amplicon for the *merA* gene for 19 *E. coli* transconjugants. The *E. coli* transconjugant containing pSRC124 was used as a representative for this 394 bp PCR product. *Salmonella* strain SRC26 was used as a positive control. Strain *E. coli* 294 Rif^r was used as a negative control (1% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

In order to confirm that the *dfrA5* gene cassette and the class 1 integron specific integrase gene *intI1* were also located on these plasmids, the linkage PCR of *dfrA5-intI1* was performed on the 19 transconjugants. The PCR amplicon was obtained as expected with a size of 555 bp (Figure 6.9A). The result of the *dfrA5-intI1* linkage PCR showed that 14 transconjugants were positive for this PCR, in common with their original *Salmonella* strains (Table 6.3). Transconjugant pSRC29 was negative for this PCR, while its *Salmonella* donor strain was positive for this PCR. All the PCR amplicons were further characterised by restriction digestion using enzyme *Ase*I. Two fragments of 131 bp and 424 bp were obtained as expected (Figure 6.9B). The enzyme digestion result confirmed that all the *dfrA5-intI1* PCR amplicons had the same RFLP profile.

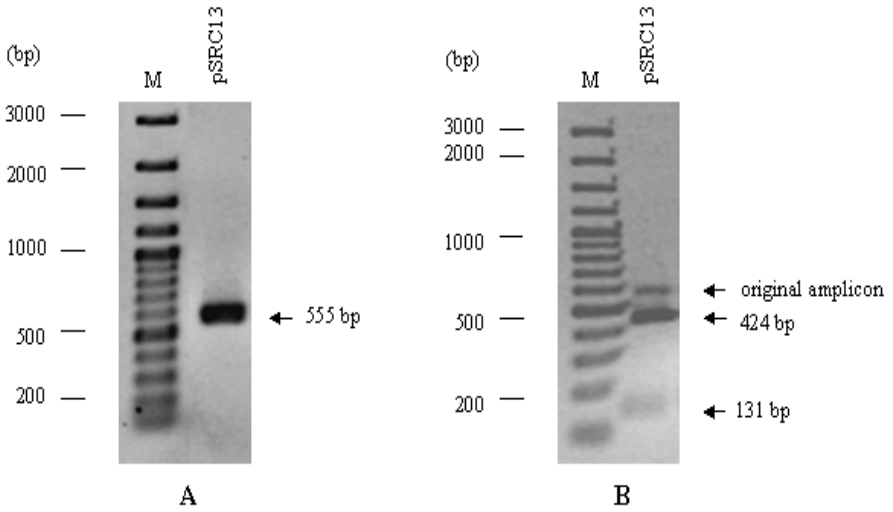


Figure 6.9A Gel electrophoresis of the *dfrA5-intI1* linkage PCR amplicon for 19 *E. coli* transconjugants. Transconjugant containing pSRC13 was chosen as a representative for the 555 bp amplicon (1.0% agarose gel). **B** Gel electrophoresis of the restriction digestion of the *dfrA5-intI1* amplicon using enzyme *Ase*I. Transconjugant containing pSRC13 was used as a representative for the digested fragments of 131 bp and 424 bp (2.0% agarose gel). The 100 bp plus DNA ladders (Fermentas) is labeled M and the corresponding sizes are given on the left.

6.3.3 Southern hybridisation results

6.3.3.1 Southern hybridisation using the *bla*$_{TEM}$ gene probe

In order to confirm that the *bla*$_{TEM}$ gene was carried by these plasmids, Southern hybridisation using the *bla*$_{TEM}$ probe was performed on the 19 *E. coli* transconjugants. Restriction enzyme *Pst*I was used to digest the chromosomal DNA. The *bla*$_{TEM}$ Southern hybridisation revealed that 10 *E. coli* transconjugants contained two *Pst*I fragments of approximately 1.0 kb and 1.2 kb. Seven transconjugants contained two *Pst*I fragements, which were 1.0 kb and 3.5 kb in size. Two *E. coli* transconjugants (pSRC27 and pSRC28) contained three fragments of 1.0 kb, 1.2 kb and 10 kb (Figure 6.10) (Table 6.3). All the 19 strains contained the same size *Pst*I fragments as their original *Salmonella* strains in the *bla*$_{TEM}$ Southern hybridisation.

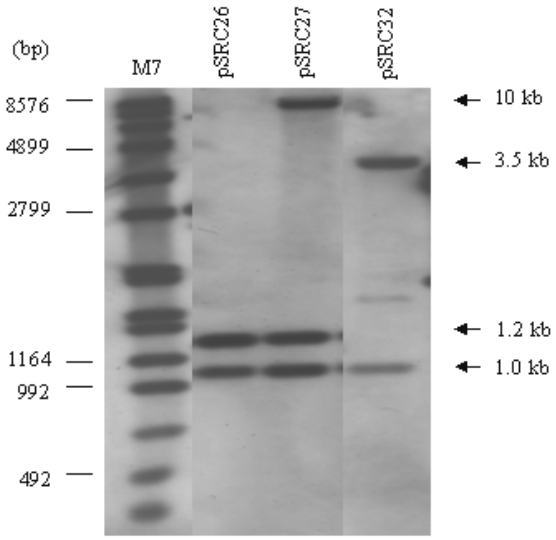


Figure 6.10 Southern hybridisation using the *bla*$_{TEM}$ gene probe on 19 *E. coli* transconjugants. The *E. coli* transconjugant containing pSRC26 showed the *Pst*I fragments of 1.0 kb and 1.2 kb. The *E. coli* transconjugant pSRC27 was used to show three *Pst*I fragments of 1.0 kb, 1.2 kb and 10 kb. The *E. coli* transconjugant pSRC32 was chosen as a representative producing the 1.0 kb and 3.5 kb fragments. DIG-labeled marker VII (Roche) is labeled M7 and the corresponding sizes are given on the left.

6.3.3.2 Southern hybridisation using the *aphAI* gene probe

Given some of the *S. enterica* serovar Typhimurium strains contained the *aphAI* gene, in order to determine its transferring, a Southern hybridisation using the *aphAI* probe was performed on the 19 transconjugants. Restriction enzyme *Pst*I was used to digest the chromosomal DNA for each transconjugant. The result confirmed that the *aphAI* gene was on an approximately 1.9 kb fragment as expected and all of the *aphAI* genes in the 11 *Salmonella* strains were transferred to their corresponding *E. coli* transconjugants (Figure 6.11) (Table 6.3).

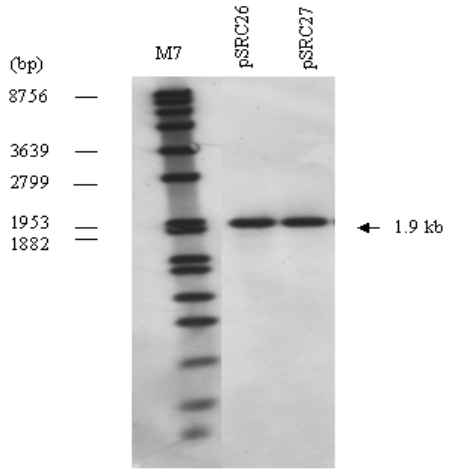


Figure 6.11 Southern hybridisation using the *aphAI* gene probe on 19 *E. coli* transconjugants. The *E. coli* transconjugants containing pSRC26 and pSRC27 showed the *Pst*I fragment of approximately 1.9 kb. DIG-labeled marker VII (Roche) is labeled M7 and the corresponding sizes are given on the left.

Table 6.3 The results of all PCR amplifications and Southern hybridisations for the 19 *E. coli* transconjugants. Genes present in the *Salmonella* donor not transferred to *E. coli* 294 Rif^r recipients are highlighted in red.

