Antibiotic resistance genes located in integrons isolated from Escherichia coli recovered from humans and animals

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Antibiotic Resistance Genes Located in Integrons Isolated from *Escherichia coli* Recovered from Humans and Animals

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ABSTRACT

Multi-drug resistant pathogens are the principal cause of failure in the treatment of bacterial infectious diseases. Accurate surveillance of antibiotic resistance genes in the community is essential to developing strategies for resistance control and prevention. In this study, a collection of 514 *Escherichia coli* strains from animal and human sources was examined for the presence of class 1, 2 and 3 integrons using a PCR-based screening method. A multiplex PCR was developed to simultaneously screen for *intI1*, *intI2* and *intI3* genes. This study characterised all gene cassettes including those that could not be PCR amplified using standard screening methods.

The frequency of class 1 and class 2 integrons detected in *E. coli* strains in this study was generally lower than that reported in previous studies. Class 1 integrons were detected in 81/514 *E. coli* strains sourced from animals and humans. Gene cassette arrays identified in class 1 integrons include *dfrA5, dfrA7, aadA1, aadA2, dfrA1/aadA1, dfrA17/aadA5* and *dfrA12/orfF/aadA2*. In addition, atypical integrons containing *dfrA5*-IS*26* and *dfrA15*-IS*26* elements were discovered. The *dfrA5*-IS*26* element, a unique class 1 integron with most of the integron 3′-conserved segment (CS) deleted by the insertion of IS*26,* was detected in 31/514 *E. coli* isolates. This novel integron-*dfrA5*-IS*26* element, which was widespread in *E. coli* isolates of bovine origin and also found in *E. coli* of human origin, may act as a conduit for the transfer of integron-related resistance genes to human pathogens. Utilisation of PCR targeting the integron-IS*26* element will allow the

characterisation cassette arrays in atypical class 1 integrons that remain undetected using currently available PCR-based screening strategies.

Seven of the 514 *E. coli* strains contained class 2 integrons and six of these harboured the gene cassette array analogous to that found in Tn*7*, *dfrA1-sat2-aadA1*. In the remaining *intI2* positive *E. coli* strain 80, in which the gene cassette region could not be PCR amplified using standard methods, the *intI2* gene was found to be located on a plasmid. The complete nucleotide sequence of this plasmid (pECTm80) was determined, revealing an intact *dfrA1-sat2* cassette array and a truncated *aadA1* gene cassette, due to the insertion of IS*1*. Open reading frames and Tn*7* transposition genes normally conserved at the 3′ end of Tn*7*-like class 2 integrons were not detected. This atypical class 2 integron is flanked by a Tn*3* family transposon or insertion sequence (IS) remnant and IS*1*. The plasmid pECTm80, of the incompatibility (Inc) group X, has the potential to facilitate the horizontal transfer of tightly-linked antibiotic resistance genes to diverse antimicrobial species. Features which contribute to the clinical relevance of this plasmid include its ability to be mobilised, the presence of genes to ensure the stable maintenance of the plasmid through successive bacterial cell divisions and the presence of a highly-regulated DNA replication system consisting of α, β and γ origins of replication.

This thesis provides a snapshot of the antibiotic resistance genes located in integrons in *E. coli* strains sourced from Australian animals and humans. The association of atypical integrons with IS elements suggests these DNA elements play an important role in the evolution of integrons.

STATEMENT OF CANDIDATE

I, Fay Ellen Dawes, declare that this thesis, submitted in fulfillment of the requirements for the award of Doctor of Philosophy, in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Fay Ellen Dawes

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ABBREVIATIONS

IntI – integron integrase

59-be – 59-base element

CS – conserved segment

Bp – base pairs

CDS – coding sequences

DNA – deoxyribonucleic acid

IS – insertion sequence

PCR – polymerase chain reaction

IRi – terminal inverted repeat at the integrase end of class 1 integrons

IRt – the inverted terminal repeat at the *tni* end of class 1 integrons

RFLP – restriction fragment length polymorphism

MCS – multiple cloning site

NBUs – nonreplicating *Bacteroides* unit

ORF – open reading frame

R – purine

Y – pyrimidine

ANGIS – Australian National Genomic Information Service

NCBI – National Centre for Biotechnology

EMAI – Elizabeth McArthur Agricultural Institute

MDU – Microbiological Diagnostic Unit

AP – alkaline phosphatase

DIG – digoxigenin

DMF – dimethylformamide

EBSL – extended-spectrum β-lactamase

IPTG – isopropyl-1-thio-β-D-galactopyranoside

LB broth – Luria-Bertani broth

MIO – motility-indole-ornithine

ONPG – o-nitrophenyl-ß-d-galactopyranoside

rpm – revolutions per minute

TAE – Tris acetate EDTA

TE buffer – Tris EDTA

TSI – triple-sugar-iron

X-gal – 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside

A – ampicillin

Cm – chloramphenicol

K – kanamycin

Rif – rifampicin

S – streptomycin

Sp – spectinomycin

Su – sulfathiazole

Tc – tetracycline

Tm – trimethoprim

Nal – nalidixic acid

MRSA – methicillin-resistant *Staphylococcus aureus*

EAEC – Enteroaggregative *E. coli*

EHEC – subclass of Shiga toxin-producing, enterohemorrhagic *E. coli*

EIEC – enteroinvasive *E. coli*

EPEC – enteropathogenic *E. coli*

ETEC – enterotoxigenic *E. coli*

NEMEC – neonatal meningitis *E. coli*

SEPEC – septicemic *E. coli*

STEC – shiga toxin-producing *E. coli*

UPEC – uropathogenic *E. coli*

VRE – vancomycin-resistant enterococci

VTEC – verocytotoxigenic *E. coli*

VCR - *Vibrio cholerae* repeat

UTI – urinary tract infection

HUS – hemolytic uremic syndrome

SIDS – sudden infant death syndrome

IncX – incompatibility group X

IMU – integron mobilisation unit

CHAPTER 1 – INTRODUCTION

1.1 – Antibiotic resistance

Antibiotic resistance is a natural phenomenon in response to the use of an antibacterial agent. To become resistant, bacteria utilise defense mechanisms whereby they inactivate or circumvent an antibacterial agent. Acceleration of the frequency and spectrum of antimicrobial resistant infections in recent years is a major public health concern (Cohen 1992; Finch 1998; Goldmann 1999; Levy 1998; Livermore 2003). The major selective pressure driving changes in the frequency of antibiotic resistance is the widespread use of antimicrobial agents in medicine, veterinary practice and farming (Austin *et al.* 1999; Courvalin and Julian 2003; Goldmann 1999). Multidrug-resistant pathogens which have accumulated resistance genes are the principal cause of failure in the treatment of infectious diseases resulting in increases in the term and magnitude of morbidity, higher rates of mortality and a greater economic burden on governments, healthcare systems and individuals (Goldmann 1999; Lipsitch *et al.* 2002).

1.2 – Response from the pharmaceutical industry

The pharmaceutical industry cannot keep pace with the extraordinary capacity of microbes to acquire antimicrobial genetic resistance determinants. New antimicrobial agents are increasingly required as the level and prevalence of resistance continues to rise. Drug discovery and development is a complex, expensive and time-consuming process involving pharmaceutical manufacturers, regulatory authorities, patent authorities and clinical and academic researchers (Projan 2003). The European

Federation of Pharmaceutical Industries and Associations in November 1999 estimated the research and development costs of taking a compound to market at $600 million. In order to maximise their investment dollar, many large pharmaceutical companies have curtailed or eliminated their antibacterial drug research, shifting their emphasis from short-course therapies targeting acute diseases in favour of long-term treatments for chronic illnesses (Projan 2003). Clearly, the balance between the development of new antibacterial agents and the accumulation of resistance determinants needs to be improved (Livermore 2003).

Current trends of increasing antibiotic resistance have prompted fears that we may be approaching a "post-antibiotic era", where common infections will be frequently untreatable. Bacteria have been able to develop resistance against virtually all known antibacterial agents, whether of natural, semi-synthetic or totally synthetic origin. Resistance has been reported to all known antibiotics including more than 50 penicillins, 70 cephalosporins, 12 tetracyclines, 8 aminoglycosides, 1 monobactam, 3 carbapenems, 9 macrolids, 2 streptogramins and 3 dihydrofolate reductase inhibitors (Neu 1992). However, most "*new antibiotics*" introduced in the last 40 years are minor chemical derivatives of compounds to which microbes are already resistant. As a consequence, bacteria readily adapt existing resistance mechanisms to inactivate these new antibiotics. The only truly *novel* class of antibacterial to be approved for the treatment of bacterial infections in several decades is the oxazolidinones (Normark and Normark 2002). The failure of newly introduced antibacterial agents to achieve a sustained effect is demonstrated by the rapid emergence of resistance to linezolid, a synthetic oxazolidinone which was approved and released by the US Food and Drug Administration in April 2000. The following year resistance was reported in clinical

isolates from patients treated with linezolid for methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) infections (Gonzales *et al.* 2001; Tsiodras *et al.* 2001). Coordinated efforts are required by the medical profession, patients, researchers, health authorities and the general public in order to prolong the life-span of existing antibiotics.

1.3 – Mechanisms of antibiotic resistance

The surveillance of resistance genes is essential to defining the frequency, distribution and dynamics of resistance. Antibiotic resistance surveillance data may be used as a basis for designing strategies for resistance control and evaluating their impact (Finch 2002). The biochemical mechanisms of bacterial resistance which are encoded by resistance genes typically involve one or more of the following (i) alteration or amplification of the drug target in the bacterial cell, (ii) enzymatic modification or destruction of the drug and (iii) limitation of drug accumulation as a result of exclusion, reduced permeability or active drug efflux (Poole 2002). The biochemical mechanisms of antibiotic resistance for trimethoprim and sulfonamides involve amplification of the drug target preventing the action of these antibiotics, which act to inhibit folate metabolism by competitively blocking the synthesis of tetrahydrofolate (Huovinen 2001; Huovinen *et al.* 1995). Detailed knowledge of resistance mechanisms may be utilised to chemically modify antibiotics in order to elude resistance (Davies 1994). Potential therapeutic strategies to combat increasing antimicrobial resistance include targeting generalised resistance mechanisms such as altered uptake by way of restricting membrane permeability or enhanced efflux systems, antibiotic target site modification and antibiotic inactivation (Poole 2002). Information about the resistance gene pool

may be used to predict the impact of newly introduced antimicrobial agents and assess the need for new therapeutic agents (Finch 2002).

1.4 – Antibiotic resistance gene stability, amplification and transmission

Gene stability is a critical, although often overlooked feature defining resistance. Resistance gene transfer elements adapt rapidly to their new hosts, as a consequence they are not readily lost even in the absence of selection (Salyers and Amabile-Cuevas 1997). The initial cost of resistance to the bacterium may be annulled by compensatory mutations in the genome or the evolution of alternative uses for resistance determinants. Additionally, bacteria may accumulate and link resistance genes within the same genetic units (e.g. plasmids, transposons or integrons) allowing resistance to a specific antibiotic to be maintained by any one of these antibiotic compounds via a co-selection process. Co-selection of antibiotic resistance also occurs when genes encoding virulence determinants, biodegradation pathways, resistance to heavy metals, disinfectants or detergents provide a selective advantage to the organism (Baker-Austin *et al.* 2006; Barbosa and Levy 2001; Normark and Normark 2002). Related antibiotics may cause cross-resistance, where resistance to a given antibiotic develops in the absence of that antibiotic, for example virginiamycin for quinupristin/dalfopristin (Barbosa and Levy 2001).

The stability of antibiotic resistance genes was demonstrated by a study conducted using 477 *Enterobacteriaceae* isolated from the Royal London Hospital. In the absence of streptomycin use, streptomycin resistance was found in 20% of *Enterobacteriaceae* isolates. Integron-associated gene cassette *aadA1*, which confers resistance to streptomycin and spectinomycin, was found in 70% of observed resistant strains.

Streptomycin therapy was common in the UK in the 1950s and 1960s, but its use is now restricted to the treatment of tuberculosis, while spectinomycin is used in humans solely for the treatment of gonococci (Chiew *et al.* 1998). Another study of 350 *E. coli* isolates, conducted at the Royal London Hospital, found the persistence of sulfonamide resistance even though a national prescribing restriction policy was implemented four years earlier. Despite these restrictions, which resulted in sulfonamide prescriptions decreasing from 320,800 per year in 1991 to 7,700 in 1999, the frequency of sulfonamide resistance remained high in 1999 (46.0%) compared to 39.7% in 1991 (Enne *et al.* 2001).

In contrast, other studies have found that the withdrawal or reduction of antibiotic use was associated with a decline in the prevalence of antibiotic resistance. For example in Finland, following nationwide reductions in the use of macrolide antibiotics in the community, there was a significant and steady decline in the frequency of erythromycin resistance among group A streptococci (Seppälä *et al.* 1997). Likewise, a European ban on avoparcin, a growth promoter added to animal feed that is known to cause cross-resistance with vancomycin, resulted in a decrease in vancomycin-resistant *enterococci* isolated from animals, food products and healthy humans (van den Bogaard *et al.* 2000).

Amplification and transmission of antimicrobial-resistant bacteria is accelerated where there is close contact between individuals, which occurs in institutions such as hospitals, day care centres, correctional facilities, homeless shelters, immigration detention centres and military barracks (Goldmann 1999). The mobility of the world's populations also facilitates the rapid dissemination of microorganisms harbouring antibiotic resistance

genes (Goldmann 1999). The spread of antibiotic resistance within a hospital, country or globally is usually achieved by clones with a high transmission ability (Normark and Normark 2002). It is not known why some multi-resistant strains achieve epidemic spread, which is vital to the accumulation of resistance determinants, while others with similar resistance profiles do not (Livermore 2003).

1.5 – Monitoring the movement of antibiotic resistance genes

The effect of agricultural antibiotic use on antibiotic resistance in human medicine is contentious, exacerbated by difficulties in defining the nature and magnitude of that contribution. Obtaining unambiguous documentation and quantification of the effects of antibiotic use in animals on human health presents a major challenge to researchers (Lipsitch *et al.* 2002). These difficulties are reflected by the paucity of data on the impact of agricultural use of antibiotics on antibiotic resistance levels (Salyers and Amabile-Cuevas 1997). Potential ways in which antibiotic use and resistance in food-producing animals can affect human health are via (i) zoonotic infection, by the ingestion of contaminated meat or food contaminated by meat-borne bacteria or animal manure, (ii) the accumulation of resistance genes on mobile elements and the movement of these genes to commensal or pathogenic bacteria, (iii) the selection for commensal bacteria inhabiting food-animals that may serve as a reservoir of resistance-encoding plasmids or other mobile elements and (iv) the emergence of resistant bacteria carried by food-producing animals and humans that are transmitted among humans (Lipsitch *et al.* 2002).

1.6 – Acquisition and emergence of resistance genes

The evolution and spread of antibiotic resistance in bacteria is a complex process involving a variety of different mechanisms. Some bacterial species show a high intrinsic resistance to specific antibiotics, while susceptible bacteria may acquire resistance by alterations in their own genome via mutations or the transfer of resistance genes located on mobile DNA elements (Normark and Normark 2002).

1.6.1 – Mutation

Mutation of chromosomal genes is generally a less common way for bacteria to become resistant to antibiotics than the acquisition of exogenous DNA (Roberts 2003). Most mutations affect one class of antibiotic, although those affecting efflux or impermeability have a pleiotropic effect. Mutations have been described in chromosomal genes, resulting in resistance to many antibiotics including β-lactams, fluoroquinolones, macrolide-lincosamide-streptogramin, rifampicin, tetracyclines, quinolones, trimethoprim, sulfonamide, and `aminoglycosides such as streptomycin (Table 1.1).

Table 1.1. Mutation of chromosomal genes inducing antibiotic resistance.

Antibiotic	Target gene acquiring mutation	Reference
Aminoglycosides (e.g. streptomycin)	16S rRNA	(Melancon *et al.* 1988)
Aminocyclitol (spectinomycin)	16S rRNA	(Sigmund *et al.* 1984)
Fluoroquinolones and quinolones	DNA gyrase DNA topoisomerase IV	(Fluit *et al.* 2001) (Drlica and Zhao 1997)
Macrolide-lincosamide-streptogramin	23S rRNA	(Sigmund *et al.* 1984; Sutcliffe and Leclercq 2002)
Rifampicin	β-subunit of RNA polymerase	(Telenti *et al.* 1993)
Sulfonamide	*dhps* dihydropteroate synthetase	(Huovinen *et al.* 1995; Pato and Brown 1963)
Tetracyclines	16S rRNA and 23S rRNA	(Ross *et al.* 1998)
Trimethoprim	*dhfr* (*dfrA*)	(Huovinen *et al.* 1995; Powell *et al.* 1991)
β-lactams	Penicillin binding proteins	(Fluit *et al.* 2001)

1.6.2 – Horizontal transfer

Comparative analyses of complete genomic sequences of Archaea and bacteria support horizontal gene transfer as an important factor in the speciation and diversification of microorganisms (Garcia-Vallve *et al.* 2000). In contrast to mutation, which causes a subtle refinement of existing functions, horizontal transfer usually involves much larger changes that allow the bacteria to carry out new functions and adapt to different environments (Lawrence 1999). Horizontal transfer allows blocks of DNA carrying multiple genes to be transferred as a single unit between unrelated species and ecosystems resulting in the rapid spread of associated genes within and between bacterial populations (Roberts 2003). Horizontal transfer of antibiotic resistance genes may occur via various genetic elements including self-replicating plasmids (transmitted by conjugation, transformation or transduction), bacteriophages, transposons, nonreplicating *Bacteroides* unit (NBUs), integrons and pathogenicity islands (Normark

and Normark 2002; Salyers and Amabile-Cuevas 1997) (Table 1.2). Conjugative plasmids, mobilisable plasmids, conjugative transposons and phages are able to transfer genes between bacterial cells, while transposons, IS elements, NBUs, and integrons move genes between DNA molecules within a bacterial cell.

Table 1.2. Characteristics of mobile DNA elements.