Isolates	PCR amplification								Southern hybridisation (kb)	
plasmid	*strA*	*strB*	*sul2*	*strA-strB*	*tetA*(A)/ Tn*1721*	*tetA*(B)	*merA*	*dfrA5 -intI1*	bla_{TEM}	*aphAI*
pSRC13	+	+	+	+	+/+	-	+	+	+	-
pSRC14	+	+	+	+	+/+	-	+	+	+	-
pSRC26	+	+	+	+	-	+	+	+	+	+
pSRC27	+	+	+	+	-	+	-	-	+	+
pSRC28	+	+	+	+	-	+	-	-	+	+
pSRC29	+	+	+	+	-	+	+	+	+	+
pSRC30	+	+	+	+	-	+	-	+	+	-
pSRC31	+	+	+	+	+/+	-	+	+	+	-
pSRC32	+	+	+	+	-	+	+	+	+	+
pSRC117	+	+	+	+	-	-	+	+	+	+
pSRC118	+	+	+	+	-	+	+	+	+	+
pSRC122	+	+	+	+	-	-	-	+	+	+
pSRC124	+	+	+	+	+/+	-	+	+	+	-
pSRC126	+	+	+	+	-	-	+	+	+	-
pSRC129	+	+	+	+	+/+	-	+	+	+	+
pSRC131	+	+	+	+	-	-	+	+	+	-
pSRC132	+	+	+	+	-	-	-	-	+	-
pSRC133	+	+	+	+	-	-	-	-	+	+
pSRC135	+	+	+	+	-	+	+	+	+	+

6.3.4 The *bla*$_{TEM}$ Southern hybridisation of three plasmids

Three large plasmids were extracted from transconjugants containing pSRC14, pSRC26, pSRC27 by the modified Kado and Liu (1981) method. The sizes of these plasmids were very large and could not be estimated by comparison with the DIG-labeled marker II (Roche). *E. coli* 294 Rif^r was used as a negative control and no detectable plasmid was extracted from this strain. *Salmonella* strain SRC26 was used as a positive control and two large plasmid bands were obtained (Figure 6.12).

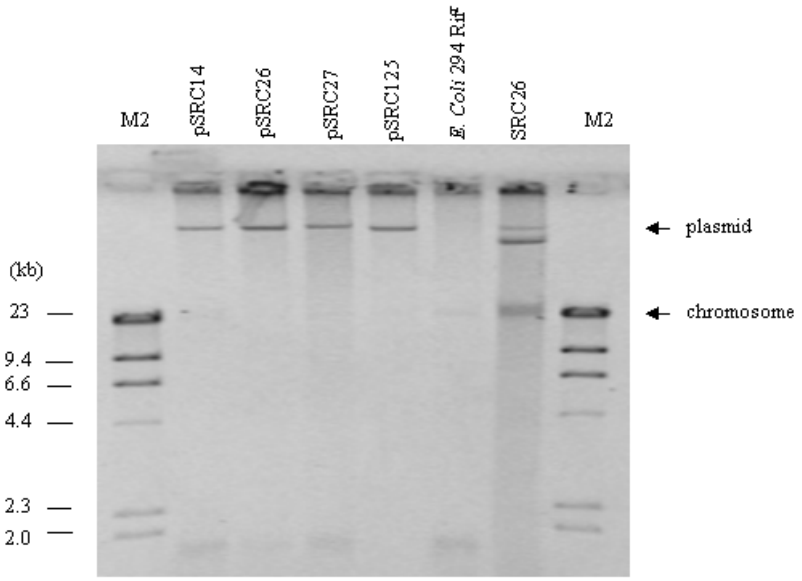


Figure 6.12 Gel electrophoresis of large plasmids extracted from three *E. coli* transconjugants containing pSRC14, pSRC26 and pSRC27. Plasmid pSRC125 (large plasmid not containing *bla*$_{TEM}$) and the recipient strain *E. coli* 294 Rif^r were negative controls. *Salmonella* strain SRC26 was used as a positive control (0.7% agarose gel). DIG-labeled marker II (Roche) are labeled M2 and the corresponding sizes are given on the left.

In order to confirm the presence of an antibiotic resistance gene cluster on these large plasmids, Southern hybridisation using the *bla*$_{TEM}$ gene probe was performed (Figure 6.13). The result confirmed that either the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$ or the

IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* antibiotic resistance gene cluster is probably located on these large plasmids.

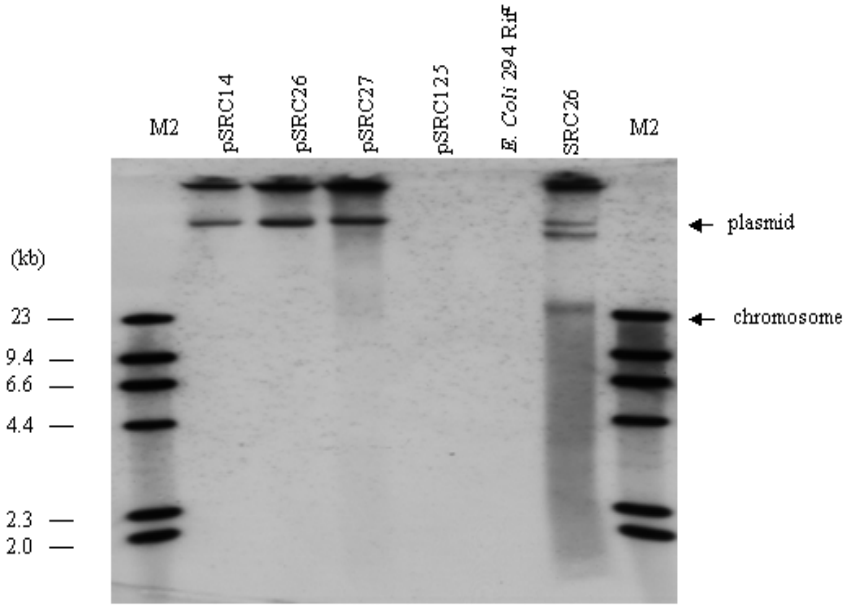


Figure 6.13 Southern hybridisation using the *bla*$_{TEM}$ probe on three large plasmids extracted from transconjugants. Plasmid pSRC125 (large plasmid not containing *bla*$_{TEM}$) and *E. coli* 294 Rif^{r} were used as negative controls. *Salmonella* strain SRC26 was used as a positive control. Chromosomal DNA runs at approximately 23 kb. DIG-labeled marker II (Roche) is labeled M2 and the corresponding sizes are given on the left.

6.4 Discussion

Specific PCR reactions were used to confirm the identity of *E. coli* plasmid transconjugants and differentiate these from the donor *Salmonella* strains. Because Salm3 and Salm4 primers do not amplify any nonspecific PCR products, the presence or absence of *Salmonella* spp. donor is unequivocally reflected by the presence or absence of the 389 bp amplicon (Ferretti et al., 2001). The primer pair of FD-uspAF/FD-uspAR is derived from the DNA sequences encoding the universal stress protein (*uspA*) to differentiate *E. coli* from other Gram-negative bacteria (Chen and Griffiths, 1998). These primers were used to verify 19 transcongants were multidrug

resistant *E. coli* strains, not donor *Salmonella* strains.

The *tetA*(A) gene in five *Salmonella* strains, which was located on the right arm of transposon Tn*1721*, was transferred to *E. coli* transconjugants with this transposon, which suggested that the *tetA*(A) gene was located on the same large plasmid with the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$-IS*26* antibiotic resistance gene cluster. During conjugation or triparental mating, five plasmid transconjugants pSRC29, pSRC30, pSRC32, pSRC118 and pSRC135 did not transfer the tetracycline resistance gene *tetA*(B), while the *tetA*(B) gene of three *Salmonella* strains SRC26, SRC27 and SRC28 was transferred to their *E. coli* transconjugants with the resistance plasmids. It is thus possible that the *tetA*(B) gene was located on the same plasmids with the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$-IS*26* gene cluster for *Salmonella* strains SRC26, SRC27 and SRC28. However, for *Salmonella* strains SRC29, SRC30, SRC32, SRC118 and SRC135, the *tetA*(B) gene may possibly be located on either the chromosome or another plasmid, such that this gene could not be transferred to *E. coli* recipient with the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$ antibiotic resistance gene cluster via conjugation or triparental mating. In conclusion, for those strains containing the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$-IS*26* gene cluster, the tetracycline resistance *tetA*(A) or *tetA*(B) was located on the same plasmid. The *tetA*(A) gene was further confirmed to be located on the right arm of Tn*1721* transposon. For those strains containing the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{\text{TEM-1}}$ gene cluster, the tetracycline resistance gene *tetA*(B) was located on the chromosome or another

plasmid. This difference is also consistent with the IS*200* patterns described in Chapter 5. Those strains containing the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*26* gene cluster belonged to the group II IS*200* profile, while strains containing the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$ gene cluster belonged to group I.

The plasmid transconjugant pSRC29 did not contain the trimethoprim resistance gene cassette *dfrA5* when compared to the *Salmonella* donor. Multidrug resistant plasmids have also been identified by Hradecka et al. (2008) in *S. enterica* serovar Typhimurium strains and 12 large plasmids were transferred from *Salmonella* donor strains to *S. enterica* serovar Typhimurium F98 recipient cells by conjugation. Three different transconjugants were obtained for one strain, one transconjugant harboured all the antibiotic resistance genes [*bla*$_{\text{TEM}}$, *cat*, *strA*, *sul2*, *tetA*(A), *aac(3)-IV*], one transconjugant contained all genes except the tetracycline resistance gene *tetA*(A) and the third contained all resistance genes except *tetA*(A) and *bla*$_{\text{TEM}}$ (Hradecka et al., 2008). Further research revealed that the *tetA*(A) gene alone or in combination with *bla*$_{\text{TEM}}$ were deleted from the R-plasmid. The donor strain contained the R-plasmid and also a virulence plasmid of 94 kb. For the transconjugant lacking *tetA*(A) gene, some additional sequences such as *rck*, *srgA*, *srgB* genes, which originated from the virulence plasmid of the donor cells were inserted to replace the *tetA*(A) gene (Hradecka et al., 2008). Hradecka et al. (2008) suggested that the exposure of *S. enterica* serovar Typhimurium recipient cells to an R-plasmid may lead to R-plasmid recombination with the virulence plasmid. Both the antibiotic resistance and virulence

genes may then be spread to the recipient cells (Hradecka et al., 2008). Such gene arrangements may have occurred in *S. enterica* serovar Typhimurium strains SRC29.

The mating and triparental mating results confirmed that for 19 *Salmonella* strains, the gene cluster IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*26* or IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$ was located on large plasmids. Plasmid incompatibility data revealed that most of these plasmids belonged to IncHI and IncI incompatibility groups (R. Levings, unpublished data). Hradecka et al. (2008) also found that 12 transconjugants belonged to two incompatibility groups, IncHI1 and IncI1, which were the same as the incompatibility groups of transconjugants in this work. The antibiotic resistance gene clusters of 4 *Salmonella* strains could not be transferred to *E. coli* by conjugation or triparental mating. The lack of transfer of plasmids from these strains might be explained because the plasmid is non-mobilisable or have different preferential conjugation temperatures. As described by Hradecka et al. (2008), some *Salmonella* strains preferred 24°C and some preferred 37°C. In this work, conjugation experiments were carried out at 37°C. Additional investigations are required for these four strains.

Apart from the identification of IncHI and IncI plasmids, IncF incompatibility R plasmids have also been identified in several *S. enterica* serovar Typhimurium. For example, a virulence plasmid of the IncFII group conferring the AMP-GEN-KAN-STR-SPT-SUL-TMP antibiotic resistance phenotype was reported in Italy (Villa and Carottoli, 2005). A 140 kb hybrid virulence resistance plasmid

pUO-StVR2 has been identified from a collection of 49 *S. enterica* serovar Typhimurium isolates in Spain. This plasmid conferred a defined resistance profile of AMP-CHL-STR-SPT-SUL-TET-TMP in combination with a *merA* gene (Herrero et al., 2006). Similar fusions of resistance and virulence genes on a single plasmid have also been described in *S. enterica* serovar Typhimurium (Llanes et al., 1999). An IncFIC plasmid harboured *spvCD-orfE* genes (virulence genes) together with resistance determinants coding for an AMP-CHL-STR-SPT-SUL-TET phenotype (Llanes et al., 1999). The acquisition of resistance genes on virulence plasmids may represent an evolutionary process resulting in the capacity to colonise novel hosts and environments (Carottoli, 2003). As plasmids are not essential for bacterial life, recombination events in these molecules are more frequent than in the main chromosome, which may underpin the appearance of new gene combinations including recombination events between different R-plasmids and serovar-specific virulence plasmids found in *S. enterica* serovars Typhimurium (Hradecka et al., 2008).

Multidrug resistant *S. enterica* serovar Typhimurium has emerged as a leading serotype causing salmonellosis in India during the last decade (Mohan et al., 1995). In the early 1980's only a relatively small proportion of strains (22%) were found to carry conjugative self-transmissible plasmids (Sharma et al., 1984). In the 1990's, Mohan et al. (1995) reported that 190 *S. enterica* serovar Typhimurium strains isolated from different regions of India were all found to carry varying molecular weight plasmids. Most of the strains carried large (114.3-171.5 kb) or medium (23.6-88.2 kb) sized

plasmids and these plasmids were non-conjugative non-autotransmissible (Mohan et al., 1995). It was suggested by Mohan et al. (1995) that genetic shift from self to non-autotransmissible plasmids might have occurred in the heterogenous plasmid pool of *S. enteirca* serovar Typhimurium strains in India. In the *S. enterica* serovar Typhimurium collection under examination in this study, some strains contain conjugative plasmids, while others contain non-autotransmissible plasmids, similar to that described by Mohan et al. (1995).

Antibiotic resistance gene clusters on large plasmids have a major impact on human health. Firstly, a large number of antibiotic resistance genes may be acquired rapidly by human pathogens in a single step. Secondly, the selection for one antibiotic resistance may lead to the proliferation of resistance to many antibiotics due to co-selection. Thirdly, if multiple drug resistant strains can be transferred rapidly from one serovar to the other, then the impact of selection for resistance in one serovar can affect resistance in other serovars as well (Holt et al., 2007). Co-selection can also occur when antibiotic resistance genes are haboured on the same plasmid as virulence genes, potentially selecting for increased virulence in the bacterial population. Conjugation is the most frequently recognized mechanism for horizontal gene transfer. Conjugation can also mediate chromosomal exchange following the integration of a self-transmissible plasmid into the bacterial chromosome (Carottoli, 2001). In this study, the conjugative transfer of a plasmid with a Tn*21*-like transposon harbouring a class 1 integron allows the movement of antibiotic resistance gene clusters from one bacterial cell to another.

The presence of the class 1 integron also facilitates capture of new antibiotic resistance gene cassettes. The characterisation of the burden of antibiotic resistance genes and an understanding of the acquisition and transfer of such genes is an important first requirement for combating the further spread of antibiotic resistance genes into commensal and pathogenic bacterial populations.

Chapter 7: Discussion

Antibiotic resistance is a global public health concern that affects the efficacy of all licensed antibiotics (Woodford et al, 2009). In recent years, rapid emergence of MDR bacteria in farm animals has caused serious concern, especially for zoonotic pathogen such as *Salmonella* and *E. coli* (Gebreyes and Altier, 2002; Pereira et al, 2007; Su et al., 2003). In this work, 136 *Salmonella* strains isolated from human sources, animals, environmental and plant sources in Australia were characterised for streptomycin resistance genes *strA* and *strB*, the sulfonamide resistance gene *sul2*, and tetracycline resistance genes *tetA*(A) and *tetA*(B). 49.3% of strains were identified to contain *strA-strB* genes, and 43.4% of strains contain the *sul2* gene. 37.5% and 25.0% of the strains contain *tetA*(A) and *tetA*(B) genes respectively.