Gene transfer element	Characteristics of DNA transfer element	Role in the dissemination/expression of resistance genes
Integron	Integrated DNA segment; contains integrase, a promoter and an integration site for gene cassettes	Forms arrays of several different resistance genes; provides a promoter for gene cassette expression
Gene cassette	Nonreplicating DNA segments containing open reading frames (ORFs), integrate into integrons	Carry resistance genes
Self-transmissible conjugative plasmid	Autonomously replicating element; carries the genes necessary for conjugal DNA transfer	Mobilisation of other elements that carry resistance genes
Mobilisable plasmid	Autonomously replicating element; carries an origin of conjugal transfer (*oriT*) that allows it to use conjugal apparatus provided by self-transmissible plasmid	Same as for self-transmissible plasmid
Conjugative transposon	DNA segments; to transfer they excise themselves to form a nonreplicating circular intermediate which is transferred by conjugation to a recipient where it integrates into the recipient's genome	Same as for self-transmissible plasmid
Transposon	Move from one DNA segment to another within the same cell	Transfer of resistance genes from chromosome to plasmid or vice versa
Insertion sequences	Associated with other mobile genetic elements; flanked by short terminal inverted repeats	Up-regulate or inactivate gene expression
Complex transposons	Contain insertion sequences with short inverted repeats at their termini; undergo replicative transposition	Transfer of resistance genes
Bacteriophage	Vector for the transfer and injection of DNA	Transfer of resistance genes/virulence factors
Nonreplicating *Bacteroides* unit	Integrated element; triggered to excise and transfer by conjugative transposons, transfer intermediate is circular non-replicative	Transfer of resistance genes

Conjugative plasmids, conjugative transposons or phages often carry transposons or integrons, which ensures the transfer of the integron or transposon. Additionally, these combinations of horizontal gene transfer elements increase the potential for gene shuffling (Toussaint and Merlin 2002). Plasmids allow their bacterial hosts to "sample" the gene pool for traits, which may provide a selective advantage under local conditions. Only plasmids harbouring genes which provide a selective advantage are retained by the bacterial host (Sherley *et al.* 2004). Plasmids may harbour a variety of mobile genetic elements including transposons, conjugative transposons, IS elements and integrons (Roberts 2003).

1.6.3 – Insertion sequence (IS) elements

IS elements have been extensively characterised and are often associated with specific antibiotic resistance genes, pathogenic and virulence factors. IS elements are small genetic elements that are flanked by short terminal inverted-repeat sequences (IR) of 10-40 bp and are able to insert at multiple sites in target DNA. They are usually phenotypically cryptic; generally only encoding genes associated with their own mobility (tyrosine and serine recombinases, and the DD-E transposases). Two copies of an IS element flanking a DNA segment may act together to mobilise the intervening sequence. As a result of integration into the chromosome, IS elements may either inactivate or up-regulate antibiotic resistance gene expression (Mahillon and Chandler 1998). In addition to their important function in the dissemination and acquisition of resistance genes, IS elements participate in chromosomal and plasmid rearrangement, integration and excision (Mahillon and Chandler 1998). IS elements are often associated with other mobile genetic elements or integrons. Significant features of the IS elements IS*26*, IS*1* and IS*91,* pertinent to this study are described in Table 1.3.

Table 1.3. Features of IS*26*, IS*1* and IS*91* (Mahillon and Chandler 1998).

IS	Target sequence	Duplication	Mechanism of transposition	Association with integrons, resistance genes or virulence genes
IS*1*	Strong preference for insertion at AT-rich target sites	9 bp repeat in target sequence	Generates simple insertions or co-integrates resulting in 2 copies of IS*1* at each junction between the target and donor.	Inserted into the *intI2* gene (DQ196320) (Coelho *et al.* 1995); Tn*7*::IS*1*-*ereA* (AY183453) (Biskri and Mazel 2003); and Tn*7*::In2-10 **(**EF042190).
IS*26*	Shows no marked target selectivity	8 bp direct target repeat	Involves formation of co-integrates. A resolution step separates donor and target replicons by homologous recombination. Transposition is accompanied by replication.	Flanks *dfrA8* and *orf1* in Tn*5091* (Sundström et al. 1995) and the class 1 integron, In53 carried on Tn*2000* (Naas et al. 2001). Inserted into the *intI1* gene in the class 1 integrons In-t3, In-111, In-e541 (Adrian *et al.* 2000; Miriagou *et al.* 2005) and the 5′ end of *intI1* (AY138987) (Segal *et al.* 2003). Implicated in the dissemination of resistance genes (Miriagou *et al.* 2005).
IS*91*	Inserts into the specific target sequence of CAAG or GAAC	No direct repeats are generated on insertion	Rolling-circle mechanism allows the movement of segments of donor DNA of variable length.	Often found in human or animal pathogenic strains in close association with functional bacterial virulence genes harboured in known virulence plasmids.

1.7 – Integrons

Integrons are DNA elements that function as gene-capture and expression systems that incorporate open reading frames (ORFs) and convert them into functional genes (Rowe-Magnus *et al.* 2002a; Rowe-Magnus and Mazel 1999). Integrons provide (i) an integration site (*attI*) for the site-specific insertion of gene cassettes (Partridge *et al.* 2000), (ii) an integrase to mediate excision and orientation-specific integration of gene cassettes (Collis *et al.* 1993) and (iii) a promoter Pc, to ensure expression of the operon (Lévesque *et al.* 1994). Integrons are capable of acquiring a wide-range of individual and unrelated genes as gene cassettes, by site-specific integration (Stokes and Hall 1989).

Gene cassettes are small non-replicating mobile DNA elements. Although they may contain a diverse range of genes, they all share a common structure. Gene cassettes usually lack a promoter and may be excised from, or integrated into, an integron. A gene cassette is comprised of a single gene and a recombination site known as a 59-base element (59-be) (or *attC* site), which is located downstream of the gene coding region. Gene cassettes vary considerably in length from 262 to 1549 bp, due to differences in the size of the genes they carry (as little non-coding sequences flank the gene) and length of the 59-be (Recchia and Hall 1995). Integrons from different isolates are highly variable with regard to the type, number and order of gene cassettes they contain (Recchia and Hall 1995).

1.7.1 – Gene cassette integration and excision

Site-specific recombination of a gene cassette is catalysed by an integrase. In class 1 integrons the integrase gene; *intI1* is located in the 5′-conserved segment (CS) of the integron. The integrase is a recombinase related to the λ integrase family of site-specific recombinases. It interacts with two recombination sites, the 59-be of the gene cassette and the *attI* site located in the 5′-CS of the integron (Hall and Vockler 1987; Ouellette and Roy 1987). The 59-bes are unique and vary in terms of both sequence and length (from 57 to 141 bp), although they all have consensus sites at their boundaries corresponding to the inverse core site RYYYAAC and the core site GTRRRRY (R, purine; Y, pyrimidine) (Stokes *et al.* 1997). Interaction of the integrase with these recombination sites results in a cross-over that is localised between the G and first T of the consensus core site, GTTRRRY, and the insertion of the gene cassette into the *att1* site of the integron (Hall *et al.* 1991). Integrated cassettes are always in the same orientation with the 5′ end of the gene closest to the 5′-CS of the integron. The

insertion of additional cassettes may result in multi-cassette arrays containing two or more cassettes. Although gene cassettes are usually found inserted into an integron or more rarely at a secondary (non-specific) location, they also transiently exist as free covalently closed circular molecules (Collis and Hall 1992). Free circular gene cassettes formed by excision from integrons may constitute important intermediates in the dissemination of cassettes (Figure 1.1).

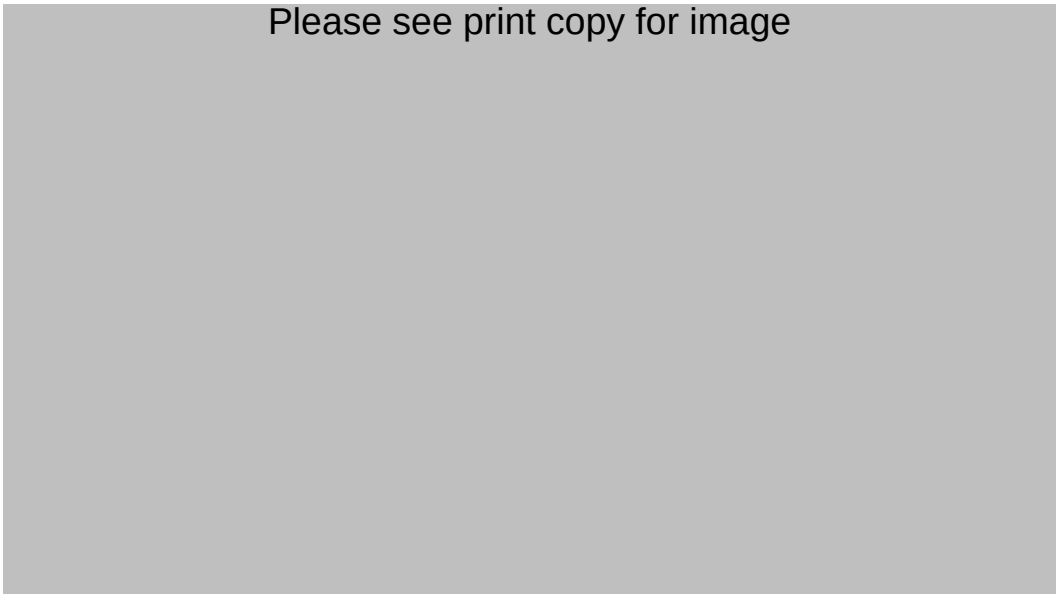

Figure 1.1. Diagram showing the integration and excision of a gene cassette in a class 1 integron at *attI1* or at a secondary site. **(A)** An integron without a gene cassette inserted. The following features are indicated: P_c, the promoter; *intI1* gene (open box); *attI1* integration site (grey box); and the integron 3′-CS (black box). The circularised form of the gene cassette is shown, which consists of a coding gene (large bold open circle) and a 59-be (small filled circle). **(B)** An integron with a gene cassette (bold line and small filled circle of the 59-be) inserted into the *attI1* site. The movement of the gene cassette is catalysed by the integrase IntI1, encoded by the *intI1* gene. **(C)** An alternate site of gene cassette insertion in the chromosome at a non-specific site near a promoter, P is indicated by x. **(D)** A gene cassette inserted at a non-specific site, x. Source: (Recchia and Hall 1995).

1.7.1.1 – Multi gene cassette arrays

The impact of integrons on bacterial evolution is evident in the stockpiling of antibiotic resistance genes and creation of multiresistant bacteria. One example of gene cassette stockpiling is evident in a pathogenic *E. coli* strain isolated from a patient hospitalised

in France. This class 1 integron named In53 contained 9 functional resistance gene cassettes including *aadB* (gentamicin), *aacA1/orfG* (amikacin, tobramycin and netilmicin), $bla_{\text{VERB-1}}$ (extended spectrum β-lactamases), *arr-2* (rifampicin), *cmlA5* (chloramphenicol), $bla_{\text{OXA-10}}$ (penicillin) and *aadA1* (spectinomycin and streptomycin) (Naas et al., 2001). The role of integrons in the accumulation and dissemination of antibiotic resistance genes has been well established (Leverstein-van Hall *et al.* 2003; Lucey *et al.* 2000; Naas *et al.* 2001; Tennstedt *et al.* 2003).

Some integrons are active transposons, for example Tn*7*, Tn*1825,* Tn*1826,* Tn*4132* and Tn*402* (Hall *et al.* 1991; Sundström *et al.* 1991; Tietze and Brevet 1991; Young *et al.* 1994), although most integrons are defective transposon derivatives (Shapiro and Sporn 1977). Evidence of extensive horizontal transfer of integron-mediated antimicrobial resistance genes among *Enterobacteriaceae* has been previously documented (Leverstein-van Hall *et al.* 2002). Horizontal transfer of integrons generally occurs when an integron harbouring gene cassette(s) is incorporated into a broad-host-range plasmid or transposon.

1.7.2 – Integron classes

Nine distinct integron classes have been identified including class 1, 2, 3, 4, 5, 6, 7, 8 and an unnumbered class (GeneBank no. AJ277063) (Arakawa *et al.* 1995; Clark *et al.* 2000; Hall *et al.* 1991; Mazel and Davies 1998; Nield *et al.* 2001; Stokes and Hall 1989). Each class of integron contains a gene that codes for a distinct, yet related integrase enzyme. The *attI* sites of the respective classes differ although the core sites (GTTRRRY) contained within these regions are conserved (Nield *et al.* 2001; Stokes *et al.* 1997). There is little similarity between the *attI1* sequence in class 1 integrons and

the corresponding regions in class 2 and 3 integrons predicted to contain *attI2* and *attI3* sites (Hall *et al.* 1999).

1.7.2.1 – Class 1 integrons

Class 1 integrons are the most common and well characterised class of integron. They are widely disseminated in animal and human clinical strains of the family *Enterobacteriaceae* (Goldstein *et al.* 2001). Class 1 integrons contain the three features that are considered definitive for all integrons: the *attI1* recombination site, integrase gene *intI1* and the promoter Pc. Class 1 integrons are bounded by terminal inverted repeats, designated IRi and IRt. IRi is located at the 5′ end of class 1 integrons (down stream of the *intI1* gene), while IRt is located at the 3′ end (adjacent to the *tni* module) (Brown *et al.* 1996; Partridge *et al.* 2001). Class 1 integrons contain a specific recombination site *attI1,* located near the *intI1* gene that is recognised by the integrase IntI1 and a promoter Pc that lies within the *intI1* gene that directs the transcription of inserted gene cassettes. Most class 1 integrons possess two CSs that flank either end of the gene cassette region. The 5′-CS contains the *intI1* gene (Hall and Vockler 1987; Ouellette and Roy 1987) and a promoter Pc directed towards the integration site (Lévesque *et al.* 1994). The 3′-CS usually contains the *qacEΔ1* (Paulsen *et al.* 1993) and *sul1* genes (Stokes and Hall 1989) and an ORF of unknown function (*orf5*) (Figure 1.2) (Stokes and Hall 1989). Originally the 3′-CS of class 1 integrons was defined as a region that consisted of the *qacEΔ1, sul1,* and *orf5* genes (Recchia and Hall 1995). A diagram showing variation in the number, type and order of gene cassette(s) in class 1 integrons with the classical *qacEΔ1, sul1,* and *orf5* genes in the 3′-CS is given in Figure 1.3.

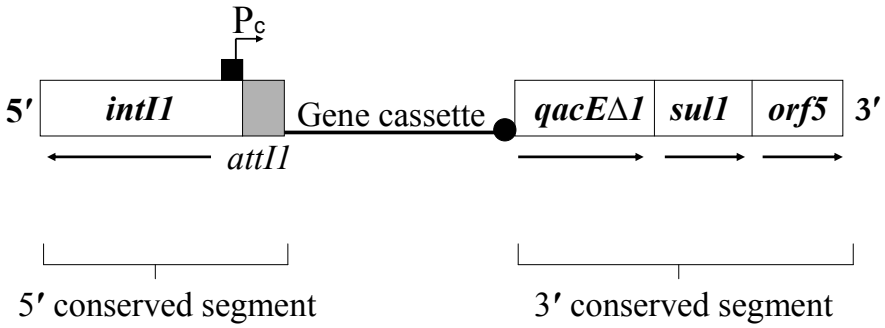

Figure 1.2. Basic structure of a class 1 integron showing the 5′- and 3′-conserved segments. The *intI1* gene (open box) and the *attI1* recombination site (grey box) are located in the 5′-CS. The *qacEΔ1* gene encoding resistance to quaternary ammonium compounds, the *sul1* gene encoding sulfonamide resistance and the ORF *orf5* are found in the 3′-CS. The gene cassette is represented by a bold line and the associated 59-be by a filled circle. The direction of gene transcription and the promoter region Pc, directed toward the integration site, are indicated. Source: (Collis *et al.* 2002; Lévesque *et al.* 1995).

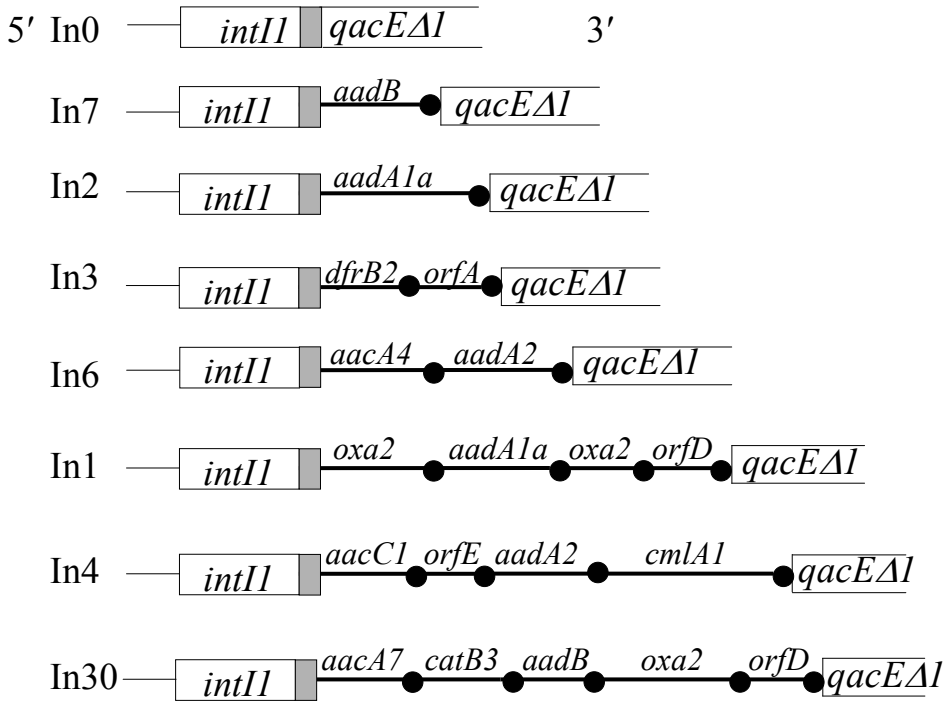

Figure 1.3. A diagram showing variation in cassette genes carried by class 1 integrons. The *qacEΔ1* gene of the 3′-CS is indicated by an open box. The *sul1* gene and *orf5* which are located downstream of the *qacEΔ1* gene are not shown. The following features are indicated: *intI1* gene (open box); *attI1* (grey box); gene cassettes (bold lines) and 59-be (filled circles). Antibiotic resistance gene cassettes include: *aadA1a* and *aadA2* (streptomycin and spectinomycin); *aacC1, aacA7, aacA4, aadB*; *cmlA1* (chloramphenicol); *dfrB2* (trimethoprim); *oxa2* (penicillin); and *catB2* and *catB3* (chloramphenicol). Source: (Recchia and Hall 1995).

However, more recent studies have revealed variation in the length of the 3′-CS, which may include an additional ORF known as *orf6* or portions of *orf6*. The integron In16 which is an active transposon, Tn*402* (also known as Tn*5090*) does not contain the 3′-CS typical of most class 1 integrons. In Tn*402* the *tni* module, consisting of the entire complement of transposition genes, *tniA*, *tniB* and *tniQ* and a resolvase gene *tniR,* is located within the integron In16, down-stream of the cassette array. The *qacE* gene of In16 is inserted into the integron in a gene cassette (Liebert *et al.* 1999; Partridge *et al.* 2001). Similarly, an extensive survey of Gram-negative bacteria isolated from an estuarine environment revealed that some class 1 integrons lacked the *qacEΔ1* and *sul1* genes, while others lacked the *sul1* gene and contained the *qacE* gene (Rosser and Young 1999).