The *strA-strB* antibiotic resistance genes from plant sources were found located on the Tn*5393* transposon (Chiou and Jones, 1996). With time however, the Tn*5393* transposon has likely spread its host range and has now also been identified from animal sources (Pezzella et al., 2004). In this study, Tn*5393* transposon was identified in 11 *S. enterica* serovar Hadar strains, among these, six strains were isolated from human sources and five were from ducks. It is very possible that Tn*5393* transposon has been passed to humans via food contamination. Tn*5393* transposon has been detected in bacteria isolated from permafrost and been located on the medium-size plasmids with a narrow host range (Petrova et al., 2008). Recently, part of Tn*5393* containing

streptomycin resistance genes *strA-strB*, IS*1133* and a truncated *tnpR*, was identified in *S. enterica* serovar Kentucky strain SGI1-K, which was isolated from a human (Doublet et al., 2009). Tn*5393* transposon has been found to localize not only on repN- and repI1-positive plasmids, but also in the main chromosome (Pezzella et al., 2004). The broad spread of the *strA-strB* gene could be accounted for by transposition of Tn*5393* to conjugative plasmids, which are then disseminated widely among Gram-negative bacteria (Chiou and Jones, 1993).

In this collection, small plasmids including RSF1010, p9123 and RSF1010-like plasmids were identified to harbour the *strB-strA-sul2* region. This region has also been detected on *E. coli* small plasmid pBP1, plasmid pIE1115 from an uncultured *Eubacterium*, the IncQ-like plasmid pMS260 isolated from *Actinobacillus pleuropneumoniae*, the multidrug resistance island of *S. enterica* serovar Typhimurium DT193, and the trimethoprim-sulfamethoxazole resistance-encoding transposon-like element of *Vibrio cholerae* (Korfmann et al., 1983; Smalla et al., 2000; Ito et al., 2004; Daly et al., 2005; Beaber et al., 2002). The common occurrence of the *strB-strA-sul2* region suggests that this highly disseminated genetic trait can be transferred between the chromosomes and plasmids of different bacteria (Chen et al., 2006).

Thirty-three strains were characterised to contain the IS*26-strB-strA-sul2-repC-repA*-IS*26* antibiotic resistance region as noted by Daly et al. (2005). Twenty-three of these were *S. enterica* serovar Typhimurium, 8 were *S. enterica*

serovar Bovismorbificans and 2 were other serovars. It is suggested by Daly et al. (2005) that the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* antibiotic resistance region derived from the *strB*-*strA*-*sul2*-*repC*-*repA* region of the RSF1010 plasmid is flanked by two inverted IS*26* elements. The IS*26* element downstream of the *repA* gene probably caused the deletion of the 5′ end of the *repA* gene of RSF1010 (Daly et al., 2005). Three different sized *repC*-IS*26* amplicons were obtained in our collection, which contained the same *repC* and IS*26* gene, but the size of the *repA* gene varied. The *repC*-IS*26* amplicon of 882 bp appeared in 6 strains and a 1274 bp amplicon was detected in 26 strains. The *repC*-IS*26* amplicon of 1123 bp was detected only in one strain. The occurrence of the same derivatives of this region in different strains suggests that the deletion of the 5′ end of the *repA* gene caused by IS*26* element happened only rarely and the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* region may have been transferred between different strains by horizontal transfer. The findings of this study suggest that the association of IS*26* to *repC* gene was potentially a critical step in the formation of this antibiotic resistance region and it may be a useful marker to detect clonal strains.

Further characterisation of 23 *S. enterica* serovar Typhimurium strains revealed that two different but related antibiotic resistance gene clusters were present in these strains. Ten strains contained the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-$bla_{\text{TEM-1}}$ gene cluster but an IS*26* element was absent downstream of the $bla_{\text{TEM-1}}$ gene. Eleven strains contained the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-$bla_{\text{TEM-1}}$-IS*26* gene cluster. The other two strains contained the gene cluster IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-$bla_{\text{TEM-1}}$-IS*26* or

IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$ respectively. DNA sequence analysis of the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster from strain SRC26 revealed that it was 99% identical to the corresponding fragments on plasmid pHCM1 (accession no. AL513383) from *S. enterica* serovar Typhi, pU302L (accession no. AY333434) from *S. enterica* serovar Typhimurium, pRSB107 (accession no. AJ851089) from an uncultured bacteria and pAKU_1 (accession no. AM412236) from *S. enterica* serovar Paratyphi A. This result suggested that this IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* antibiotic resistance gene cluster might move as one genetic element between different plasmids and bacteria (Chen et al., 2007).

For the further characterisation of the 11 strains containing the IS*26-strB-strA-sul2-repC-repA*-IS*26-bla*$_{TEM-1}$-IS*26* gene cluster, three derivatives were observed downstream of the *bla*$_{TEM-1}$-IS*26* region. Strain SRC26 harboured a nearly complete In4 type class 1 integron (IS*6100*-orf5-*sul1-qacE△1-dfrA5-intI1*) with only 25 bp of the IS*6100* inverted repeat missing. Strain SRC129 contained *qacE△1-dfrA5-intI1* genes downstream of the *bla*$_{TEM-1}$-IS*26* region where only 225 bp of the *qacE△1* was retained. Strain SRC14 contained *dfrA5-intI1* genes downstream of the *bla*$_{TEM-1}$-IS*26* region. Doublet et al. (2008) identified different left insertion sites of the IS*26* element in the region encompassing the *res* gene to the 5′-CS of the integron in variant SGI1 MDR regions (Figure 7.1). In the SGI1-Q1 structure, the IS*26* element was inserted downstream of the *intI1* gene and deleted part of this gene. In the SGI1-Q2

structure, the IS*26* element was inserted downstream of the inverted repeat of the *intI1* gene. In the SGI1-Q3 structure, the IS*26* element was inserted downstream of the *res* gene and deleted part of this gene.

Identification of the derivatives in this project suggest that the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-bla_{TEM-1}-IS*26* gene cluster has possibly inserted into an existing class 1 integron (In4 type), which is located on a Tn*21*-like transposon. Insertion has deleted the 3′-CS of the integron to form the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-bla_{TEM-1}-IS*26*-*dfrA5*-*intI1* antibiotic resistance region. Plasmid conjugation experiments confirmed that the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-bla_{TEM-1}-IS*26* or IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-bla_{TEM-1} antibiotic resistance gene clusters for 19 strains were located on plasmids.

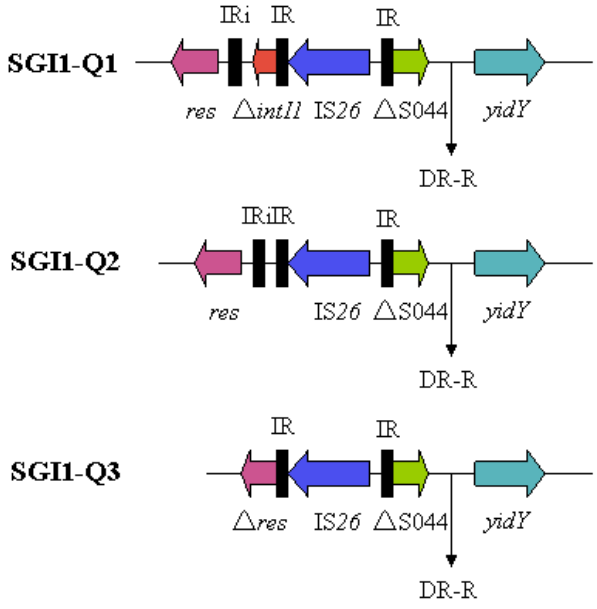


Figure 7.1 Schematic representations of the SGI1-Q1, SGI1-Q2 and SGI1-Q3 MDU regions. Genes and their transcriptional directions are indicated by arrows. Inverted

repeats are indicated by vertical black bars. DR-R represents the right 18-bp direct repeat at the end of SGI1 (adapted from Doublet et al., 2008).

Four steps are hypothesized for the formation of the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*26* gene cluster located on plasmids (Figure 7.2). Firstly, RSF1010 was the possible ancestor supplying the *strB*-*strA*-*sul2*-*repC*-*repA* genes to form the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* antibiotic resistance region. Though two IS*26* elements are in opposite orientation, the involvement of IS*26* may have contributed to the gene assembly, mobilisation and rearrangements including deletions and inversions within this locus (Miriagou et al., 2006). Secondly, the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26* antibiotic resistance region was preceded by a putative transposase gene *tnpB* and this gene was also noted in the RSF1010 plasmid sequence, adjacent to the *strB* gene (Daly et al., 2005). The *bla*$_{\text{TEM-1}}$ gene was possibly derived from Tn*3* transposon. This IS*26*-mediated co-integration leads to the formation of the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*26* antibiotic resistance gene cluster. Thirdly, this gene cluster disrupted the existing class 1 integron (In4 type) and inserted into the Tn*21*-like transposon. The insertion of the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{\text{TEM-1}}$-IS*26* gene cluster deleted the 3′-CS of the In4 type class 1 integron in different lengths. Fourthly, the Tn*21*-like transposon was transferred into a plasmid backbone by transposition. These multi-resistance regions could further evolve, responding to selective pressure leading to the integration of novel gene cassettes and the recruitment of other IS*26*-associated segments by homologous recombination. The transposon Tn*4352B* (IS*26*-*aphAI*-IS*26*) may be inserted upstream

of the antibiotic resistance gene cluster in this way (Szczepanowski et al., 2005). The IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* gene cluster, inserted between the integron and the *tniA* module in Tn*21*-like transposon, may have occurred before or after the acquisition of Tn*21* by a plasmid backbone (Szczepanowski et al., 2005).

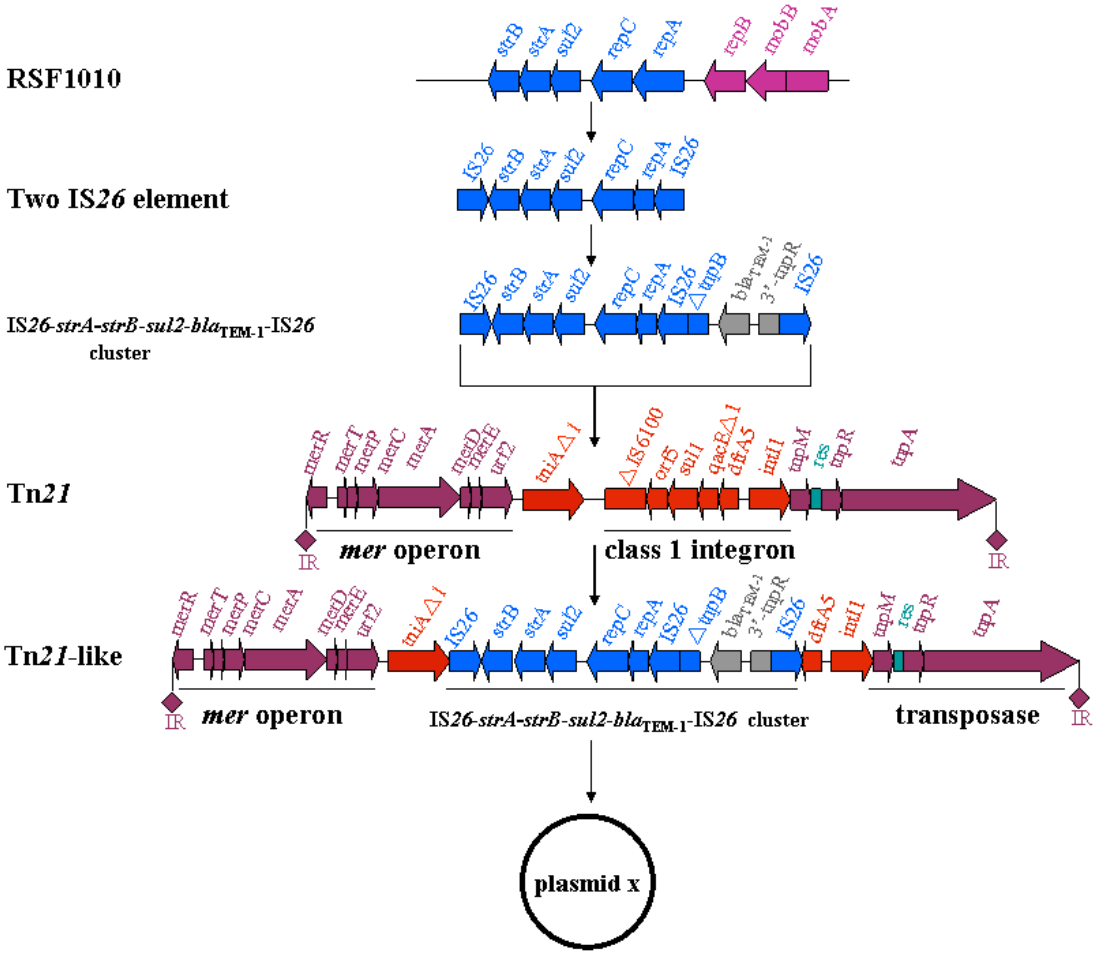


Figure 7.2 The hypothetical evolutionary process for the formation of the IS*26*-*strB*-*strA*-*sul2*-*repC*-*repA*-IS*26*-*bla*$_{TEM-1}$-IS*26* antibiotic resistance gene cluster harboured on plasmids.

Recently, Doublet et al. (2009) have identified variants of SGI1 contained large IS*26*-composite transposons, which were inserted by a transposition event into the SGI1 chromosomal backbone. These IS*26*-composite transposons contained an In4-type integron, a mercury resistance operon and parts of Tn*1721* and Tn*5393*. It was

suggested by Doublet et al. (2009) that the genomic island SGI1 appears to be a hotspot of acquisition of antibiotic resistance genes by the transposition of In4-type integrons or transposon structures.

Resistance has arisen to all antibiotics introduced into general clinical practice and is likely to arise to any new antibiotic introduced in the future. It is therefore imperative to consider what can be done to minimise the development and transfer of antibiotic resistance gene clusters. Methods can be developed to minimise antibiotic resistance. Firstly, despite the high cost of each new antibiotic developed, these new antibiotics need to be reserved for clinical and not for animal use. Secondly, the level of use of all antibiotics should be reduced to the minimum requirement compatible with clinical imperatives on a global basis. Thirdly, worldwide education is needed to emphasize the consequences of overuse and misuse of antibiotics. Fourthly, more studies are needed concerning the evolution and spread of antibiotic resistance bacteria. Finally, vaccines that can protect against the most common nosocomial infections need to be developed to reduce disease burden and hence antibiotic use (Bennett, 2008). Recently, Woodford et al. (2009) have developed a new strategy called ‘antisense’ or ‘antigene’ agents, which may interrupt the expression of antibiotic resistance genes and restore susceptibility to key antibiotics.

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http://www.ch.embnet.org/software/ClustalW.html

http://blast.ncbi.nlm.nih.gov/Blast.cgi

Appendix I

LB Media

Tryptone	10 g
Yeast	5 g
NaCl	5 g
Add MQ water to	1000 ml

LB Agar

Tryptone	10 g
Yeast	5 g
NaCl	5 g
Agar	15 g
Add MQ water to	1000 ml

1 X PBS Buffer

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.31 g
Add MQ water to	1000 ml

1 M Tris Buffer

Tris	121.14 g
Add MQ water to	1000 ml

EDTA Buffer (0.5 M, pH=8.0)

EDTA	186.1 g
Add MQ water to	1000 ml

NaOH pellets were used to regulate the pH to 8.0.