1.7.2.2 – Class 2 integrons

Class 2 integrons contain a putative but defective integrase gene *intI2*, as the nucleotide sequence is interrupted by an internal stop codon (TAA) at amino acid position 179. The *intI2* gene is located 5′ to the first gene cassette and its gene product, IntI2, has 46% homology with IntI1 (Hansson *et al.* 2002).

Class 2 integrons are widely distributed globally in a variety of bacterial species and sources. Class 2 integrons have been isolated from France, Spain, Ireland, Nigeria, Chile, Korea, Greece, Czech Republic, Germany, USA and Australia. Class 2 integrons have been found in several species of Gram-negative bacteria isolated from human, animal and environmental sources. Bacteria harbouring class 2 integrons include *E. coli*, *Salmonella typhimurium* (Goldstein *et al.* 2001; Miko *et al.* 2003), *Salmonella enterica* (Miko *et al.* 2003), *Morganella morganii*, *Providencia stuartii, Proteus*

vulgaris (Barlow *et al.* 2004), *Acinetobacter baumannii* (Gonzalez *et al.* 1998; Oh *et al.* 2002), *Proteus mirabilis* (Jones *et al.* 2003), *Shigella sonnei* (McIver *et al.* 2002; Oh *et al.* 2003), *Klebsiella* spp. (White *et al.* 2001), *Vibrio cholerae* (Ahmed *et al.* 2006) and *Bacteroides* species.

Class 2 integrons are typically located in a unique site near the left end of the non-replicative transposon Tn*7* or related transposons including Tn*1825,* Tn*1826,* and Tn*4132* (Figure 1.4) (Hall *et al.* 1991; Sundström *et al.* 1991; Teitze and Brevet 1991; Young *et al.* 1994). The almost ubiquitous Tn*7*-like integron contains the gene cassette array *dfrA1-sat2-aadA1* (Barlow *et al.* 2004; DeLappe *et al.* 2003; Jones *et al.* 2003; Miko *et al.* 2003; Sáenz *et al.* 2004; White *et al.* 2001; Yu *et al.* 2003).

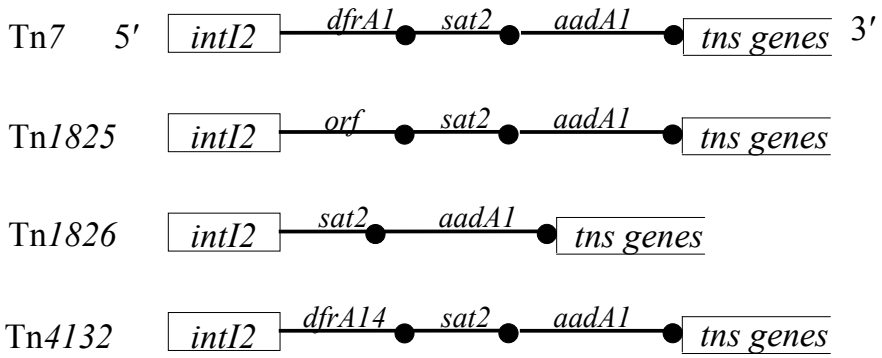

Figure 1.4. Genetic organisation of Tn*7* and the Tn*7*-like class 2 integrons Tn*1825*, Tn*1826* and Tn*4132*. Gene cassettes shown include *dfrA1* and *dfrA14* (trimethoprim); *sat2* (streptothricin); *aadA1* (streptomycin and spectinomycin) and an *orf* of unknown function. The class 2 integrase gene *intI2* is indicated by an open box as are the transposition genes *tnsA, tnsB, tnsC, tnsD* and *tnsE* (open box labelled *tns* genes).

In addition to the Tn*7*-like integron, class 2 integrons have been identified with variation in the gene cassettes they harbour. The following gene cassettes have been reported in class 2 integrons: *ereA* (conferring resistance to erythromycin) (Biskri and Mazel 2003), *catB2* (chloramphenicol), *aadB* (gentamycin, tobramycin and kanamycin)

(Ramírez *et al.* 2005), *dfrA14* of Tn*4132* (trimethoprim) (Young *et al.* 1994), *sat1* and *sat2* (streptothricin), and *estX* (encoding a putative esterase). The *sat1* gene cassette is an *estX-sat2* gene fusion (Partridge and Hall 2005). Class 2 integrons associated with IS*1* that show variation from the Tn*7*-like gene cassettes array of *dfrA1-sat2-aadA1* are illustrated in Figure 1.5.

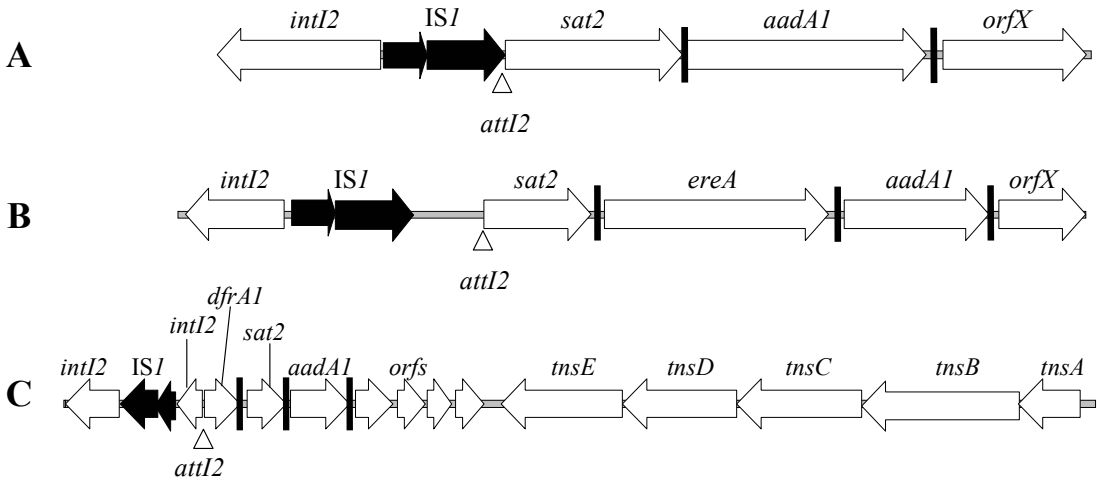

Figure 1.5. Diagram of various class 2 integrons associated with the insertion sequence IS*1*. IS*1* is inserted into the *intI2* gene of the following class 2 integrons: **(A)** Class 2 integron with the gene cassettes *sat2*, *aadA1* and *orfX* also named *ybeA*, which is part of a truncated cassette (Coelho *et al.* 1995) (GenBank no. DQ196320), **(B)** Tn*7*::IS*1*-*ereA* (Biskri and Mazel 2003) (GenBank no. AY183453) and **(C)** Tn*7*::In2-10 **(**GenBank no. EF042190). The following features are indicated: *intI2* gene; IS*1* *insA* and *insB* genes (black); *sat2* cassette gene (streptothricin resistance); *aadA1* cassette gene (spectinomycin and streptomycin resistance); *ereA* cassette gene (erythromycin resistance); *dfrA1* cassette gene (trimethoprim resistance); Tn*7* transposition genes *tnsABCDE*; ORFs of unknown function; 59-bes (vertical bold lines); and *attI2* cassette integration site (arrow head). The direction of gene transcription is indicated.

1.7.2.2.1 – Mechanism of Tn*7* transposition

The mechanism of Tn*7* transposition, which may involve one of two distinct Tn*7* target-site selection pathways, has been extensively characterised. The two transposition pathways work to minimise the deleterious effects of Tn*7* insertion and ensure the dissemination of Tn*7* between diverse bacterial hosts. Tn*7* encodes 3 core transposition proteins (TnsA, TnsB and TnsC) and two target site selecting proteins

(TnsD or TnsE) (Rogers *et al.* 1986; Waddell and Craig 1988). TnsD-directed transposition involves Tn*7* insertion at the *attTn7* site, a unique region in the *E. coli* chromosome (Lichtenstein and Brenner 1981; Lichtenstein and Brenner 1982). *attTn7* is located within the transcriptional termination signal of the *glmS* gene (Gay *et al.* 1986; McKown *et al.* 1988). The alternative pathway of Tn*7* transposition, directed by the protein TnsE, involves Tn*7* preferentially inserting into non-specific sites in conjugating plasmids (Wolkow *et al.* 1996) or in chromosomal DNA (Peters and Craig 2000).

1.7.2.3 – Class 3 integrons

The first class 3 integron was discovered in the *Serratia marcescens* strain TN9106, isolated from a patient with a urinary tract infection (UTI) hospitalised in Japan. This class 3 integron contained the bla_{IMP} and *aacA4* gene cassettes. The bla_{IMP} gene encodes a broad spectrum metallo-β-lactamase conferring resistance to carbapenems, while *aacA4* confers resistance to aminoglycosides. The class 3 integron is flanked by a copy of the terminal inverted repeat (IRi) on one side and a resolvase-encoding *tniR* gene on the other (Figure 1.6) (Arakawa *et al.* 1995; Collis *et al.* 2002). The class 1 integron, Tn*402* is also flanked by IRi and *tniR* genes, which suggest class 3 integrons are part of a related transposable element. IntI3 is 59% identical to IntI1 and preliminary experiments indicate it is an active integrase. *In vitro* studies demonstrated that IntI3 was able to recognise and recombine with both types of integron recombination sites; the *attI3* site in the integron and various cassette associated 59-bes. IntI3 also catalysed the integration of circularised gene cassettes into the *attI3* site and secondary sites and their excision (Collis *et al.* 2002).

A class 3 integron was discovered in *Klebsiella pneumoniae* (strain FFUL 22K), isolated in 1999 from the urine of an intensive care patient in Portugal. This class 3 integron consisted of an *intI3* integrase gene, an *attI3* recombination site, two promoter regions, and two gene cassettes. The integron contains the bla_{GES-1} gene encoding an extended spectrum β-lactamase and a second gene cassette that is a fusion between bla_{OXA-10} and *aac(6')-1b* conferring resistance to kanamycin (Figure 1.6B) (Correia *et al.* 2003).

Two class 3 integrons carrying gene cassettes of unidentified functions have been detected in *Delftia* spp. isolated from North American environmental aquatic samples (Xu *et al.* 2007). The identification of class 3 integrons from North American water samples demonstrates a wider geographical and ecological distribution than previously described. In contrast to the previously characterised class 3 integrons isolated from clinical sources, the environmental class 3 integrons detected in *Delftia* spp. did not contain antimicrobial resistance gene cassettes (Figure 1.6C and 1.6D).

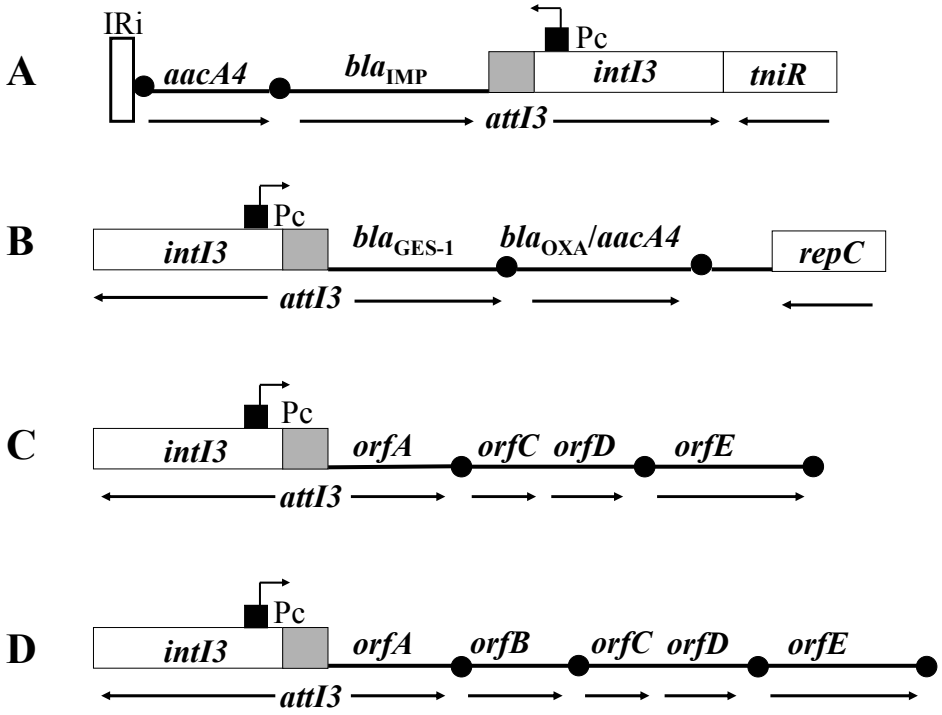

Figure 1.6. Diagram showing variation in the gene cassettes carried by class 3 integrons. **(A)** A class 3 integron isolated from the *Serratia marcescens* strain TN9106 carried on a large transferable plasmid. **(B)** Integron In3-p22K9 located on a 9.4 kb plasmid (p22K9) isolated from the *Klebsiella pneumoniae* strain FFUL 22K. **(C)** Class 3 integron detected in *Delftia* spp. isolated from environmental aquatic samples carrying the gene cassettes *orfA*, *orfC*, *orfD* and *orfE*. **(D)** Class 3 integron detected in *Delftia* spp. isolated from environmental aquatic samples carrying gene cassettes *orfA*, *orfB*, *orfC* and *orfD* and *orfE*. The following features are indicated: *aacA4*, bla_{IMP}, bla_{GES-1} and the fused bla_{OXA}/*aacA4*gene cassettes (bold lines). (Note: *aacA4,* which collectively describes *aac(6')-1b* and *aac(6')-II,* is substituted for *aac(6')-1b* in part B of this figure); 25-bp inverted repeat, IRi (open vertical bar); the integrase gene *intI3*, the resolvase-encoding gene *tniR* and the plasmid replication gene *repC* (open box). 59-bes are indicated by small filled circles and the *attI3* sites by grey boxes. The position of promoters and the direction of gene transcription are indicated. Figures were adapted from (Collis *et al.* 2002; Correia *et al.* 2003; Xu *et al.* 2007).

1.7.2.4 – Class 4 integrons

Class 4 Integrons, also known as chromosomal integrons or "super-integrons", were discovered in the *Vibrio cholerae* genome, located on the smaller of the two circular chromosomes (Barker *et al.* 1994; Heidelberg *et al.* 2000; Mazel and Davies 1998). Since their initial discovery, class 4 integrons have been identified in (i) *Vibrionaceae* and their close relatives, including *V. mimicus, V. metschnikovii, V. parahaemolyticus,*

L. pelagia and V. fischeri (Rowe-Magnus *et al.* 2001), (ii) several species of the Genus *Pseudomonas* including *P. alcaligenes, P. mendocina* and *P. stutzeri* (Vaisvila *et al.* 2001), and (iii) distinct genera of the γ-proteobacteria including *Shewanella* and *Xanthomonas*. Super-integron signatures have also been identified by PCR for a gamut of γ-proteobacteria including *V. harveyi, V. marinus, V. hollisae, V. anguillarum, V. salmonicida, Alteromonas macleodii, Phytobacterium phosphoreum, Moritella marina,* several *Xanthomonas* spp., and *Pseudomonas pseudoalcaligenes.* In addition to the chromosomal location classification of class 4 integrons, the mobile STX element integron of *Vibrio cholerae* has also been designated class 4 (Hochhut *et al.* 2001).

Class 4 integrons have been described as a chromosomal array of a large number of gene cassettes that can be mobilised by a site specific integrase (IntI4) provided by an integron (Mazel and Davies 1998). The large array of gene cassettes found in *V. cholerae* spans 126 kb and contains at least 179 gene cassettes (Heidelberg *et al.* 2000; Rowe-Magnus and Mazel 1999). The super integron gene cassette array of *P. alcaligenes* is five times smaller than that of *V. cholerae* and contains 33 gene cassettes (Vaisvila *et al.* 2001). The 59-bes identified in *V. cholerae* (Barker *et al*., 1994), designated VCRs for *Vibrio cholerae* repeats display a high degree of sequence relatedness (95-97%), unlike their counterparts in multi-resistant integrons (Clarke *et al.* 1997). Some VCRs associated with gene cassettes are closely related to the 59-bes of conventional integrons such as those of *bla*P3 and *dfrA6* (Mazel and Davies 1998; Rowe-Magnus and Mazel 1999).

Super-integrons are distinguished from conventional integrons by their size, placement of promoters, chromosomal location, and the nature of the gene cassettes they carry

(Heidelberg *et al.* 2000). Clearly, the 179 cassettes of *V. cholerae* cannot be expressed from one promoter, especially given the genes inside the cassettes are not all orientated in the same direction (Barker *et al.* 1994). Sequence similarity and experimental data suggest that gene cassettes of super-integrons encode diverse functions including the transport of small molecules, restriction modification, sulfate-binding activity, plasmid specific roles and psychrophilic lipase activity (Barker and Manning 1997; Barker *et al.* 1994; Rowe-Magnus *et al.* 2001). DNA sequencing of both chromosomes of *V. cholerae* revealed that class 4 integrons also carry gene cassettes that encode gene products involved in antibiotic resistance including a chloramphenicol acetyl transferase, glutathione transferase and a fosfomycin resistance protein (Heidelberg *et al.* 2000).

It has been proposed that multidrug-resistant integrons arose from super-integrons by the entrapment of *intI* genes and their related *attI* sites on mobile genetic elements such as transposons (Rowe-Magnus and Mazel 1999). The gene cassette reservoirs of super integrons provide a source of gene cassettes that are recruited by multi-resistant integrons (Rowe-Magnus *et al.* 2002b). This phenomena was demonstrated by the transfer of a novel but phenotypically silent chloramphenicol acetyltransferase gene from the *V. cholerae* super integron to a class 1 multi-resistant integron (Figure 1.7) (Rowe-Magnus *et al.* 2002b).

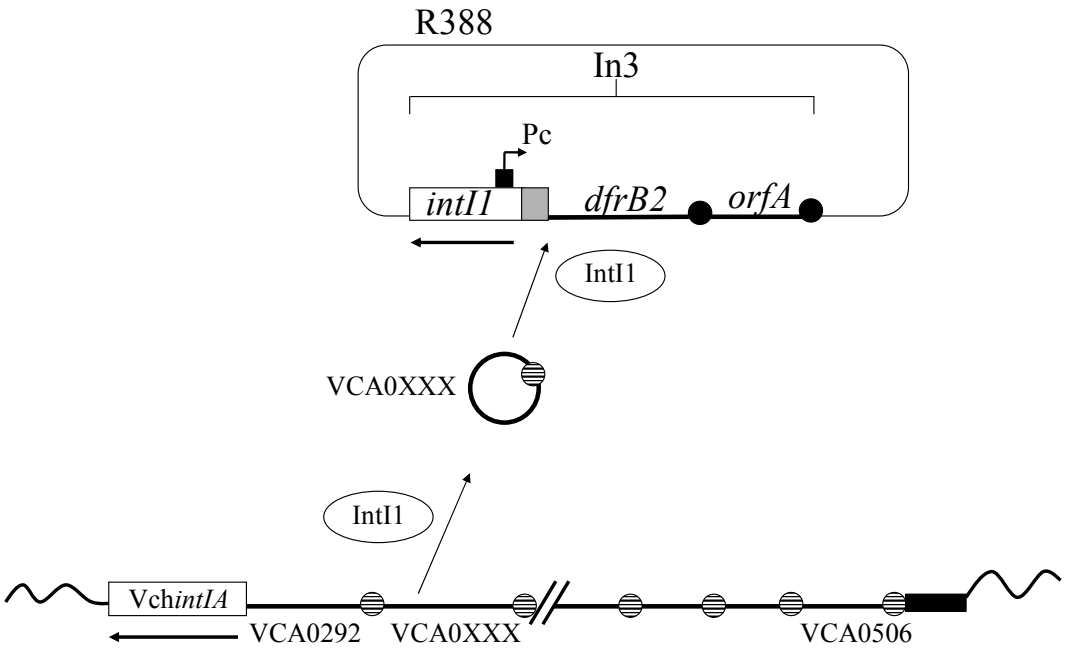

Figure 1.7. Diagram showing the recruitment of genes by a class 1 integron (In3 located on the plasmid R388) directly from a super integron in *V. cholerae*. Only a portion of the *V. cholerae* super integron on chromosome II is shown. The wavy black lines and the black filled box represent chromosomal DNA that is not part of the super integron. Bold lines represent ORFs of gene cassettes. The *V. cholerae* signature 59-be sites, also known as VCRs, are shown as striped filled circles while the 59-be typically found in multi resistant integrons are shown as black filled circles. IntI1 catalyses the random excision of a gene cassette from the super integron and the subsequent integration of the circular free cassette at the *attI1* site of the class 1 integron. The ORFs of the super integron gene cassettes are numbered from VCA0292 to VCA0506 and a random representative gene cassette numbered VCA0XXX is recruited and incorporated into In3. Diagram was adapted from (Rowe-Magnus *et al.* 2002b).

1.7.2.5 – Other integron classes

An additional class of chromosomal integron within the genus *Vibrio* was identified by Southern analysis of genomic DNA with VCR-specific probes. Many *V. cholerae* serotype fragments that hybridise with VCR probes show a high degree of restriction fragment length polymorphism (RFLP). Partial sequence of the integrase gene from the *Vibrio mimicus* strain V800, showed significant variation to justify the classification of this fifth class of integrase (Clark *et al.* 2000).

Environmental integrons isolated from soil samples collected from the Balmain Power Station and Homebush Bay (Sydney, Australia) and Pulgamurtie, Sturt National Park (Western New South Wales, Australia) contained novel integrases designated class 6 (*intI6*), class 7 (*intI7*), and class 8 integrons (*intI8*). Characteristic integron features found in these environmental DNA samples include the presence of novel integrase genes, gene cassettes that encode ORFs of unknown functions, sequences suggestive of *attI* sites and presumptive Pc promoter sites (Figure 1.8) (Nield *et al.* 2001).

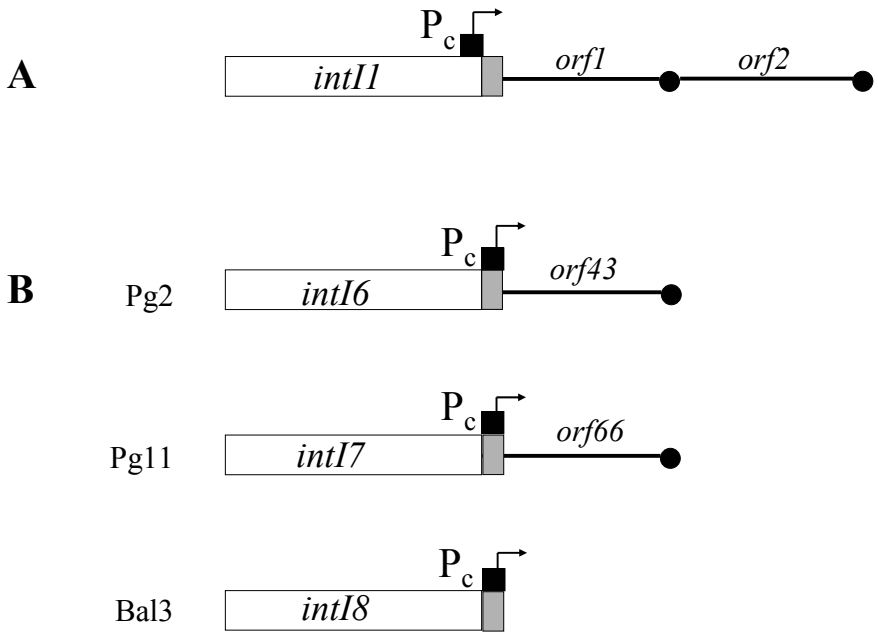

Figure 1.8. Comparison of a class 1 integron with environmental integrons. **(A)** Class 1 integron containing a two cassette array. **(B)** Integrons derived from environmental soil samples obtained from Pulgamurtie (sample Pg2, class 6 integron and sample Pg11, class 7 integron) and Balmain (sample Bal3, class 8 integron). Features indicated include *intI* genes (open boxes); Pc promoters; putative *attI* sites (grey boxes); gene cassettes (bold lines); and 59-bes (filled circles). No ORF was detected in Bal3. Source: (Nield *et al.* 2001).

1.8 – *Escherichia coli*

Escherichia coli (*E. coli*) is a prime candidate species to study antibiotic resistance as it is an enteric commensal with a very wide natural distribution and a common cause of

human infection (Selander *et al.* 1987). Moreover, *E. coli* are routinely exposed to various antibiotic agents allowing for the selection of resistant strains, which may act as a reservoir of antibiotic resistance genes. Outbreaks caused by multiple antibiotic-resistant *E. coli* have been reported in several European hospitals since the early 1990's (O'Neill *et al.* 1990; Olesen *et al.* 1994; Olesen *et al.* 1995).

E. coli are a predominant facultative anaerobe of the human colonic flora that typically colonise the human infant gastrointestinal tract within the first hours of life. Non-pathogenic *E. coli* usually remain confined to the intestinal lumen where they are beneficial to their host. However, non-pathogenic strains of *E. coli* may cause infection in debilitated or immunosuppresed patients or when gastrointestinal barriers are breached, for example during surgery. In addition to the non-pathogenic strains, several highly adapted *E. coli* clones have evolved the ability to cause a broad spectrum of human diseases in even the most robust of our species. Infection with pathogenic *E. coli*, which may be confined to the mucosal surface or disseminated throughout the body, may cause one of three general clinical syndromes including UTI, septicemia and meningitis, and enteric/diarrhoeal disease (Nataro and Kaper 1998). Pathogenic *E. coli* strains are classified by the expression of certain virulence factors, which contribute to the establishment of infection and distinguish them from non-pathogenic *E. coli* strains (Table 1.4).

There is evidence that toxins produced by *E. coli* may be involved in the mechanism of death in sudden infant death syndrome (SIDS) (Goldwater and Bettelheim 2002; Weber *et al.* 2008). Cultures from SIDS infants contained significantly more *E. coli* than those from infants whose deaths were of non-infective cause (Weber *et al.*

2008). In addition, the most consistent characteristic of SIDS deaths, the correlation of incidence with age, provides support for the idea that bacterial toxins/infection could have a causal role in some SIDS cases. Most SIDS deaths occur between 8 and 10 weeks of age (uncommon after 6 months of age) and infants at this age have low serum immunoglobulin concentrations, which normally protect against bacterial infection and bacterial toxins (Morris 1999).

Table 1.4. The major categories of pathogenic *E. coli* strains and their associated diseases (Nataro and Kaper 1998; Ørskov and Ørskov 1985; Sussman 1997).

***E. coli* pathotype**	**Associated disease**
Uropathogenic *E. coli* (UPEC)	UTI, pyleonephritis
Neonatal meningitis *E. coli* (NEMEC)	Newborn meningitis
Septicemic *E. coli* (SEPEC)	Septicemia
Enterotoxigenic *E. coli* (ETEC)	Travelers diarrhoea, infantile diarrhoea
Enteroinvasive *E. coli* (EIEC)	*Shigella*-like dysentry
Enteroaggregative *E. coli* (EAEC)	Mucoid diarrhoea, watery diarrhoea
Enteropathogenic *E. coli* (EPEC)	Infantile diarrhoea
Shiga toxin-producing *E. coli* (STEC)	Bloody diarrhoea, hemorrhagic colitis
Enterohemorrhagic *E. coli* (EHEC)	Hemolytic uremic syndrome (HUS)

1.8.1 – Pathogenic *E. coli* strains and associated diseases

1.8.1.1 – Diarrhoeal diseases

Enterovirulent *E. coli* (ETEC, EIEC, EAEC, EPEC and EHEC), which are collectively referred to as the EEC group, cause the illness commonly known as gastroenteritis in humans. Diarrhoeal diseases caused by EEC strains are generally contracted by ingestion of contaminated food or water. ETEC are characterised by their ability to colonise the small bowel mucosal surface and secrete enterotoxins. ETEC are the main cause of infant diarrhoea in developing countries and diarrhoea in travellers visiting these countries. EAEC strains are defined as *E. coli* that do not secrete either the heat-labile or heat-stable enterotoxins and display aggregative adherence to Hep-2 cells.

Following infection with enteroinvasive *E. coli* (EIEC), the bacteria invade the epithelial cells of the intestine and cause an illness known as bacillary dysentery. Infantile diarrhoea is the illness generally associated with EPEC infection, as infants in the developing world are often affected. EPEC strains are characterised by their attaching-and-effacing histopathology, which involves the effacement of microvilli and adherence between the bacterium and the epithelial cell membrane. Infection with the enterohemorrhagic *E. coli* (EHEC) strain O157:H7 is responsible for the acute disease, hemorrhagic colitis. This *E. coli* strain produces large amounts of potent toxins (verotoxins) that cause severe damage to the lining of the intestine (FDA 2009; Nataro and Kaper 1998).

1.8.1.2 – Hemolytic uremic syndrome

HUS, one of the most frequent causes of acute renal failure in children, is commonly associated with EHEC (Varade 2000). Hemorrhagic colitis, a diarrhoeal illness commonly caused by EHEC O157:H7 and other serotypes including O111 and O103, precedes HUS in 90% of cases. There is no specific therapy for HUS although treatment includes supportive care such as blood transfusion and dialysis. Treatment of hemorrhagic colitis with antibiotics is controversial; some studies suggest antibiotics prevent the development of HUS, while others suggest they facilitate its development via the release of verotoxin from lysed bacteria and the alteration of gut flora (Nataro and Kaper 1998; Varade 2000).

1.8.1.3 – Septicemia

E. coli is the most frequently encountered bacterial species causing septicemia (bloodstream infection), a potentially life-threatening condition. Mortality rates attributed to septicemia of 10% to 50% have been confirmed by several European

studies (Fluit *et al.* 2000). An increase in the incidence of septicemia in 80 American medical centers from 1980 to 1992 was documented by the National Nosocomial Infectious Surveillance System report (Harley *et al.* 1985; Jarvis and Martone 1992; Pittet *et al.* 1994). Concomitant with the changing epidemiology and incidence of bloodstream pathogens has been the widespread emergence of microbial resistance to many antibiotic drugs (Cohen 1992; Gold and Moellering 1996). This is especially evident among *Enterobacteriaceae*, for example *E. coli,* where some species express ESBLs which confer resistance to most penicillins, cephalosporins and monobactams (Burwen *et al.* 1994; Jarlier *et al.* 1996). *E coli* that produce an emerging and important class of ESBL known as the CTX-M-type extended-spectrum β lactamases, are prevalent worldwide (Pitout and Laupland 2008). The Antimicrobial Availability Task Force of the Infectious Diseases Society of America identified ESBL-producing *E coli* as one of the drug-resistant bacteria in urgent need of new anti-infective therapies (Talbot *et al.* 2006).

1.8.1.4 – Urinary tract infections

E. coli has been identified as the main etiological agent responsible for UTIs and pyelonephritis (Kahlmeter 2000). Acute pyelonephritis characteristically causes some scarring of the kidney with each infection of the renal parenchyma. Pyelonephritis is potentially fatal when conditions such as abscess formation, necrotising infections or sepsis syndromes develop (Shoff *et al.* 2009). In the past decade, uropathogens such as *E. coli* and other *Enterobacteriaceae* have become increasingly more resistant to many commonly used antibiotics. The ECO-SENS project, an international epidemiological survey to investigate the prevalence and susceptibility of pathogens causing community acquired uncomplicated UTIs, reports high resistance to ampicillin, sulfonamides,

trimethoprim, trimethoprim-sulfamethoxazole and lower resistance to amoxicillin-clavulanic acid, quinolones, and cephalosporins in *E. coli* (Kahlmeter 2000). The extensive use of quinolones to treat *E. coli* infections has led to problems with resistance, while other antimicrobials used specifically to treat UTIs, such as mecillinam, nitrofurantoin, and fosfomycin, show low levels of resistance despite many years of use (Kahlmeter 2000). Prior to 1990 resistance of *E. coli* to fluroquinolones, potent broad-spectrum antibiotics structurally related to nalidixic acid, was exceptionally rare. However, resistant strains have emerged following the frequent use of norfloxacin and ciprofloxacin in the prophylaxis of neutropenia, cirrhosis and the treatment of UTIs. Increased use of fluroquinolones has been associated with the incidence of ciprofloxacin resistance in both community acquired and nosocomial infections (Goettsch *et al.* 2000; Pena *et al.* 1995).

1.8.1.5 – Meningitis

Bacterial meningitis is the cause of considerable morbidity and mortality in childhood. Although bacterial meningitis can be successfully treated with antibiotics, it is considered a serious infection that can result in severe neurological sequelae (Grimwood 2001). A ten-year Australian study from 1992 through to 2001 conducted by the Australasian Study Group for Neonatal Infections found that *E. coli* and Group B *Streptococcus* were the predominant causative organisms for meningitis (May *et al.* 2005). Changes in the epidemiology of bacterial meningitis have been attributed to intrapartum antibiotic prophylaxis and the introduction of new vaccines for *Haemophilus influenzae* type b, *Neisseria meningitidis* and *Streptococcus pneumoniae* (Daley and Isaacs 2004; Theodoridou *et al.* 2007).

1.8.2 – Gene cassettes detected in *E. coli*

Gene cassettes conferring resistance to various antibiotics have been detected in *E. coli* including streptomycin and spectinomycin, *aadA* (Lanz *et al.* 2003); trimethoprim, *dfrA* (Adrian *et al.* 1998); gentamycin, tobramycin and kanamycin, *aadB*, (Naas *et al.* 2001; Shaw *et al.* 1993); erythromycin, *ereA* (White *et al.* 2001); streptothricin, *sat2* (White *et al.* 2001); gentamicin, astromicin, and sisomicin, *aacC1* (*aac(3)-Ia*) (Lévesque *et al.* 1995; Shaw *et al.* 1993); rifampicin, *arr2* (Naas *et al.* 2001); chloramphenicol, *cmlA5* (Maguire *et al.* 2001); β-lactamases, $bla_{\text{VEB-1}}$ (Girlich *et al.* 2001), $bla_{\text{OXA-1}}$ (Sallen *et al.* 1995), $bla_{\text{OXA-5}}$ (Gassama *et al.* 2004), and $bla_{\text{OXA-10}}$ (Naas *et al.* 2001); and extended spectrum β-lactamases $bla_{\text{GES-1}}$ (Poirel *et al.* 2000) and $bla_{\text{GES-2}}$ (Poirel *et al.* 2001).

1.9 – Aims

The main aim of this study is the monitoring and surveillance of antibiotic resistant genes located on integrons in *E. coli* sourced from humans and animals. As part of the integron-associated gene cassette characterisation, this thesis examines evidence regarding the dissemination of integrons in *E. coli* from animal and human hosts. This study endeavored to discover the identity of the gene cassettes harboured by all integron positive isolates, including those that could not be amplified using standard PCR conditions. *Chapter 1* reviews the importance of monitoring antibiotic resistance genes, the role of integrons in the emergence of antibiotic resistant pathogens and the suitability of *E. coli* for this study. Materials and methods used in this study are covered in *Chapter 2*. *Chapter 3* describes the development of a multiplex PCR (m-PCR) to screen a collection of 514 *E. coli* strains for class 1, 2 and 3 integrase genes and by inference detect integrons in these strains. Integron screening results are also presented in *Chapter 3*. *Chapter 4* presents the characterisation of gene cassette arrays

in class 1 integrons while *Chapter 5* presents the characterisation of gene cassette arrays in class 2 integrons. *Chapter 6* provides concluding remarks regarding these results and their implications in relation to the antibiotic resistance surveillance and the evolution of integrons.

CHAPTER 2 - MATERIALS AND METHODS

2.1 – Media and bacterial strains

The composition of all media and buffers are described in Appendix I. *E. coli* were routinely grown at 37°C on Luria Bertani (LB) agar or in LB broth (Sambrook *et al.* 1989). All liquid cultures were aerated during incubation in an orbital-shaking incubator (Paton Industries) at 180 rpm. The culture medium was supplemented with antibiotics when required, at the following concentrations: ampicillin (A) 100 μg/ml; chloramphenicol (Cm) 10 μg/ml; kanamycin (K) 10 μg/ml; tetracycline (Tc) 20 μg/ml; trimethoprim (Tm) 50 μg/ml; rifampicin (Rif) 100 μg/ml; streptomycin (S) 25 μg/ml; spectinomycin (Sp) 50 μg/ml and sulfathiazole (Su) 550 μg/ml. LB agar plates to be used for blue-white screening were prepared by the addition of 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (X-gal) to a final concentration of 50 μg/ml [prepared in dimethylformamide (DMF)] and isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 20 mM (prepared in sterile dH_2O).

The following positive control *E. coli* strains and their resident plasmids were used for the integrase m-PCR: UB1637 (pR388) which carries the class 1 integron In3, DH5α (pMAQ612) which contains *intI2* from Tn*7* and DH5α (pSMB731) which harbours a class 3 integron. The features of *E. coli* strains used as positive controls for integron screening and laboratory strains used for cloning, conjugation and transformation are given in Table 2.1.