TE Buffer (pH=8.0)

1M Tris	10 ml
0.5M EDTA	2 ml
Add MQ water to	1000 ml

5 X TBE Buffer

Tris	54 g
Boric acid	27.5 g
0.5 M EDTA (pH=8)	20 ml
Add MQ water to	1000 ml

1 X TBE Buffer

5 X TBE Buffer	200 ml
Add MQ water to	1000 ml

0.5 X TBE Buffer

5 X TBE Buffer	200 ml
Add MQ water to	2000 ml

100bp^{+} molecular weight marker

DNA ladder	20 µl
Loading dye	20 µl
MQ water	80 µl

0.2% HCl

32% HCl	5 ml
Add MQ water to	800 ml

Denaturing solution

NaOH	20 g
NaCl	87.66 g
Add MQ water to	1000 ml

Neutralizing solution

NaCl	87.66 g
Tris	121.14 g
Add MQ water to	1000 ml

10% w/v SDS

sodium dodecyl sulphate (SDS)	100 g
Add MQ water to	1000 ml

20 X SSC

NaCl	175.3 g
Trisodium citrate	88.2 g
Add MQ water to	1000 ml

2 X SSC

20 X SSC	100 ml
Add MQ water to	1000 ml

2 X SSC, 0.1% SDS

20 X SSC	100 ml
10% SDS	10 ml
Add MQ water to	1000 ml

TE saturated phenol/chloroform/isoamylalcohol (25:24:1) (300 ml)

phenol pH=8.0	150 ml
chloroform	144 ml
isoamylalcohol	6 ml

The above solution was mixed thoroughly, then 100 ml of TE buffer (pH=8.0) was added and mixed thoroughly again. The bottle was wrapped in alfoil and stored in fridge.

TE saturated chloroform/isoamylalcohol (24:1) (300 ml)

chloroform	288 ml
isoamylacohol	12 ml

The above solution was mixed thoroughly, then 100 ml of TE buffer (pH=8.0) was added and mixed thoroughly again. The bottle was wrapped in alfoil and stored in fridge.

Developer (Kodak)

Kodak GBX developer	206 ml
Add MQ water	740 ml

Mixed thoroughly, then wrapped in alfoil and stored in dark room.

Fixer (Kodak)

Kodak GBX fixer	206 ml
Add MQ water	740 ml

Mixed thoroughly, then wrapped in alfoil and stored in dark room.

Lysis solution (pH=12.6)

1M Tris	50 ml
10% SDS	300 ml
Add MQ water	650 ml

NaOH pellets were used to regulate the pH to 12.6.

Phenol/chloroform (1:1)

Phenol	200 ml
Chloroform	200 ml

Mixed thoroughly, then wrapped in alfoil and stored in dark room.

10% Sucrose/TE

Sucrose	100 g
TE buffer	1000 ml

Use 800 ml TE buffer to solve 100 g sucrose first, add the rest TE buffer to 1000 ml.

10% SDS/TE

SDS	100 g
TE buffer	1000 ml

Used 800 ml TE buffer to solve 100 g SDS first, add the rest TE buffer to 1000 ml.

RNaseA (10 mg/ml)

RNaseA	10 mg
MQ water	1 ml

Mixed thoroughly.

Proteinase K (10 mg/ml)

Proteinase K	10 mg
MQ water	1 ml

Made freshly.

Lysozyme (50 mg/ml)

Lysozyme	50 mg
MQ water	1 ml

Made freshly.

20% glycerol

Glycerol	200 g
MQ water	1000 ml

Need to be autoclaved.

10% glycerol

Glycerol	100 g
MQ water	1000 ml

Need to be autoclaved

10% glycerol/1% Casamino

Glycerol	100 g
Casamino	10 g
MQ water	1000 ml

Need to be autoclaved

Loading dye (100 ml)

Bromophenol blue	0.25 ml
Ficoll	15 g
MQ water	84.75 ml

Stir for a whole day

Appendix II

Table A PCR screening results of *sul2*, *sul2-strA*, *strA*, *strB*, *strA-strB*, Tn*5393*, RSF1010, *tetA*(A), *tetA*(B) and the right arm of Tn*1721*.

SRC#	Serovar	Source	Resistance Phenotype	*sul2*	*sul2-strA*	*strA*	*strB*	*strA-strB*	Tn*5393* (IS*1133*)	RSF1010	*tet*(A)	*tet*(B)	Tn*1721*
12	Typhimurium PT135	human, F (99)	ApSu	+	-	-	-	-	-	-	-	-	-
13	Typhimurium PT135	human, F (99)	ApSmSuTcTp	+	+	+	+	+	-	-	+	-	+
14	Typhimurium PT44	human, F (99)	ApSmSuTcTp	+	+	+	+	+	-	-	+	-	+
15	Typhimurium PT9	human, F (99)	SmSu	+	+	+	+	+	-	+	-	-	-
16	Typhimurium Ptu	human, M (99)	ApCmGmSpSuTcTp	+	-	+	-	-	-	-	+	-	+
26	Typhimurium PT1var2	bovine (99)	ApKmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
27	Typhimurium PT135	equine (99)	ApCmGmKmSmSpSuTcTp	+	+	+	+	+	+	-	-	+	-
28	Typhimurium PT135	equine (99)	ApCmGmKmSmSpSuTcTp	+	+	+	+	+	-	-	-	+	-
29	Typhimurium PT141	porcine (99)	ApGmKmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
30	Typhimurium PT141 v4	porcine (99)	ApGmKmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
31	Typhimurium PT44	bovine (99)	ApSmSuTcTp	+	+	+	+	+	-	-	+	-	+
32	Typhimurium PT68	bovine (99)	ApKmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
33	Typhimurium PT9	bovine (99)	SmSu	+	+	+	+	+	-	+	-	-	-
56	Typhimurium 104L	human, M Timor (ND)	ApCmGmKmSmSpSuTcTp	+	+	+	+	+	-	+	-	+	-
113	Typhimurium PT12	bovine (00)	Tc	-	-	-	-	-	-	-	+	-	+
114	Typhimurium PT135	avian (00)	Sm	-	-	-	-	-	-	-	-	-	-
115	Typhimurium PT135	chicken (00)	TcTp	-	-	-	-	-	-	-	+	-	+
116	Typhimurium PT135	bovine (00)	ApSmSuTcTp	+	+	+	+	+	-	-	+	-	+
117	Typhimurium PT141	porcine (ND)	ApGmKmSmSuTp	+	+	+	+	+	-	-	-	-	-
118	Typhimurium PT141	porcine (00)	ApGmKmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
119	Typhimurium PT208 v1	porcine (00)	ApCmGmKmSmSpSuTc	+	-	-	-	-	-	-	-	-	-
120	Typhimurium PT29	porcine (00)	ApKmSmSuTp	+	+	+	+	+	-	-	-	-	-
121	ND	porcine (00)	ApGmKmSmSuTp	+	+	+	+	+	-	-	-	-	-
122	Typhimurium PT29	porcine (00)	ApKmSmSuTp	+	+	+	+	+	-	-	-	-	-
123	Typhimurium PT29	porcine (00)	ApKmSmSuTcTp	+	+	+	+	+	-	-	+	-	+
124	Typhimurium PT44	bovine (00)	ApSmSuTcTp	+	+	+	+	+	-	-	+	-	+
125	Typhimurium PT44	bovine (00)	KmSuTcTp	-	-	+	+	+	-	-	-	+	-
126	Typhimurium PT44	bovine (00)	ApSmSuTp	+	+	+	+	+	-	-	-	-	-
127	Typhimurium PT44	bovine (00)	KmSuTcTp	-	-	+	+	+	-	-	-	+	-
128	Typhimurium PT44	bovine (00)	ApSmSuTcTp	+	+	+	+	+	-	-	+	-	+
129	Typhimurium PT44	bovine (00)	ApKmSmSuTcTp	+	+	+	+	+	-	-	+	-	+

SRC#	Serovar	Source	Resistance Phenotype	*sul2*	*sul2-strA*	*strA*	*strB*	*strA-strB*	Tn*5393* (IS*1133*)	RSF1010	*tet*(A)	*tet*(B)	Tn*1721*
130	Typhimurium PT64	bovine (00)	SmSu	+	+	+	+	+	-	+	-	-	-
131	Typhimurium PT9	bovine (00)	ApSmSuTp	+	+	+	+	+	-	-	-	-	-
132	Typhimurium PT9	bovine (00)	ApSmSu	+	+	+	+	+	-	-	-	-	-
133	Typhimurium PT9	bovine (00)	ApKmSmSu	+	+	+	+	+	-	-	-	-	-
134	Typhimurium PT99	pheasant (00)	Tc	-	-	-	-	-	-	-	+	-	+
135	Typhimurium RDNC	porcine (ND)	ApKmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
136	Typhimurium RDNC	porcine (00)	ApKmSmSuTp	+	+	+	+	+	-	-	-	-	-
137	Typhimurium	ND/ND	Tc	-	-	-	-	-	-	-	+	-	+
17	Bovismorbificans	bovine (99)	ApCmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
36	Bovismorbificans PT13	human, F (01)	ApKmSmSuTcTp	+	+	+	+	+	-	-	+	-	+
59	Bovismorbificans PT13	bovine (01)	ApKmSmSuTcTp	-	-	-	-	-	-	-	-	-	-
60	Bovismorbificans PT13	bovine (01)	ApCmKmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
61	Bovismorbificans PT11	bovine (01)	ApKmSmSuTcTp	+	+	+	+	+	-	-	+	-	+
62	Bovismorbificans PT32	bovine (01)	ApCmKmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
79	Bovismorbificans PT14	bovine (00)	ApCmKmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
80	Bovismorbificans PT24	bovine (00)	ApCmKmSmSuTcTp	+	+	+	+	+	-	-	-	+	-
81	Bovismorbificans 24	abattoir effluent (00)	ApGmTc	-	-	-	-	-	-	-	+	-	+
82	Bovismorbificans 32	bovine (00)	ApSmSuTp	+	+	+	+	+	-	-	-	-	-
83	Bovismorbificans u	chicken (00)	CmSmSpSuTc	-	-	-	-	-	-	-	-	+	-
24	Sofia	chicken (99)	SmSpSuTp	-	-	-	-	-	-	-	-	-	-
25	Sofia	chicken (99)	ApTc	-	-	-	-	-	-	-	+	-	+
52	Sofia	human,M (01)	Tp	-	-	+	+	+	-	-	-	-	-
53	Sofia	human, F (01)	Km	-	-	-	-	-	-	-	-	-	-
75	Sofia	chicken (01)	Tp	-	-	-	-	-	-	-	-	-	-
76	Sofia	chicken (01)	SuTp	+	-	-	-	-	-	-	-	-	-
77	Sofia	chicken (01)	SuTcTp	-	-	-	-	-	-	-	+	-	+
105	Sofia	canine (00)	Tc	-	-	-	-	-	-	-	-	-	-
106	Sofia	chicken (00)	ApSmSuTc	+	+	+	+	+	-	-	+	-	+
107	Sofia	chicken (00)	SmSu	+	+	+	+	+	-	-	-	-	-
108	Sofia	chicken (00)	SuTcTp	-	-	-	-	-	-	-	+	-	+
109	Sofia	chicken (00)	SmSuTcTp	+	+	+	+	+	-	-	+	-	+
110	Sofia	chicken (00)	ApSmSuTc	+	+	+	+	+	-	-	+	-	+
111	Sofia	chicken (00)	SuTcTp	-	-	-	-	-	-	-	+	-	+
07	Hadar	human, M Indo (99)	ApSmTc	-	-	+	+	+	+	-	+	-	+
08	Hadar PT10	human, M o/s (99)	ApTc (NaCp')	-	-	+	+	+	+	-	+	-	+
09	Hadar PT2	human, F/ (99)	SmTc	-	-	+	+	+	+	-	+	-	+
39	Hadar PT10	human, M Indo (01)	SmTc (NaCp')	-	-	+	+	+	+	-	+	-	+

SRC#	Serovar	Source	Resistance Phenotype	*sul2*	*sul2-strA*	*strA*	*strB*	*strA-strB*	Tn*5393* (IS*1133*)	RSF1010	*tet*(A)	*tet*(B)	Tn*1721*
40	Hadar PT11	human,F Bali (01)	Tc (NaCp')	-	-	-	+	-	-	-	+	-	+
41	Hadar PT11	human, M (01)	CmKmSmSpSuTc (NaCp')	-	-	-	+	-	-	-	+	-	+
42	Hadar PT14	human,F Bali (01)	Tc (Na)	-	-	-	+	-	-	-	+	-	+
43	Hadar PT2	human, F (01)	SmTc (Na)	-	-	+	+	+	+	-	+	-	+
44	Hadar PT22	human, F (01)	SmTc (Na)	-	-	+	+	+	+	-	+	-	+
64	Hadar PT11	duck (01)	SmTc	-	-	+	+	+	+	-	+	-	+
65	Hadar PT14	duck (01)	SmTc	-	-	+	+	+	+	-	+	-	+
66	Hadar PT2	duck (01)	SmTc	-	-	+	+	+	+	-	+	-	+
67	Hadar PT22	duck (01)	SmTc	-	-	+	+	+	+	-	+	-	+
68	Hadar PT33	duck (01)	SmTc	-	-	+	+	+	+	-	+	-	+
20	Infantis	chicken (99)	SmSpSuTc	-	-	-	-	-	-	-	-	-	-
46	Infantis	human, F (01)	SmSpSuTp	-	-	-	-	-	-	-	-	-	-
47	Infantis	human, M (01)	ApTcTp	-	-	-	-	-	-	-	-	-	-
70	Infantis	feline (01)	SmSpSuTc	-	-	-	-	-	-	-	-	+	-
71	Infantis	chicken (01)	SmSpSuTc	-	-	-	-	-	-	-	-	+	-
72	Infantis	chicken (01)	ApSmSpSuTc	-	-	-	-	-	-	-	-	+	-
92	Infantis	canine (00)	SmSpTcTp	-	-	-	-	-	-	-	-	+	-
93	Infantis	chicken (00)	ApCmSpSuTc	-	-	-	-	-	-	-	-	+	-
94	Infantis	chicken (00)	CmSmSpSuTc	-	-	-	-	-	-	-	-	+	-
95	Infantis	chicken (00)	SmSpSuTc	-	-	-	-	-	-	-	-	+	-
96	Infantis	chicken (00)	SmSuTc	-	-	-	-	-	-	-	-	+	-
10	Kiambu	human, M (99)	ApCmSmSpSuTcTp	-	-	-	-	-	-	-	-	-	-
11	Kiambu	human, M (99)	ApCmSmSpSuTcTp (NaCp')	-	-	-	-	-	-	-	-	-	-
21	Kiambu	chicken (99)	SuTcTp	-	-	-	-	-	-	-	-	-	-
97	Kiambu	chicken (00)	SuTp	-	-	-	-	-	-	-	-	-	-
98	Kiambu	chicken (00)	SuTcTp	-	-	-	-	-	-	-	-	-	-
99	Kiambu	chicken (00)	ApCmSmSpSuTcTp	-	-	-	-	-	-	-	-	-	-
100	Kiambu	chicken (00)	SmSuTcTp	-	-	+	+	+	-	-	-	-	-
34	Agona	human, F Africa (01)	CmSmSpSuTcTp	+	-	+	+	+	-	-	+	-	+
35	Agona	human, M Malay (01)	SmSuTc (Na)	+	+	+	+	+	-	+	+	-	-
57	Agona	porcine (01)	ApGmTc	-	-	-	-	-	-	-	-	+	-
58	Agona	porcine (01)	Tc	-	-	-	-	-	-	-	+	+	+
04	Derby	human, M Malay (99)	CmGmSmSpSuTcTp	-	-	-	-	-	-	-	-	-	-
18	Derby	porcine (99)	ApTc	-	-	-	-	-	-	-	-	+	-
37	Derby	human, F (01)	SuTc	+	-	-	-	-	-	-	+	-	+
63	Derby	porcine (01)	SmTc	-	-	-	-	-	-	-	-	+	-
85	Derby	porcine (00)	SmTc	-	-	-	-	-	-	-	-	+	-