Table 2.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant features: genotype/phenotype	Source/reference
E. coli strain UB1637 (pR388:In3)	Contains plasmid pR388:In3, which harbours a class 1 integron, *intI1* and integron cassette (Tm^R, Su^R)	(Maguire *et al.* 2001) Sourced from J. Iredell[A]
E. coli strain DH5α (pMAQ612:Tn*7*)	Contains plasmid pMAQ612:Tn*7* which harbours a class 2 integron, *intI2* and integron cassette from Tn*7*, cloned into pUC18 (A^R)	(Dillon *et al.* 2005) Sourced from J. Iredell[A]
E. coli strain DH5α (pSMB731)	Contains plasmid pSMB731 which harbours a class 3 integron, *intI3* and integron cassette (K^R)	Originally described isolate (Arakawa *et al.* 1995), sourced from J. Iredell[A]
E. coli strain JM109	Endonuclease (*endA*) and recombination (*recA*) deficient. e14$^-$(McrA–) *recA1 endA1 gyrA96 thi-1 hsdR17* (*r*K– *m*K+) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*qZΔ*M15*]. The *lacI*qZΔ*M15* gene on the F′ episome, allows for blue-white screening for recombinant plasmids	http://www.stratagene.com
E. coli strain 294	Used as the recipient for conjugal transfer (Nal^R, Rif^R)	(Talmadge and Gilbert 1980)
pUC18	Contains the *lacZ* gene fragment (containing MCS) capable of α complementation with a host bacterium carrying the *lacZ*DM15 mutation; and the *bla* gene conferring A^R	Fermentas GenBank/EMBL accession number L09136
pRK600	pRK2013 *npt* : : Tn*9*, (Cm^R); provides the *tra* functions for plasmid mobilisation	(Finan *et al.* 1986)

Abbreviations: Tm, trimethoprim; Su, sulfathiazole; A, ampicillin; K, kanamycin; Nal, nalidixic acid; Rif, rifampicin; Cm, chloramphenicol and MCS, multiple cloning site. [A]Originally gifts from S. Partridge, R.M. Hall and H. Stokes.

2.2 – *E. coli* strains

2.2.1 – Strain selection

E. coli strains examined for integron carriage were isolated from 1945 to 2004 from cattle, swine, sheep, dogs, a parrot, native animals, humans and the environment, in the absence of antibiotic selection (Table 2.2). The selection of strains for examination of integron carriage was based largely on availability, while trying to obtain isolates from

a wide variety of sources. It was important to include isolates from both humans and food-producing animals in the collection to be screened, to provide a basis for the comparison of integron-related resistance genes in these hosts. *E. coli* isolates from clinical cases of human infection were selected for screening on the basis of having resistance to at least one antibiotic. There was no selection criteria applied to the other *E. coli* strains and all strains received were screened for class 1, 2 and 3 integrons. None of the *E. coli* strains sourced from either animals or humans were from a known outbreak of infection.

E. coli isolates from cattle and swine with clinical cases of infection, which had been submitted to the diagnostic laboratory at the Elizabeth McArthur Agricultural Institute (EMAI, NSW Department of Primary Industries, Camden, NSW, Australia), were obtained from Dr. M. Hornitzky. There were 320 Bovine isolates obtained from EMAI, some of which were multiple colonies from the same sample. Mixed colonies identified by serotyping were subsequently 16 streaked to obtain single colonies and only one colony designated, "A" was screened. Serotyping and PCR amplification of the *E. coli*-specific gene, *uspA,* revealed some strains including D91, D157, D161, D172 and D173 were not *E. coli*. Thus, excluding multiple colonies of the same strain and colonies that were not *E. coli*, there were 176 bovine-derived isolates from EMAI in the collection. There were 20 *E. coli* strains derived from swine exhibiting symptoms of disease and 4 strains from healthy swine. *E. coli* strains obtained from the Microbiological Diagnostic Unit (MDU, Public Health Laboratory, Department of Microbiology and Immunology, University of Melbourne, Victoria, Australia) that were sourced from animals include diagnostic specimens from dogs (n=46), native animal specimens (n=54), an ovine specimen, a diagnostic specimen from cattle and a parrot specimen.

The canine-derived *E. coli* strains were sub-cultured from the collection of Prof. G. F. Browning (Faculty of Veterinary Science, University of Melbourne, Victoria, Australia), while the native animal-derived strains were originally from Dr. D. M. Gordon (School of Botany and Zoology, The Australian National University, ACT, Australia).

All of the *E. coli* strains sourced from human specimens (n=204) were obtained from MDU. Of the *E. coli* strains obtained from human specimens, 192 were collected as part of the work of MDU from 1994 to 2003, and 12 were archived strains from patients in the UK, USA, Denmark, Austria, Germany, Indonesia, Thailand and Japan (collected from 1945 to the 1980's). *E. coli* strains sourced from human specimens were collected from healthy humans or patients suffering various ailments including (i) gastro-intestinal symptoms such as diarrhoea, bloody diarrhoea, gastroenteritis, infantile enteritis, enteritis and appendicitis (ii) UTI, (iii) septicemia, (iv) meningitis, (v) HUS and (vi) babies who had died from SIDS. The 24 *E. coli* strains isolated from healthy infants were part of a previous study of antibiotic resistance in verocytotoxigenic *E. coli* (Bettelheim *et al.* 2003). These strains were collected between 1989 and 1992 from infants less than one year of age who had not suffered from gastrointestinal symptoms or been treated with antibiotics in the two weeks prior to collection. In addition to isolates collected from animals and humans, 6 *E. coli* strains isolated from the Tasmanian environment were obtained from MDU. Details of the 514 *E. coli* strains screened for class 1, 2 and 3 integrons are given in Appendix III.

Table 2.2. *E. coli* strains examined for class 1 integron carriage.

Source of strains			Number of strains
Animal	Diagnostic specimens	Bovine	177
		Canine	46
		Porcine	20
	Healthy	Native animals	54
		Porcine	4
		Ovine	1
	Unknown symptoms	Porcine	1
		Parrot	1
	Total animal-derived strains		**304**
Human	Diagnostic specimens	UTI	96
		SIDS	21
		Diarrhoea	22
		Bloody diarrhoea	7
		Suspected diarrhoea	5
		Gastroenteritis	4
		Infantile gastroenteritis	3
		Enteritis	1
		Suspected traveller's diarrhoea/ infantile enteritis	1
		Septicemia	5
		HUS	2
		Suspected HUS	1
		Neonatal meningitis	1
		Appendicitis	1
	Healthy	Infants	24
		Human	2
	Unknown symptoms	Human	8
	Total human-derived strains		**204**
	Environmental		**6**
	Total number of strains		**514**

Abbreviations: SIDS, sudden infant death syndrome; UTI, urinary tract infection; and HUS, hemolytic uremic syndrome.

2.3 – Characterisation of *E. coli* strains

Dr K. Bettleheim and A. Kruzevski (MDU) performed the phenotypic characterisation of *E. coli* strains. The strains were identified as *E. coli* based on their reaction in triple-sugar-iron (TSI) agar (Oxoid), motility-indole-ornithine (MIO) medium (Oxoid), o-nitrophenyl-ß-d-galactopyranoside (ONPG) (Oxoid) and urease broth (Oxoid).

E. coli strains were O- and H-serotyped as previously described (Bettelheim and Thompson 1987; Chandler and Bettleheim 1974).

2.4 – Antimicrobial testing

E. coli isolates were tested for their sensitivity to several antibiotics by the plate-replicator method, which was performed by Dr K. Bettleheim and A. Kruzevski (MDU). The following antibiotics were used: ampicillin (32 μg/ml), streptomycin (25 μg/ml), tetracycline (20 μg/ml), chloramphenicol (10 μg/ml), sulfathiazole (550 μg/ml) trimethoprim (50 μg/ml), kanamycin (10 μg/ml), nalidixic acid (50 μg/ml), spectinomycin (50 μg/ml), gentamicin (2.5 μg/ml) and ciprofloxacin (2 μg/ml). The antibiotics were incorporated in lysed blood isosensitest agar (Oxoid) and cultures were tested using a Clements antibiotic sensitivity replicator with 32 prongs. *E. coli* and controls were cultured in double strength nutrient broth (Oxoid) with rotary aeration at 100 rpm for 1.5 h at 37°C. The cultures were diluted 1:10 in tryptone water and added to the antibiotic sensitivity replicator, which was filled with 0.5 ml nutrient agar containing 0.05% agar. The replicator was used to inoculate *E. coli* onto the antibiotic agar plates, which were incubated at 37°C overnight. Sensitivity to a specific antibiotic was demonstrated by absence of *E. coli* growth on the plate. Strains of *Salmonella* Heildeberg and *Salmonella* Hadar with known resistances were used as positive controls, while a sensitive strain of *Salmonella* Typhimurium was used as a negative control. All integron-positive strains were tested against all antibiotics listed.

2.5 – DNA manipulations

2.5.1 – Agarose gel electrophoresis

Agarose gels were routinely prepared in 1X TAE buffer (Appendix I) with 0.7-1.5% electrophoresis grade agarose. DNA samples were typically electrophoresed for 1-2 h at 60-80 V using BioRad electrophoresis apparatus. Gels containing electrophoresed DNA were stained by immersion in a solution of ethidium bromide (1 μg/ml) for 30 min. Ethidium bromide-stained bands were visualised with ultra-violet light. The size of digested DNA fragments were estimated by comparison with λ bacteriophage DNA digested with *Hind*III restriction enzyme or GeneRuler 100bp DNA Ladder Plus (Fermentas, Australia).

2.5.2 – Restriction endonuclease analysis of DNA

Restriction endonucleases used in this study were purchased from either Roche Diagnostics (Australia) or Fermentas Life Sciences (USA). Digests were performed according to manufacturer's instructions. The composition of reagents for digestion consisted of 2-10 μl of DNA, 1 μl of restriction enzyme, 2 μl of the appropriate restriction enzyme buffer, and autoclaved Milli Q water to a total volume of 20 μl. Digests were incubated at 37°C for 1-2 h. When required, the enzyme was inactivated by incubation at 65°C for 10 min.

2.5.3 – Extraction of DNA from agarose gels

DNA was extracted from agarose gels using the Wizard SV Gel and PCR Clean-Up System (Promega). Briefly, the electrophoresed DNA band was excised from the gel and placed in a 1.5 ml microcentrifuge tube. Membrane Binding Solution (Appendix I) was added to the gel slice (10 μl per 10 mg of gel slice), vortexed and incubated at

50-65°C until the gel dissolved. The dissolved gel mixture was transferred to a silica-based column and incubated at room temperature, to allow DNA to bind to the column. Contaminants were removed by centrifugation at 10,000 *x g* and successive wash steps; firstly with 700 µl then 500 µl of Column Wash Solution (Appendix I). Purified DNA was eluted from the column in 50 µl of nuclease free water.

2.5.4 – Crude DNA extraction

DNA to be used as a PCR template was extracted from *E. coli* isolates using a simple boiling method. Briefly, *E. coli* were grown overnight on LB agar. The following day 2-3 colonies were harvested and resuspended in 1 ml of sterile Milli Q water. The bacterial suspension was centrifuged at maximum speed for 5 min and the cell pellet was resuspended in 500 µl of Milli Q water. The washed bacterial cell suspension was heated at 100°C for 7 min to release genomic and plasmid DNA. The boiled preparation was centrifuged at maximum speed for 5 min and the supernatant containing DNA was transferred to an Eppendorf tube for storage at 4°C or -20°C. A volume of 3-5 µl of this DNA preparation was used to provide a template for PCR (per 50 µl reaction mix).

2.5.5 – Plasmid DNA extraction

Small-scale plasmid DNA extractions (1-20 µg) were performed using Wizard SV Miniprep DNA Purification System (Promega), which utilises an alkaline lysis system. Bacterial cells were harvested by centrifugation from a 4 ml overnight culture. The resulting pellet was resuspended in 250 µl of Cell Resuspension Solution (Appendix I). Cells were lysed with 250 µl of Cell Lysis Solution (Appendix I). Hydrolysis of cross-linked proteins from the bacterial cell wall was achieved by the addition of 10 µl

of alkaline protease. Centrifugation at 16,000 *x g* for 10 min was performed to pellet cellular debris. The supernatant was loaded onto a silica based column and centrifuged at 16,000 *x g* for 5 min. DNA present in the supernatant bound to the silica matrix of the column while contaminants were removed by subsequent wash steps; firstly with 750 µl then 250 µl of Column Wash Solution (Appendix I). DNA was eluted from the column with 50 µl of nuclease free water.

Large-scale plasmid extractions were performed using the plasmid maxi kit while medium-scale extractions were performed using the plasmid midi kit (Qiagen). Both systems use an anion exchange resin packed into a disposable tip to bind DNA, which is washed and then eluted. Bacterial cells were harvested by centrifugation at 6,000 *x g* for 15 min at 4°C and resuspended in buffer P1 (Appendix I). Bacterial cells were lysed by the addition of a NaOH/SDS buffer P2 (Appendix I) and incubation at room temperature for 5 min. The lysed bacterial suspension was neutralised and DNA was precipitated the addition the acidic potassium acetate buffer, P3 (Appendix I). DNA precipitation was enhanced by using chilled P3 and incubation on ice for 30 min. Following centrifugation at 20,000 *x g* for 30 min at 4°C to pellet the cellular debris, the supernatant was removed and a second centrifugation was performed for 15 min. For low-copy number plasmids, for example the plasmid isolated from *E. coli* strain 80, an additional isopropanol precipitation step was performed to reduce the large sample volume (2.5 L of media for a maxi kit) and facilitate loading of the column. The DNA was pelleted by centrifugation at 15,000 *x g* for 30 min, redissolved in TE (Appendix I) and then loaded onto the anion-exchange resin. DNA bound to the column was washed free from contaminating RNA, proteins and low-molecular weight impurities using a medium-salt wash buffer, QC (Appendix I) and eluted in a high salt buffer, QF

(Appendix I). DNA was concentrated and desalted by a second isopropanol precipitation and centrifuged at 15,000 *x g* for 30 min. The DNA pellet was washed with 70% ethanol, air dried and redissolved in a suitable volume of autoclaved Milli Q water.

2.5.6 – Chromosomal DNA extraction

Chromosomal DNA was extracted from *E. coli* strains using the DNeasy Tissue Kit (Qiagen, Australia). The DNeasy Tissue Kit uses silica-gel-membrane technology. Bacterial cells were harvested by centrifugation from a 4 ml overnight culture and the resulting pellet was resuspended in 180 µl of Buffer ATL. Bacterial cells were lysed by the addition 20 µl of Proteinase K and incubation at 55°C for 1 hr with shaking. RNA was removed by incubation of the sample with of 4 µl of RNase (100 mg/ml) at room temperature for 2 min. Proteins were denatured by the addition of 200 µl of AL buffer and incubation at 70°C for 10 min. Following the addition of 200 µl of ethanol (96-100%), the lysate was loaded onto the DNeasy spin column. DNA present in the lysate selectively bound to the silica matrix of the column and contaminants were removed by centrifugation. Subsequent wash steps; firstly with 500 µl of AW1 then 500 µl of AW2 were performed to remove remaining contaminants and enzyme inhibitors. Purified DNA was eluted from the column with 100 µl of nuclease free water.

2.6 – Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify the integrase genes *intI1*, *intI2, intI3* and the *uspA* gene to detect integrons in *E. coli* isolates and to amplify the gene cassette regions of integrase positive isolates. Other applications of PCR in this

study include the detection of IS*26*-integron elements in "non-amplifiable" class 1 gene cassette arrays, analysis of Tn*7* insertion and the production of various digoxigenin (DIG)-labelled probes for Southern-blot analysis.

2.6.1 – General PCR parameters

PCR primers were designed using Primer3 program (Rozen and Skaletsky 2000) available via BioManager, Australian National Genomic Information Service (ANGIS) (http://www.angis.org.au/). The sequence of oligonucleotide primers used for PCR and their associated GenBank accession numbers are listed in Appendix II. All primers were synthesised by Sigma (Australia). When necessary, PCR amplification was optimised by varying the concentration of template DNA, concentration of $MgCl_2$, annealing temperature or other cycling parameters. The composition of reagents used in a typical PCR amplification is given in Table 2.3. All PCR apparatus including PCR tubes, PCR rack, Eppendorf tubes, pipettes, filter tips, pen, gloves and tip discard container were exposed to UV light for 30 min prior to PCR. PCR reactions were setup in a Biohazard safety hood, keeping all reagents on ice. All PCR amplifications were carried out using the Palm Cycler (Corbett Research, Australia).

PCR cycling conditions used in this study are outlined in Appendix II. Published PCR parameters were used when available. General PCR cycling conditions were used as a basis to develop primer/template/amplicon size specific PCR parameters (Table 2.4). The annealing temperature was routinely set at $5^{o}C$ below the lowest melting point of the primer pair. The annealing temperature was modified if necessary, as indicated by low specificity or low yield of amplification. Temperature gradient PCR was performed

to determine optimal annealing temperature. The extension time was routinely set at 1 min for amplification of sequences less than 2 kb (Innis and Gelfand 1990).

Table 2.3. Concentration of reagents used in a typical PCR amplification.

Reagent	**Final Concentration**
Template DNA	8 ng
10X PCR buffer (containing 15 mM $MgCl_2$)	1X
dNTPs	200 μM each dNTP
5′ primer	0.25 μM
3′ primer	0.25 μM
Taq DNA polymerase	2.5 U
Autoclaved Milli Q H_2O	To final volume of 50 μl

Table 2.4. Generalised PCR cycling conditions used as a basis to develop specific PCR parameters.

Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	94°C	2.5 to 5 min	1
Denaturation	94°C	1 min	30-35
Annealing	5°C lower than primer melting temp.	1 min	
Extension	72°C	Variable[A]	
Final extension	72°C	10 min	1

[A]Variable depending on the length of sequence to be amplified.

2.6.2 – Class 1 integron-related PCR

Gene cassette regions of class 1 integrons were amplified using the primer pair L1 and R1 and PCR parameters described previously (Lévesque *et al.* 1995). In most instances the primer pair L1 and JL-D7 was used to amplify IS*26*-integron elements, although in one case where IS*26* was inserted in the reverse direction L1 and JL-D2 were used. Amplification of the *intI1* gene (partial sequence) and the transposase genes of Tn*21, tnpM*, *tnpR*, and *tnpA* (partial sequence) was achieved using the primer pair FD-tnpA

and FD-IntI1. The following PCR conditions were used to amplify the IS*26*-integron element and the Tn*21* transposase genes: 94^{o}C for 3 min, followed by 30 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min and 72^{o}C for 4 min, and finally 72^{o}C for 10 min.