SRC#	Serovar	Source	Resistance Phenotype	*sul2*	*sul2-strA*	*strA*	*strB*	*strA-strB*	Tn*5393* (IS*1133*)	RSF1010	*tet*(A)	*tet*(B)	Tn*1721*
86	Derby	porcine (00)	ApCmTc	-	-	-	-	-	-	-	+	-	+
87	Derby	porcine (00)	SmTcTp	-	-	-	-	-	-	-	+	-	+
88	Derby	porcine (00)	ApGmTc	-	-	-	-	-	-	-	-	+	-
45	Havana	human, M (01)	SmSpSuTc	-	-	-	-	-	-	-	-	+	-
69	Havana	porcine (01)	ApGmKmSmSpTc	+	-	-	-	-	-	-	-	-	-
90	Havana	porcine (00)	ApCmSuTcTp	-	-	-	-	-	-	-	+	-	+
91	Havana	porcine (00)	GmSmSpSuTc	+	-	-	-	-	-	-	-	-	-
23	Senftenberg	porcine (99)	ApCmSmSpSuTcTp	+	+	+	+	+	-	-	-	+	-
102	Senftenberg	porcine (00)	ApGmKmSmSpSuTcTp	+	-	-	-	-	-	-	-	-	-
103	Senftenberg	porcine (00)	ApGmKmSmSpSuTc	+	-	-	-	-	-	-	-	-	-
104	Senftenberg	meat, bone meal (00)	Sm	-	-	-	-	-	-	-	-	-	-
49	Paratyphi B dT^+	human, F (01)	ApCmSmSpSuTc	-	-	-	-	-	-	-	-	-	-
50	Paratyphi B dT^+	human, M (01)	ApCmSmSpSuTc	-	-	-	-	-	-	-	-	-	-
101	Paratyphi B dT^+	fish tank water (00)	ApCmSmSpSuTc	-	-	-	-	-	-	-	-	-	-
54	Stanley	human, M Thai (01)	CmGmKmSmSpSuTc (Cp')	-	-	+	+	+	-	-	+	-	+
55	Stanley	human, F Thai (01)	SmSuTc	+	+	+	+	+	-	+	+	-	-
112	Stanley	porcine (00)	ApGmSuTcTp	-	-	-	-	-	-	-	-	+	-
02	Blockley	human, F Bali (99)	KmSmTc (NaCp')	-	-	+	+	+	-	-	+	-	+
03	Blockley	human, M Thai (99)	CmKmSmSpSuTcTp (NaCp')	-	-	+	+	+	-	-	+	-	+
22	Ohio	porcine (99)	CmGmKmSmSpSuTc	+	-	-	-	-	-	-	-	-	-
74	Ohio	porcine (01)	ApCmGmKmSmSpSuTc	+	-	-	-	-	-	-	-	-	-
01	Singapore	human, F (01)	CmSmSpSuTcTp (Na)	+	+	+	+	+	-	-	+	-	+
51	Singapore	human, M (01)	CmSmSpSuTcTp (NaCp')	+	+	+	+	+	-	-	+	-	+
05	Cerro	human, F Thai (99)	ApCmSuTcTp (NaCp')	-	-	-	-	-	-	-	-	-	-
06	Enteritidis PT1	human, F HK (99)	SmSuTc (NaCp')	+	+	+	+	+	-	+	+	-	-
19	Emek	waste effluent (99)	CmSuTcTp (NaCp')	-	-	-	-	-	-	-	-	-	-
38	Dusseldorf	human, M Malay (01)	ApCmSuTcTp (Na)	-	-	-	-	-	-	-	-	-	-
48	Montevideo	human, M (01)	GmKmSmSpTc (NaCp')	-	-	-	-	-	-	-	-	-	-
73	Kentucky	imported spice, (01)	ApGmSmSpSuTc (Na)	-	-	+	+	+	+	-	+	-	-
78	Anatum	porcine (00)	Tc	-	-	-	-	-	-	-	-	+	-
84	Bredeney	porcine (00)	SuTcTp	-	-	-	-	-	-	-	-	-	-

Table B PCR amplification of IS*26*-*aphAI*, *aphAI*-*strB*, IS*26*-*strB*, IS*26*-*tnpB*, *tnpB*-*bla*$_{TEM-1}$, *bla*$_{TEM-1}$-IS*26* and *dfrA5*-*intI1*.

No	Serovar	Source	Resistance Phenotype	PCR amplification						
				*IS*26-*aphAI*	*aphAI*-*strB*	*IS*26-*strB*	*IS*26-*tnpB*	*tnpB*-*bla*$_{TEM-1}$	*bla*$_{TEM-1}$-IS*26*	*dfrA5*-*intI1*
SRC30	Typhimurium PT141var4	P	ApGmKmSmSuTcTp	-	-	+	+	+	-	+
SRC29	Typhimurium PT141	P	ApGmKmSmSuTcTp	+	+	+	+	+	-	+
SRC32	Typhimurium PT68	B	ApKmSmSuTcTp	+	+	+	+	+	-	+
SRC117	Typhimurium PT141	P	ApGmKmSmSu...Tp	+	+	+	+	+	-	+
SRC118	Typhimurium PT141	P	ApGmKmSmSuTcTp	+	+	+	+	+	-	+
SRC120	Typhimurium PT29	P	ApKmSmSuTp	+	+	+	+	+	-	+
SRC122	Typhimurium PT29	P	ApKmSmSuTp	+	+	+	+	+	-	+
SRC123	Typhimurium PT29	P	ApKmSmSuTcTp	+	+	+	+	+	-	+
SRC135	Typhimurium RDNC	P	ApKmSmSuTcTp	+	+	+	+	+	-	+
SRC136	Typhimurium RDNC	P	ApKmSmSuTp	+	+	+	+	+	-	+
SRC13	Typhimurium PT135	H	ApSmSuTcTp	-	-	+	+	+	+	+
SRC14	Typhimurium PT44	H	ApSmSuTcTp	-	-	+	+	+	+	+
SRC31	Typhimurium PT44	B	ApSmSuTcTp	-	-	+	+	+	+	+
SRC124	Typhimurium PT44	B	ApSmSuTcTp	-	-	+	+	+	+	+
SRC126	Typhimurium PT44	B	ApSmSuTp	-	-	+	+	+	+	+
SRC128	Typhimurium PT44	B	ApSmSuTcTp	-	-	+	+	+	+	+
SRC131	Typhimurium PT9	B	ApSmSuTp	-	-	+	+	+	+	+
SRC132	Typhimurium PT9	B	ApSmSu	-	-	+	+	+	+	-
SRC26	Typhimurium PT1var2	B	Ap.KmSmSuTcTp	+	+	+	+	+	+	+
SRC129	Typhimurium PT44	B	ApSmSuTcTpKm	+	+	+	+	+	+	+
SRC133	Typhimurium PT9	B	ApKmSmSu	+	+	+	+	+	+	-
SRC27	Typhimurium PT135	E	ApCmGmKmSmSpSuTcTp	+	+	+	+	+	+	-
SRC28	Typhimurium PT135	E	ApCmGmKmSmSpSuTcTp	+	+	+	+	+	+	-