2.6.3 – Class 2 integron-related PCR

The gene cassette regions of class 2 integron positive strains were amplified using the primers hep51 and hep74 (White *et al.* 2001) and conditions previously described (White *et al.* 2000). Tn*7* was identified by PCR of the Tn*7* specific gene, *tnsC,* using the primers FD-tnsCF and FD-tnsCR. PCR cycling conditions used for amplification of the *tnsC* gene were 94°C for 1.5 min; 30 cycles at 94°C, 55°C, 72°C each for 45 sec duration; and 72°C for 10 min. The exact insertion site of the class 2 integron in the *E. coli* chromosome was characterised by PCR of the left and right end of Tn*7* at the *phoS/glmS* gene junction using the following PCR cycling conditions 94°C for 3 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C 4 min for the left end of Tn*7* and 1 min for the right left end of Tn*7*; and 72°C for 10 min. PCR primers used for amplification of the left end of Tn*7* and the *phoS* gene were FD-phoSF and FD-intI2 (Mazel *et al.* 2000). PCR primers used for amplification of the right end of Tn*7* and the *glmS* gene were FD-Tn7F and FD-glmSR.

2.6.4 – Expand Long Template PCR system

The Expand Long Template PCR System (Roche) was used to amplify long genomic DNA fragments. This system uses an enzyme mix of Taq DNA polymerase and Tgo DNA polymerase, which has proof-reading activity. The PCR cycling conditions and reagent concentrations used for the Expand Long Template PCR System were performed according to manufacturer's instructions.

2.6.5 – Second-step PCR

Second-step PCR was one strategy utilised to try and amplify "non-amplifiable" class 1 gene cassette arrays. This method involved using a 2 μl aliquot of the amplification products resulting from the first round of PCR, for a subsequent round of PCR using the same PCR primers.

2.6.5.1 – Core-sample PCR

Core sample PCR was performed to amplify a specific band from a mixture of discrete-sized bands produced by PCR (Coyne *et al.* 2001). This method involves taking a core sample of a DNA band from a gel and using it directly as a substrate for a second round of PCR. Replicate samples of PCR products were electrophoresed on 1-2% agarose and stained with ethidium bromide. A representative lane was removed and notched to allow reorientation with the remainder of the gel. The band of interest was identified and marked without exposing the corresponding DNA in replicate lanes to UV light, as this could result in DNA cross-linking and prevent amplification. The marked stained segment was aligned with the remainder of the gel and core samples were taken using 200 μl yellow pipette tip (Gilson, USA) with 5 mm removed from the end. The gel from which the core samples had been removed was visualised under UV light, to ensure the correct regions were sampled. Core samples were used as a template for subsequent PCR. Agarose cores were added directly to the PCR reaction mix allowing 10 μl per core sample. At the completion of a typical PCR a 10 min cycle at 72°C was incorporated to melt the agarose. For electrophoretic analysis, the PCR product was loaded prior to submersion of the gel in TAE buffer (Appendix I).

2.7 – Restriction fragment length polymorphism

Gene cassette regions of class 1 and class 2 integrons were characterised according to their restriction profile, following digestion with *Rsa*I and *Alu*I. The nucleotide sequence of a randomly selected representative amplicon from each distinct RFLP class was determined. RFLP analysis of IS*26*-*dfrA5*-integrase elements was performed by digestion with *Rsa*I and integrase-*tnpA* elements with *Eco*RI. The RFLP profile of PCR amplification products of the left end of Tn*7* and the *phoS* gene was determined following digestion with *Alu*I.

2.8 – DNA sequence determination and analysis

2.8.1 – Cycle-sequencing conditions

DNA templates were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Rotkreuz, Switzerland), and DNA molecules labelled with multiple fluorescent dyes were analysed with a capillary sequencer (Applied Biosystems, 3130 Genetic Analyzer), according to the manufacturer's instructions. A typical cycle-sequencing reaction contained the concentration of reagents shown in Table 2.5. The cycle-sequencing reaction was performed in a Palm Cycler (Corbett Research, Australia) using the cycling parameters described in Table 2.6.

PCR products to be sequenced were pooled and purified using Wizard SV gel and PCR Clean-Up system (Promega, Australia). The concentration of purified DNA was estimated by comparison with *Hind*III digested λ DNA of known concentration. DNA sequencing primers were designed using the Primer3 program available via ANGIS (http://www.angis.org.au) and synthetised by Sigma (Australia).

Table 2.5. Concentration of reagents used in a typical sequencing reaction.

Reagent	**Volume**	**Final concentration/ amount**
Template:		
PCR product 100-200 bp	0.5-5.5 µl	1-3 ng
PCR product 200-500 bp		3-10 ng
PCR product 500-1000 bp		5-20 ng
Plasmid double-stranded		150-200 ng
5X sequencing Buffer	2 µl	1X
3′ or 5′ Primer (1pm/ µl)	2 µl	0.2 ρm/µl
Terminator v3.1 Ready reaction mix	0.5 µl	N/A
Milli Q H_2O	To final volume of 10 µl	N/A

Table 2.6. Standard cycling parameters used for the sequencing reaction.

Step	**Temperature**	**Duration**	**Cycles**
Denaturation	96°C	10 sec	
Annealing	50°C	5 sec	25
Extension	60°C	4 min	

2.8.2 – Purification of sequencing extension products

Extension products were purified to remove unincorporated dye terminators prior to capillary electrophoresis. DNA was precipitated by the addition of 2 µl of 3M sodium acetate (pH 4.5) and 50 µl of 95% ethanol. The ethanol/DNA mix was briefly vortexed, incubated on ice for 10 min and centrifuged at 16,000 *x g* for 20 min. The supernatant was aspirated and the pellet rinsed with 250 µl of 70% ethanol and air-dried. Samples were dissolved in 6 µl of formamide DNA sequence loading dye (Appendix I), vortexed and incubated at 90°C for 2 min then placed on ice.

2.8.3 – Analysis of DNA sequence data

Raw electropherograms were checked and sequence data was assembled into a contiguous DNA fragment using ContigExpress by Vector NTI Advance 10 (Invitrogen,

VIC, Australia). The coding sequences (CDS) were identified using the Genefinder program GeneMarkS (Besemer *et al.* 2001) available at http://opal.biology.gatech.edu/GeneMark/genemarks_ev.cgi. Nucleotide BLAST and protein database searches were performed to identify the predicted CDS using the National Centre for Biotechnology (NCBI) databases available at http://www.ncbi.nlm.nih.gov. The Artemis program was used to visualise annotated features (Rutherford *et al.* 2000). Comparative DNA analysis was performed between the DNA sequence of a plasmid from *E. coli* strain 80 and the sequence of a *Salmonella* derived plasmid, pOU1114, the using BLAST algorithm results in conjunction with the Artemis Comparison Tool program (Carver *et al.* 2005).

2.8.4 – DNA sequencing of plasmids from *E. coli* strains D22 and 80

Plasmid DNA was isolated from *E. coli* strains D22 and 80 and transformed by electroporation into *E. coli* strain JM109 (Sambrook *et al.* 1989). The sequence of the integron and flanking regions was determined for *E. coli* strain D22 and the entire plasmid sequence was determined for *E. coli* strain 80. Transformed plasmid DNA was extracted using either the plasmid midi kit or maxi kit (Qiagen). To facilitate the sequencing of the plasmid from *E. coli* strain 80, plasmid DNA was digested with *Hind*III and the resulting fragments of 4 kb, 5 kb and 12 kb were cloned into pUC18 (Fermentas, QLD, Australia). Sequencing of the plasmid from *E. coli* strain 80 proceeded preferentially from the original plasmid and from the cloned plasmid as required.

2.9 – Cloning

A plasmid harbouring a class 2 integron was extracted from *E. coli* strain 80 and digested with *Hind*III. The resulting fragments of 12 kb, 5 kb and 4 kb were cloned into pUC18.

2.9.1 – Digestion of vector and insert DNA

Plasmid DNA from *E. coli* strain 80 (2.5 μg) was digested with *Hind*III in a 50 μl volume and incubated at 37°C for 2 h. Approximately 2.7 μg of pUC18 was digested with *Hind*III in a 50 μl volume incubated at 37°C for 1 h. *Hind*III present in digested plasmid DNA was heat inactivated at 65°C for 10 min prior to ligation.

2.9.2 – Ligation reaction

The concentration of vector and insert DNA was estimated by comparison with λ DNA digested with *Hind*III of a known concentration. Vector DNA, insert DNA, 10X ligase buffer, T_4 ligase (Fermentas) and water were added to a microfuge tube in the quantities outlined in Table 2.7. Two different ratios of vector to insert were trialed; Reaction A used 1:1 ratio while ligation B used 1:2 ratio. Controls for the ligation included a control for uncut vector in the ligation mix, a control for the ligation reaction, and an uncut vector control. The ligation mix was vortexed and briefly centrifuged, then incubated overnight at 14°C in a water bath. T_4 ligase was heat inactivated at 65°C for 10 min prior to transformation by electroporation or heat-shock.

Table 2.7. Concentrations of reagents used for the ligation reaction.

Reagent	Ligation A	Ligation B	Control[A]	Control[B]	Control[C]
pUC18/*Hind*III	2 µl	2 µl	2 µl	2 µl	Uncut vector 2 µl
Insert/*Hind*III	4 µl	8 µl	none	none	none
Ligase 10X buffer	1 µl	1 µl	1 µl	1 µl	1 µl
T_4 ligase	1 µl	1 µl	none	1 µl	none
Milli Q water	to 30 µl	to 30 µl	to 30 µl	to 30 µl	to 30 µl

[A]Control for uncut vector in vector preparation, [B]control for the ligation reaction and [C]uncut vector control.

2.10 – Southern hybridisation

Southern hybridisations were performed to establish the genomic location of integrons. This involved observing the hybridisation of a DIG-labelled integrase probe to plasmid DNA extractions compared with that of total genomic DNA. The primers L2 and L3 (Maguire *et al.* 2001) were used to synthesise the class 1 integrase (*intI1*) probe, while the primers Int2.F and Int2.R (Mazel *et al.* 2000) were used for the class 2 integrase (*intI2*) probe. Detailed analysis of chromosomally located class 2 integrons was performed to determine if the site of insertion was the *phoS/glmS* gene junction. In this Southern, the hybridisation of a DIG-labelled *intI2* probe to total genomic DNA was compared with that of a *phoS/glmS* probe.

2.10.1 – DNA transfer

The transfer of DNA to a positively charged membrane (Roche, Australia) was accomplished by the techniques described by Southern (1975). Restricted DNA was electrophoresed at 60 V and visualised with ethidium bromide staining. Partial DNA fragmentation was achieved in situ by acid depurination in 250 mM HCL for 10 min. DNA in the gel was subsequently denatured by immersion in denaturation solution for

30 min. The gel was rinsed briefly in dH_2O and submerged in two volumes of neutralisation buffer prior to transfer (Appendix I).

Apparatus for the transfer of DNA was assembled as follows. A wick consisting of three layers of 3 mm Whatman paper presoaked in 20X SSC (Appendix I) was placed over a platform, supported within a large reservoir. The reservoir was filled with 20X SSC to just below the platform. The gel was inverted and carefully placed on the wick. A mask of plastic was used to frame the gel to prevent contact of the wick with the blotting paper; thus ensuring the buffer moved through the gel and not around it. The nylon membrane was moistened in milli Q water, presoaked 20X SSC and then placed on the gel. This was followed by three layers of Whatman paper presoaked in 2X SSC and a 10 cm stack of dry blotting paper. At each stage of assembly air bubbles were excluded from wetted surfaces with a pasture pipette. A glass plate and a 500 g weight were centered on top of the absorbent paper. DNA was allowed to transfer overnight by capillary action.

2.10.2 – Labelling the probe

Incorporation of DIG-11-dUTP into the integrase gene was achieved using the PCR DIG probe synthesis kit (Roche). The composition of PCR reagents for the synthesis of the DIG-labelled integrase hybridisation probe is given in Table 2.8 and PCR cycling conditions in Table 2.9. Evaluation of the probe labelling efficiency was determined by agarose gel electrophoresis; the DIG labelled probe being larger than the unlabelled PCR product. The DIG labelled probe was purified using the Wizard SV gel and PCR Clean-Up system (Promega, Australia) prior to estimation of its concentration by comparison with *Hind*III digested λ DNA of known concentration.

Table 2.8. Reagents used in PCR for the synthesis of the DIG-labelled probe.

Reagent	**Final Concentration**
Template DNA: *E. coli* strain D87 (class 1), *E. coli* strain 64 (class 2)	8 ng
PCR buffer 10X conc. 15 mM $MgCl_2$	1X
PCR DIG labelling mix	200 mM dNTPs
5′ primers: L2 for class 1 integrase Int2.F for class 2 integrase	0.25 μM
3′ primers: L3 for class 1 integrase Int2.R for class 2 integrase	0.25 μM
Taq DNA polymerase	2.5 U
dH_2O	To final volume of 50 μl

Table 2.9. PCR cycling parameters for synthesis of the DIG-labelled probe.

Step	**Temperature**	**Duration**	**Cycles**
Denaturation	94°C	5 min	1
Denaturation	94°C	1 min	
Annealing	53°C	1 min	30
Extension	72°C	1 min	
Extension	72°C	5 min	1

2.10.3 – Hybridisation and detection

Without prior washing, DNA was immobilised on the membrane by UV-cross linking in the Stratagene Stratalinker 1800, using automatic mode. The membrane was rinsed in Milli Q water and thoroughly dried at room temperature. The membrane was placed in a Hybaid bottle with 20 ml DIG Easy Hyb prehybridisation solution (Roche, Australia) per 10 cm^2 of membrane surface area and incubated at 42°C for 2 h in a rotating Hybaid, Mini Oven Mk II. The DIG-labelled probe was denatured by heating at 95°C for 10 min and placed immediately on ice. The hybridisation solution consisting of

25 ng of denatured probe per ml of DIG Easy Hyb was added to a Hybaid bottle containing the membrane. Overnight incubation at 42°C was performed to allow hybridisation of the labelled probe to complementary DNA bound to the membrane.

Several washes were performed at room temperature to remove non-specific binding to the membrane. This included two washes in 2X SSC for 5 min followed by two washes in 0.5X SCC (Appendix I) for 15 min. The membrane was equilibrated in maleic wash buffer (Appendix I) for 1 min prior to blocking in blocking solution (Appendix I) for 30-60 min with gentle agitation at room temperature.

The hybridised probe was detected with anti-DIG-alkaline phosphatase (AP) Fab fragments. The membrane was incubated with Anti-DIG-AP antibody diluted 1:20,000 in blocking buffer for 30 min with gentle agitation. Unbound antibodies were removed by washing the membrane with 2 changes of maleic wash buffer for 15 min each. Following equilibration in detection buffer (Appendix I) for 2 min, the membrane was placed in a plastic bag and covered with CDP-*Star* Chemiluminescent substrate solution (Sigma, Australia) diluted 1:200 in detection buffer. The membrane was incubated at room temperature for 5 min prior to exposure to Lumi-Film chemiluminescent detection film (Kodak, Australia). The film was developed by immersion in developing solution (Kodak) until bands were visible and then in a fixative solution (Kodak, Australia) for 2 min. The membrane was re-exposed against the film for various time periods to optimise detection.

2.10.4 – Stripping membrane for re-probing

To remove the DIG-labelled probe, the membrane was washed in dH_2O for 1 min and then incubated in pre-warmed alkaline probe stripping solution (Appendix I) for two 10 min washes at 37°C. The stripped membrane was rinsed in 2X SSC prior to re-probing or stored in 2X SSC in a sealed plastic bag at 4°C.

2.11 – Microbiological techniques

2.11.1 – Preparation of chemically competent cells

A fresh colony of the strain to be transformed was inoculated into a 2 ml starter culture and incubated at 37°C overnight with rotary aeration at 180 rpm. The following day 200 µl of starter culture was added to 200 ml of pre-warmed LB and incubated at 37°C until it reached an A_{560} of 0.5. Bacterial cells were harvested by centrifugation at 5,000 *x g* for 10 min at 4°C and the pellet was incubated on ice for 10 min. Cells were resuspended in 100 ml of chilled 0.1 M $MgCl_2$, centrifuged and then resuspended in 10 ml of chilled 0.1 M $CaCl_2$. Chemically competent bacterial cells were stored at 4°C for up to a week or resuspended in an equal volume of 50% glycerol and stored at -80°C.

2.11.1.1 – Chemical transformation

For each plasmid transformation, 5µl of plasmid DNA was added to a 200 µl aliquot of competent *E. coli* cells and incubated on ice for 1 h. Cells were heat-shocked by incubation at 37°C for 5 min. The transformation mix was centrifuged at 16,000 *x g* for 1 min and the pellet was carefully resuspended in 500 µl of LB and incubated at 37°C for 1 h without shaking. Aliquots of transformed cells (50 µl, 100 µl, 150µl and 200 µl) were plated on to selective agar and incubated overnight at 37°C.

2.11.2 – Preparation of electro-competent cells

A fresh colony of the strain to be transformed was inoculated into a 5 ml starter culture and incubated at 37°C overnight with rotary aeration at 180 rpm. The following day the starter culture was added to 500 ml of pre-warmed LB and incubated at 37°C until it reached an A_{600} of approximately 1.0. The bacterial culture was chilled on ice for 30 min and then the cells were harvested by centrifugation at 5,000 *x g* for 10 min at 4°C. The cells were washed twice with 80 ml of chilled sterile distilled water, and then washed twice with 15 ml of chilled 10% glycerol. At each wash step care was taken to remove all of the supernatant and to resuspend the cells gently but completely. Finally, the cell pellet was resuspended in 600 µl of chilled 10% glycerol and stored at -80°C in 40 µl aliquots.

2.11.2.1 – Electroporation

Electroporation of extracted plasmid DNA was used to determine if integrons with "unamplifiable" gene cassettes were located on transformable plasmids. Seven randomly selected *E. coli* strains with differing serotypes and resistance phenotypes containing class 1 integrons that were sourced from cattle and one *E. coli* strain (strain 80) containing a class 2 integron were tested in this manner. Plasmid-associated *intI* genes were detected by the electroporation of plasmid DNA isolated from these strains into *E. coli* JM109, followed by selection with trimethoprim, sulfathiazole, or streptomycin and subsequent *intI* PCR of DNA from the resultant colonies.

Approximately 25 ng (0.5-2 µl from a Wizard SV Miniprep DNA purification) of plasmid DNA was added to a 40 µl aliquot of freshly thawed electrocompetent cells on ice. Controls for electroporation include (i) pUC18 plasmid DNA and

(ii) electrocompetent cells without plasmid DNA. The cells and plasmid DNA were gently mixed and added to the bottom of a chilled 0.2 cm electroporation cuvette. The cells were electroporated in the Gene-pulser electroporation apparatus set at 2.5 kV, 25 μFD, and the pulse controller at 200 omega. The electroporated cell-plasmid mix was immediately added to 0.5 ml of pre-warmed LB and incubated at 37°C for 2 h with shaking to allow for plasmid expression. Aliquots of 50 μl, 100 μl, and 200 μl were plated onto LB agar supplemented with the appropriate antibiotics for selection and incubated overnight at 37°C.

2.11.3 – Bacterial conjugation

E. coli strain 80 was examined for the ability to transfer a resident class 2 integron-containing plasmid to a recipient *E. coli* strain via conjugation. *E. coli* strain 294 (Rif^R, Nal^R) (Talmadge and Gilbert 1980) was used as the recipient for conjugal transfer, which was performed with and without the aid of the helper plasmid pRK600 (Finan *et al.* 1986). Liquid cultures of donor, recipient and helper strains were prepared by inoculating 2 ml of LB broth with one to two colonies, followed by overnight incubation at 37°C. Equal volumes (100 μl) of donor, recipient and helper strain cultures (as required) were spread on a LB plate and incubated at 37°C overnight. The following day the resulting bacterial colonies were harvested, serially diluted in sterile saline (0.7%) and 20 μl volumes were plated out in triplicate in the presence of selection for rifampicin (100 μg/ml) and trimethoprim (50 μg/ml) and incubated overnight at 37°C.

CHAPTER 3 – DEVELOPMENT OF A MULTIPLEX POLYMERASE CHAIN REACTION AND SCREENING FOR CLASS 1, 2 AND 3 INTEGRONS

3.1 – Introduction

Multi-drug resistant bacteria are the primary cause of failure in the treatment of infectious diseases (Goldmann 1999). The role of integrons in the emergence of multi-drug resistant bacteria via the stockpiling of gene cassettes containing antibiotic resistance genes has been well established (Leverstein-van Hall *et al.* 2003; Naas *et al.* 2001; Tennstedt *et al.* 2003). Definitive features of integrons include an integration site (*attI*) for the site-specific insertion of gene cassettes (Partridge *et al.* 2000), an integrase to mediate excision and orientation-specific integration of gene cassettes (Collis *et al.* 1993) and a promoter Pc, to ensure expression of the operon (Lévesque *et al.* 1994). Class 1, 2 and 3 integrons all contain an *intI* gene that codes for a distinct, yet related, integrase enzyme (Nield *et al.* 2001).

Class 1 integrons are the most common and well characterised class of integron and are widely disseminated in animal and human clinical strains of the family *Enterobacteriaceae* (Goldstein *et al.* 2001). Class 2 integrons, which contain a putative but defective integrase gene *intI2* (Hansson *et al.* 2002) and are typically located at the left end of Tn*7* or related transposons, are also widely disseminated (Hall *et al.* 1991;

Sundström *et al.* 1991; Teitze and Brevet 1991; Young *et al.* 1994). Only four class 3 integrons have been identified to date (Arakawa *et al.* 1995; Correia *et al.* 2003; Xu *et al.* 2007).

E. coli strains examined for integron carriage were isolated from cattle, swine, sheep, dogs, a parrot, native animals, humans and the environment, in the absence of antibiotic selection (Table 2.2). It was important to include *E. coli* isolates from both animals and humans in the collection to be screened, to provide a basis for the comparison of integron-related resistance gene carriage in these hosts. *E. coli* isolates from clinical cases of human infection were selected for screening on the basis of having resistance to at least 1 antibiotic. There was no selection criteria applied for the other *E. coli* strains and all strains received were screened for class 1, 2 and 3 integrons. None of the *E. coli* strains sourced from either animals or humans were from a known outbreak of infection.

E. coli strains were collected from animals showing symptoms of disease and from healthy native animals and swine. Most of the *E. coli* strains sourced from human specimens were collected as part of the work of MDU from 1994 to 2003. Human-derived *E. coli* strains were collected from healthy humans and from patients suffering various ailments including gastro-intestinal symptoms, UTI, septicemia, meningitis, HUS and babies who had died from SIDS. Six *E. coli* strains were isolated from the Tasmanian environment (Appendix III).

A m-PCR was developed to facilitate screening of *E. coli* isolates for integrons, a prerequisite to examining the molecular epidemiology of antibiotic resistance genes carried by integrons. M-PCR is a rapid technique for screening a large number of

isolates for multiple genes. Screening of *E. coli* isolates for class 1, 2, and 3 integrons involved the identification of integron positive isolates via PCR amplification of the class specific integrase genes *intI1*, *intI2* and *intI3*, respectively. The gene cassette arrays of integron positive isolates were PCR-amplified, analysed by RFLP and amplicons from representative isolates were sequenced.

3.2 – Stages in the development of the multiplex PCR

The initial aim of this study involved the development of a m-PCR to allow the simultaneous detection of the integrase genes *intI1*, *intI2* and *intI3* and an *E. coli*-specific gene (*uspA*). Amplification of an *E. coli*-specific gene was included in the m-PCR to serve as an internal positive control for the PCR reaction and confirm the identification of *E. coli* isolates and the integrity of *E. coli* DNA. The differentiation of *E. coli* from other Gram-negative bacteria by PCR amplification of the nucleotide sequences flanking the universal stress protein A (*uspA*) gene has been described (Chen and Griffiths 1998). Amplification of the *uspA* gene using previously published primer pairs EC1 and EC2 generated an 884 bp product (Chen and Griffiths 1998). M-PCR conditions developed to simultaneously detect the genes encoding class 1 (*intI1*), class 2 (*intI2*) and class 3 (*intI3*) integrons have been previously described (Table 3.1; B. Dillon and J. Iredell, unpublished data). The following primer pairs were used for PCR amplification: Int1F and Int1R (Koeleman *et al.* 2001), Int2.F and Int2.R (Mazel *et al.* 2000) and Int.3F and Int.3R (Mazel *et al.* 2000) (Appendix II). This m-PCR is expected to generate amplification products of 160 bp (*intI1*), 794 bp (*intI2*) and 1023 bp (*intI3*).

Table 3.1. Class 1, 2 and 3 integrase m-PCR conditions described by B. Dillon and J. Iredell.

Step	Temperature	Duration	Cycles
Hot start	94°C	4 mins	1
Denaturation	94°C	1 min	
Annealing	54°C	1 min	35
Extension	72°C	1 min	
Extension	72°C	10 min	1

Forward and reverse primers FD-uspAF and FD-uspAR were developed based on the *uspA* gene sequence (Appendix II). These primers produce an amplicon of 334 bp, which is a suitable size for incorporation into the integron m-PCR. However, the addition of the *uspA* gene detection to the integron m-PCR resulted in the inability to simultaneously detect *intI1*, *intI2* or *intI3* and *uspA*, when using the conditions and primers described above. A temperature gradient (46.7°C to 63.1°C) of the integron/*uspA* m-PCR revealed there was no temperature at which all four PCR products were amplified (Figure 3.1).

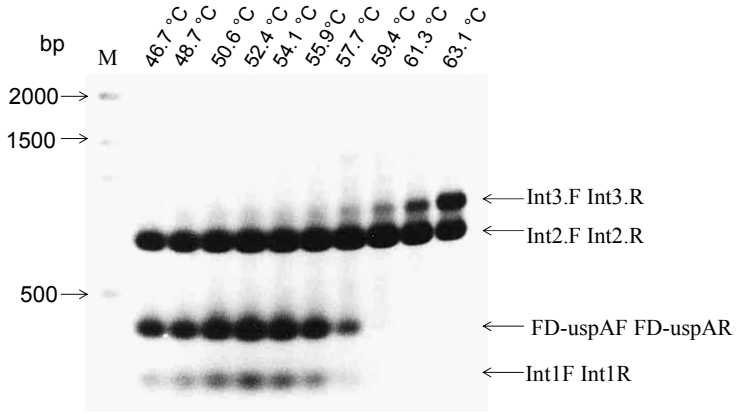

Figure 3.1. Agarose gel electrophoresis of amplification products generated using the integrase/*uspA* m-PCR performed at temperatures varying from 46.7°C to 63.1°C. M-PCR primer pairs and conditions as described by J. Iredell and B. Dillon in Table 3.1. PCR primer pairs are Int1F Int1R, Int2.F Int2.R, Int3.F Int3.R and FD-uspAF FD-uspAR. Lane M, GenRuler 100 bp DNA Ladder Plus. Template DNA controls for m-PCR included DNA extracted from *E. coli* UB1637 (pR388:In*3*), control for class 1 integrase (*intI1*); *E. coli* DH5α (pMAQ612:Tn*7*), control for class 2 integrase (*intI2*); and *E. coli* DH5α (pSMB731), control for class 3 integrase (*intI3*).

3.2.1 – Touchdown PCR

Touchdown PCR involves decreasing the annealing temperature by 1°C every second cycle to a 'touchdown' annealing temperature, which is then used for the final PCR cycles. This technique was performed in an attempt to provide conditions suitable for the amplification of all PCR products for the integrase/*uspA* m-PCR with varying annealing temperatures. The annealing temperatures using touchdown parameters commenced at 65°C for the first 10 cycles, decrementing to 60°C by cycle 15 as previously described (Paton and Paton 1998). These touchdown parameters were not suitable for the integrase/*uspA* m-PCR as the amplification of *intI2* and *intI3* genes was favoured at the expense of the amplification of the *intI1* and *uspA* genes (results not shown).

3.2.2 – Long-range temperature gradient

Long-range temperature gradient PCR was performed using the conditions described and the primers Int1F Int1R, Int2.F Int2.R, Int3.F Int3.R and FD-uspAF FD-uspAR (Table 3.2). These parameters resulted in amplification of all m-PCR products between 54.0°C and 56.7°C and preliminary screening of *E. coli* isolates commenced at 56°C (Figure 3.2).

However, these results were not reproducible and preliminary screening ceased, in order to identify the cause of PCR failure. Possible causes of inconsistent m-PCR results include the use of different brands of *Taq* DNA polymerase or degradation of template DNA. PCR reactions were performed to identify the source of PCR failure. In addition to the problem of inconsistent m-PCR results, the amplification of *intI1* consistently

produced a weak band (160 bp) that was often indistinguishable from un-reacted primer-dimers.

Table 3.2. Cycling parameters for long-range temperature gradient m-PCR.

Step	Temperature	Duration	Cycles
Denaturation	94°C	4 mins	1
Denaturation	94°C	30 sec	
Annealing	Gradient 54.0°C-61.9°C	30 sec	
Extension	72°C	30 sec, incrementing 10 sec/cycle from cycle 15	35
Extension	72°C	5 min	10

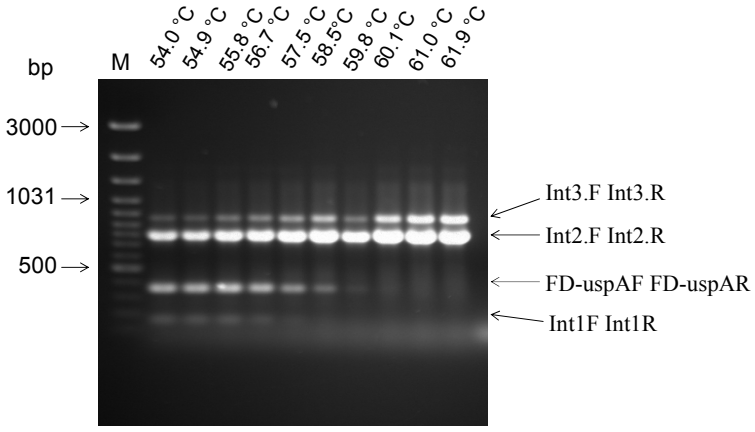

Figure 3.2. Agarose gel electrophoresis of amplification products generated for the integrase/*uspA* m-PCR at temperatures ranging from 54.0°C to 61.9°C using long-range conditions. M-PCR primer pairs are shown. Long-range parameters are described in Table 3.2. Lane M, GenRuler 100 bp DNA Ladder Plus. DNA controls for m-PCR included DNA extracted from *E. coli* UB1637 (pR388:In*3*), control for class 1 integrase (*intI1*); *E. coli* DH5α (pMAQ612:Tn*7*), control for class 2 integrase (*intI2*); and *E. coli* DH5α (pSMB731), control for class 3 integrase (*intI3*).

3.2.3 – Redesigning primers and optimising conditions for multiplex PCR

The inability to produce consistent results using the long-range temperature parameters and weak amplification of the *intI1* gene were paramount in the decision to redesign the

integrase/*uspA* m-PCR. The primers for *intI1* (Int1F and int1R) were replaced by the previously published primer pair L2 and L3, which produced a 295 bp PCR amplicon (Maguire *et al.* 2001). New PCR primers for the *uspA* gene that would generate an amplicon between 400 bp to 600 bp were also required to replace FD-uspAF and FD-uspAR, which generate a 334 bp amplicon. The combination of the *uspA* gene PCR primers EC2 (Chen and Griffiths 1998) and FD-uspAF (this study) produced a suitably sized amplicon of 449 bp. Temperature gradient PCR was performed using the following combination of primers, L2 L3, EC2 FD-uspAF, Int2.F Int2.R, and Int3.F Int3.R to yield amplification products of 295, 449, 749 and 1023 bp respectively. M-PCR parameters used for the integrase/*uspA* PCR were based on conditions previously described for primers Int2.F Int2.R and Int3.F Int3.R (Mazel *et al.* 2000) and L2 L3 (Maguire *et al.* 2001) (Table 3.3). The temperature gradient PCR revealed all multiplex amplicons were produced at annealing temperatures ranging from 60.1°C to 53.0°C (Figure 3.3). These PCR primers and cycling parameters for the integrase/*uspA* m-PCR at the empirically derived annealing temperature of 57°C proved to be reproducible and were used to screen the entire *E. coli* collection of 514 strains.

Table 3.3. Cycling parameters for the temperature gradient integrase/*uspA* m-PCR.

Step	**Temperature**	**Duration**	**Cycles**
Denaturation	94^{o}C	4 min	1
Denaturation	94^{o}C	30 sec	30
Annealing	53^{o}C-62.8^{o}C	1 min	
Extension	72^{o}C	1 min	
Extension	72^{o}C	10 min	1

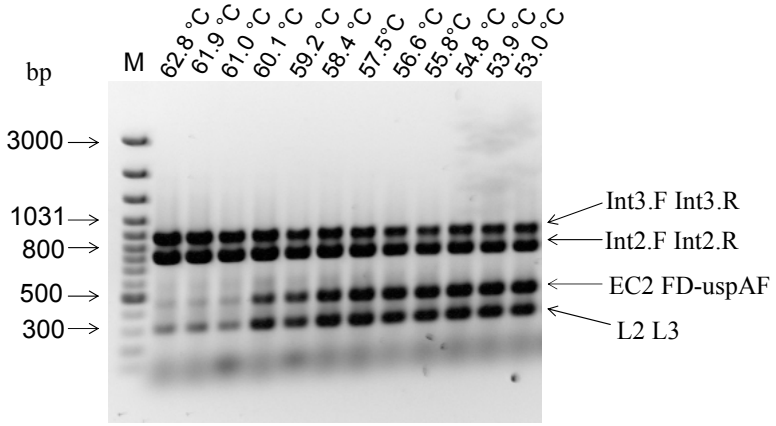

Figure 3.3. Agarose gel electrophoresis of amplification products generated for the integrase/*uspA* m-PCR using cycling parameters described in Table 3.3. PCR primer pairs and temperatures are indicated. Lane M, GenRuler 100 bp DNA Ladder Plus. Template DNA to be used as positive controls were extracted from the following *E. coli* strains: class 1 integrase, UB1637 (pR388:In*3*); class 2 integrase, DH5α (pMAQ612:Tn*7*); and class 3 integrase, DH5α (pSMB731).

3.3 – Multiplex PCR screening results for *intI1, intI2* and *intI3*

A summary of the m-PCR screening results for class 1, 2, and 3 integrase genes is shown in Table 3.4. Of the 304 animal-derived *E. coli* strains, 32 (10.5%) were positive for the *intI1* gene indicating by inference that these strains contained class 1 integrons. Animal-derived *E. coli* strains harbouring the class 1 integrase gene (*intI1*) were from bovine (n=27), canine (n=2) and porcine (n=3) diagnostic specimens. Of the 204 *E. coli* strains sourced from humans, 49 (24.1%) were positive for the *intI1* gene. Human-derived *E. coli* strains that were positive for the *intI1* gene were from the following sources: UTI (n=29), healthy infants (n=8), diarrhoea (n=3), bloody diarrhoea (n=3), suspected diarrhoea (n=1), enteritis (n=1), septicemia (n=2), HUS (n=1) and unknown symptoms (n=1). The *intI2* gene was detected in 7/514 (1.4%) *E. coli* strains indicating by inference the presence of class 2 integrons. Human-derived *E. coli* strains containing the *intI2* gene were isolated from the following sources: UTI (n=3),

diarrhoea (n=3) and a healthy infant (n=1). None of the *E. coli* strains isolated from the Tasmanian environment (n=6), native animals (n=54) or babies that had died from SIDS (n=21) contained class 2 integrase genes. The *intI3* gene was not detected in any of the *E. coli* strains. M-PCR amplicons of representative isolates and positive controls for each integron class are shown in Figure 3.4.

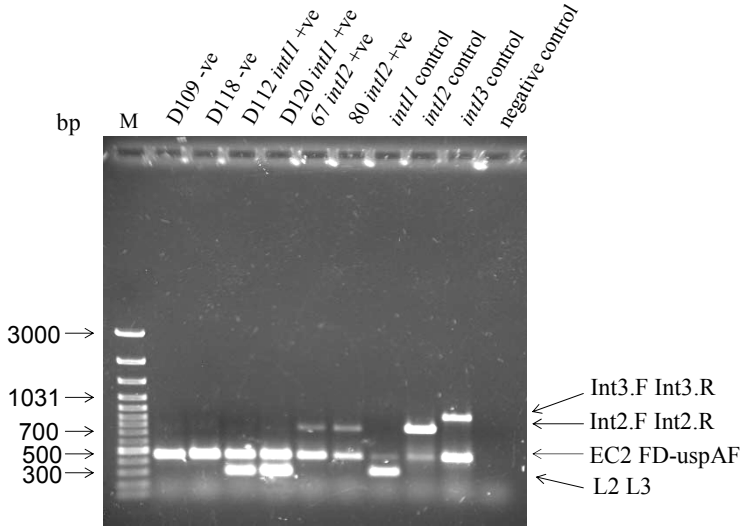

Figure 3.4. Agarose gel electrophoresis of amplicons produced using the integrase/*uspA* m-PCR to screen for *intI1*, *intI2*, *intI3* and *uspA* genes. Lane M, GenRuler 100 bp DNA Ladder Plus. Screening results of representative isolates are shown. Template DNA to be used as positive controls was extracted from the following *E. coli* strains: class 1 integrase *(intI1)*, UB1637 (pR388:In*3*); class 2 integrase *(intI2)*, DH5α (pMAQ612:Tn*7*); and class 3 integrase *(intI3)*, DH5α (pSMB731). The negative control for the m-PCR contained no DNA.

Table 3.4. m-PCR screening of *E. coli* isolates for *intI1*, *intI2*, and *intI3*[A] genes.

Source of *E. coli* strains			**No. of Strains**	**Class 1 integrase**	**Class 2 integrase**
Animal	Diagnostic specimens	Bovine	177	27	0
		Canine	46	2	0
		Porcine	20	3	0
	Healthy	Native animals	54	0	0
		Porcine	4	0	0
		Ovine	1	0	0
	Unknown symptoms	Porcine	1	0	0
		Parrot	1	0	0
Number of animal-derived *E. coli* strains			**304**	**32 (10.5%)**	**0**
Human	Diagnostic specimen	UTI	96	29	3
		SIDS	21	0	0
		Diarrhoea	22	3	2
		Bloody diarrhoea	7	3	0
		Suspected diarrhoea	5	1	1
		Gastroenteritis	4	0	0
		Infantile gastroenteritis	3	0	0
		Enteritis	1	1	0
		Suspected Traveller's diarrhoea/Infantile enteritis	1	0	0
		Septicemia	5	2	0
		HUS	2	1	0
		Suspected HUS	1	0	0
		Neonatal meningitis	1	0	0
		Appendicitis	1	0	0
	Healthy	Infant	24	8	1
		Human	2	0	0
	Symptoms unknown	Human	8	1	0
Number of human-derived *E. coli* strains			**204**	**49 (24.0%)**	**7 (3.4%)**
***E. coli* strains from Tasmanian environment**			**6**	**0**	**0**
Total number of *E. coli* strains			**514**	**81 (15.8%)**	**7 (1.4%)**

[A]The *intI3* gene was not detected in any of the *E. coli* strains.

3.4 – PCR amplification of gene cassettes

The gene cassette regions of class 1 integrase positive strains were amplified with the primer pair L1 and R1 (Lévesque *et al.* 1995) and class 2 integrase positive strains with hep74 and hep51 (White *et al.* 2001) (Table 3.5). Class 1 gene cassette arrays varied in size from approximately 0.75 kb to 1.9 kb. The most common size of gene cassette array amplicon was 1 kb (n=30). One isolate, 04004, harboured a gene cassette array of approximately 1 kb and a second of 1.7 kb. All class 2 gene cassette arrays were approximately 2.2 kb. Gene cassette regions were not amplified using standard PCR cycling conditions from 27 *intI1* positive bovine-derived *E. coli* strains, 6 *intI1* positive human-derived *E. coli* strains and 1 *intI2* positive human-derived *E. coli* strain (Table 3.5).

3.4.1 – Investigation of "non-amplifiable" class 1 gene cassette arrays

It was not possible to amplify the gene cassette arrays in a subset of *E. coli* isolates that were positive for class 1 or 2 integrase genes using standard PCR conditions. The *E. coli* strains in which the gene cassettes could not be amplified involved 40% (33/81) of class 1 and (1/7) class 2 integrons. These *E. coli* strains with "non-amplifiable" gene cassette arrays were isolated from human specimens (*E. coli* strains: 20, 24, 59, 11604, 6877, 6878, and 80) and all 27 bovine diagnostic specimens. The reason for this phenomenon was examined using several strategies including (i) second-step PCR, (ii) core sampling, (iii) Expand long-template PCR system and (iv) the use of primers targeting various regions of class 1 integrons.

Table 3.5. PCR amplification of gene cassette regions.

Approximate size of cassette array amplicon (kb)	No. of isolates	Integron class	Isolate source
0.75	9	1	4 human, UTI 2 healthy infant 1 porcine, diagnostic 2 canine, diagnostic
0.75	2	1	1 human, UTI 1 human, diarrhea
1	30	1	17 human, UTI 2 human, septicemia 1 human, diarrhoea 1 human, bloody diarrhoea 1 human, enteritis 1 human, HUS 6 healthy infants 1 porcine, diagnostic[A]
1	1	1	1 porcine, diagnostic
1.5	2	1	2 human, UTI
1.7	3	1	2 human, diarrhoea 1 human, UTI 1 porcine, diagnostic[A]
1.9	1	1	1 human, UTI
2.2	6	2	3 human, UTI 2 human, diarrhoea 1 healthy infant
Unable to amplify	6	1	3 human, UTI 2 human, bloody diarrhoea 1 human, diarrhea
Unable to amplify	27	1	bovine diagnostic
Unable to amplify	1	2	1 human, diarrhoea

[A]This *E. coli* strain contains two cassette arrays.

The annealing sites of the primer pairs used for PCR investigation of the "non-amplifiable" isolates are shown in Figure 3.5. Refer to Appendix II for cycling conditions and the exact primer positions and corresponding GenBank accession numbers. Inconsistent results and multiple amplification products were produced using second-step PCR, core sampling and the Expand long-template PCR system (results not shown). This was thought to be due to non-specific primer annealing, followed by the high level of amplification using these methods.

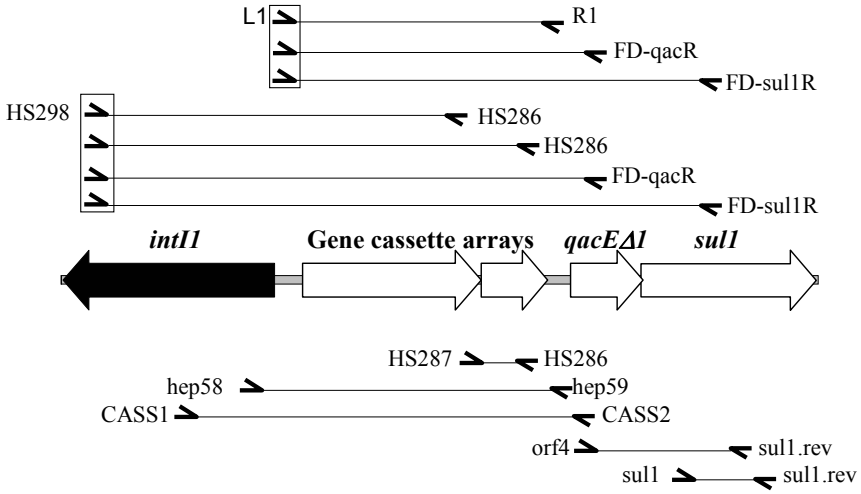

Figure 3.5. The location of primer annealing sites used to investigate "non-amplifiable" gene cassette arrays are indicated by small arrows. Primer pairs are joined by a thin horizontal line. Primers include: HS286 and HS287 (Stokes *et al.* 2001); HS298 (Nield *et al.* 2001); hep58 and hep59 (White *et al.* 2000); orf4, sul1.rev, CASS1 and CASS2 (Rosser and Young 1999); and FD-qacR and FD-sul1R (this study). Arrows indicate the direction of gene transcription. The following features are indicated: class 1 integrase gene, *intI1* (black arrow), gene cassette arrays, *qacEΔ1* and *sul1* genes (unfilled arrows).

Difficulty in amplifying the gene cassette regions should not have been caused by the inability of the forward primer L1 to anneal to template DNA, given successful screening for the integrase gene utilised the primers L2 and L3, with L1 being the reverse complement of L3. The standard gene cassette reverse primer, R1, binds in a region upstream of *qacEΔ1*. Absence of or variation in the 3′-CS of these class 1

integrase positive isolates may explain why the gene cassettes were not amplified, and with this in mind various primer pairs were used to investigate this phenomenon.

All integron-positive isolates including those that proved "non-amplifiable" were screened for the *sul1* gene using the primers sul1 and sul1.rev. None of the "non-amplifiable" isolates were positive for the *sul1* gene. PCR of the "non-amplifiable" isolates using the primer pairs described failed to produce amplicons, which supports the absence, truncation or sequence variation of the 3′-CS, specifically the *sul1* and *qacE*Δ*1* genes.

3.5 – Discussion

A m-PCR was developed and used to screen a collection of 514 *E. coli* strains for the *intI1*, *intI2* and *intI3* genes and by inference detect class 1, 2 and 3 integrons. The *intI1* gene was detected in 10.5% of the animal-derived *E. coli* strains and 24.1% of the human-derived strains. The *intI2* gene was detected in 1.4% of *E. coli* strains, while the *intI3* gene was not detected in any of the *E. coli* strains.

Inability to detect the *intI3* gene in the current study was not surprising, as only four class 3 integrons have been found to date and none of these have been detected in *E. coli* nor were they found in isolates from Australia. Class 3 integrons have been detected in *Serratia marcescensi* (Arakawa et al. 1995) and *Klebsiella pneumoniae* (Correia *et al.* 2003) sourced from patients hospitalised in Japan and Portugal, respectively and in *Delftia* spp. from North American aquatic samples (Xu *et al.* 2007).

The frequency of class 1 and class 2 integrons detected in *E. coli* strains of animal and human origin in this and other studies is given in Table 3.6. A complex interaction of

many factors influence the frequency of integron-carriage reported, which include the criterion for *E. coli* strain selection, the date and country of strain isolation. An increase in the frequency of integron detection would be expected in *E. coli* strains selected on the basis of resistance to antibiotics. Variation in the frequency of integron-carriage would also be expected in *E. coli* isolated from hosts from different countries and dates, which are subjected to diverse antibiotic selection pressures.

The frequency of class 1 integrons in *E. coli* strains sourced from sick cattle (15%), swine (1.5%) and dogs (4.3%) and healthy animals (0%) in this study is lower than previously reported. A wide variation in the frequency of class 1 and 2 integrons was observed in *E. coli* sourced from sick humans. Frequencies varied from 3.7% (USA) to 62% (Europe) for class 1 integrons and from 0.33% (Australia) to 14% (India) for class 2 integrons. Integron carriage in *E. coli* sourced from healthy humans reported in this study (31% for class 1 and 0.33% for class 2) was similar to that for Spain (26% for class 1 and 1% for class 2) (Table 3.6).

To my knowledge, this is the first time that *E. coli* isolated from babies that had died from SIDS have been screened for integron carriage. It was of interest to determine the integron-related antibiotic resistance gene profile in these *E. coli* strains, as infection has been implicated in SIDS. There is evidence that toxins produced by *E. coli* may be involved in the mechanism of death in SIDS babies (Goldwater and Bettelheim 2002; Weber *et al.* 2008). None of the *E. coli* strains collected from babies that had died from SIDS (n=21) contained integrons, which may reflect an absence of antibiotic selective pressures in these young infants.

Table 3.6. The frequency of class 1 and class 2 integrons detected in *E. coli* sourced from animals and humans.

Strain source	Country of *E. coli* isolation	Strain selection criterion	No. of strains positive/total no. of strains (%)			Reference
			Class 1 integron	Class 2 integron	Class 1 and class 2 integrons	
Sick cattle	Australia	Random	27/177 (15)	0/177 (0)	0/177 (0)	This study
Sick cattle	USA	Random	42/56 (75)	13/56 (23)	11/56 (20)	(Goldstein *et al.* 2001)
Sick swine	Australia	Random	3/20 (1.5)	0/20 (0)	0/20 (0)	This study
Sick swine	Canada	ETEC[A]	67/112 (60)	ND[B]	ND	(Maynard *et al.* 2003)
Sick swine	USA	Random	25/29 (86)	0/29 (0)	0/29 (0)	(Goldstein *et al.* 2001)
Sick dogs	Australia	Random	2/46 (4.3)	0/46 (0)	0/46 (0)	This study
Sick dogs and cats	Germany	Random	20/228 (9)	10/228 (4.4)	ND	(Kadlec and Schwarz 2008)
Sick animals[C]	USA	Random	35/193 (18)	ND	ND	(Singh *et al.* 2005)
Sick animals[D]	Switzerland	Random	130/263 (49.4)	ND	ND	(Lanz *et al.* 2003)
Healthy native animals	Australia	Random	0/54 (0)	0/54 (0)	0/54 (0)	This study
Healthy swine	Australia	Random	0/4 (0)	0/4 (0)	0/4 (0)	This study
Healthy swine	Netherlands	MDR[E]	15/21 (71)	ND	ND	(Box *et al.* 2005)
Healthy calves	Netherlands	MDR	35/45 (78)	ND	ND	(Box *et al.* 2005)

Strain source	Country of *E. coli* isolation	Strain selection criterion	No. of strains positive/total no. of strains (%)			Reference
			Class 1 integron	Class 2 integron	Class 1 and class 2 integrons	
Sick human	Australia	DR[F]	40/170 (24)	6/170 (3.5)	ND	This study
Sick humans	USA	Random	3/81 (3.7)	ND	ND	(Singh *et al.* 2005)
Sick humans	Chile	Random	35/128 (27)	10/128 (8)	17/128 (13)	(Reyes *et al.* 2003)
Sick humans, UTI	India	Random	21/58 (36)	8/58 (14)	1/58 (1.7)	(Mathai *et al.* 2004)
Sick humans, UTI	Australia	Random	36/90 (40)	3/90 (0.33)	5/90 (0.55)	(White *et al.* 2001)
Sick children, diarrhoea	Nigeria	CDEC[G]	11/21 (52)	1/21 (4.8)	0/21 (0)	(Okeke *et al.* 2002)
Sick humans[H]	Europe[I]	Random	44/77 (62)	ND	ND	(Martinez-Freijo *et al.* 1998)
Healthy humans	Australia	DR	8/26 (31)	1/26 (0.4)	ND	This study
Healthy humans	Spain	Random	26/100 (26)	1/100 (1)	2/100 (2)	(Vinué *et al.* 2008)

[A]ETEC, Enterotoxigenic *E. coli* with the serotype O149:H91.
[B]ND, not determined.
[C]Sick animals are not specified.
[D]Sick animals include swine, dogs, cats, cows and laying hens.
[E]MDR, multi-drug resistance defined as resistance to 5 or more antibiotics.
[F]DR, defined as drug resistance to at least 1 antibiotic.
[G]CDEC, Cell-detaching *E. coli.*
[H]Sick humans from hospital intensive care and surgical units.
[I]European countries, included in this study are Austria, Belgium, France, Germany, Italy, Poland, Portugal, Spain, and the UK.

There is a paucity of data on integron-carriage in bacteria isolated from native animals with no known previous exposure to antibiotics. Most previous studies have investigated strains isolated from humans exhibiting symptoms of disease, food-producing animals, companion animals or exotic animals. It is of consequence that integrons were not detected in any of the *E. coli* isolates from Australian native animals but were detected in farm animals (cattle and swine) and companion animals (dogs) in this study. Our findings are concordant with another study, which found the prevalence of integrons isolated from *E. coli* in various animal populations correlated with their proximity to humans; integrons were not detected in wild animals with little or no human contact but were detected in farm animals and companion animals (Skurnik *et al.* 2006).

In this study integrons were not detected in *E. coli* isolated from the Tasmanian environment. In contrast, integron gene cassettes were abundant in DNA extracted from soil, sediment, biomass, and water samples collected from natural marine, freshwater, and terrestrial locations in Australia and Antarctica (Stokes *et al.* 2001). Integrons have also been detected from contaminated sites, for example, heavy-metal-contaminated mine tailings in Colorado (class 1) (Nemergut *et al.* 2004) and irrigation water and sediments from the Rio Grande River, Colorado (Class 1 and 2) (Roe *et al.* 2003).

3.6 – Conclusion

The frequency of class 1 and class 2 integrons detected in *E. coli* strains in this study were generally lower than that reported in previous studies. The absence of integrons in *E. coli* sourced from babies that had died from SIDS, native animals, and the Tasmanian environment, may reflect a lack of antibiotic selection pressures in these

sources and underlines the importance of exposure to antibiotics in the acquisition and/or maintenance of integron-related antibiotic resistance genes in *E. coli*.

In 40% of the class 1 integron positive *E. coli* strains and 14% of class 2 integron positive *E. coli* strains the gene cassette regions could not be amplified with standard PCR primers (L1 and R1 for class 1 and hep74 and hep51 for class 2) and cycling conditions. PCR strategies employed to amplify the gene cassette regions in "non-amplifiable" strains including utilising different PCR primers in the integron 3′-CS and varying cycling parameters, have thus far been unsuccessful.

Chapters 4 and *5* will investigate further the phenomenon of "non-amplifiable" gene cassettes. These chapters will also present the characterisation of PCR amplified gene cassette arrays by RFLP and DNA sequencing of gene cassette arrays in a randomly chosen representative *E. coli* strain from each RFLP group. *Chapter 4* will present the characterisation class 1 integron gene cassettes while *Chapter 5* will present the results for class 2 integrons.

CHAPTER 4 – MOLECULAR CHARACTERISATION OF CLASS 1 INTEGRONS: WIDESPREAD DETECTION OF A UNIQUE CLASS 1 INTEGRON-IS*26* ELEMENT IN *ESCHERICHIA COLI* ISOLATED FROM CATTLE AND HUMANS

4.1 – Introduction

The emergence and spread of antibiotic resistance is an escalating global health concern. Multi-drug resistant bacteria are the principal cause of failure in the treatment of infectious diseases, resulting in increases in the term and magnitude of morbidity, higher rates of mortality, and a greater health cost burden (Goldmann 1999). National and international government-commissioned reviews have concluded that the use of antimicrobial agents in food-producing animals leads to the emergence of antibiotic-resistant bacteria and the spread of these bacteria or their resistance genes to humans may occur via the food-chain (JETACAR 1999; WHO 2001). The role of class 1 integrons in the emergence of multi-resistant bacteria via the stockpiling of resistance determinants has been well established (Leverstein-van Hall *et al.* 2003; Naas *et al.* 2001; Tennstedt *et al.* 2003). Monitoring and surveillance of antibiotic resistance genes in the community is essential for developing strategies to minimise the spread of antimicrobial resistance and evaluating their impact.

Class 1 integrons usually contain two CSs, which flank the variable gene cassette region. Essential features of the 5′-CS are the integrase gene (*intI1*) (Collis *et al.* 1993), a promoter region, containing the potential promoter Pc (Lévesque *et al.* 1994) and an integration site (*attI*) (Partridge *et al.* 2000). The 3′-CS of class 1 integrons typically contains the *qacEΔ1* gene (Paulsen *et al.* 1993), the sulfonamide resistance gene, *sul1* (Stokes and Hall 1989; Sundström *et al.* 1988) and an ORF, *orf5*, of unknown function (Stokes and Hall 1989). However, absence of the 3′-CS of class 1 integrons has been described. The integron In16 which is an active transposon, Tn*402* (also known as Tn*5090*) does not contain the 3′-CS typical of most class 1 integrons. In Tn*402* the *tni* module, consisting of the transposition genes, *tniA*, *tniB* and *tniQ* and a resolvase gene *tniR,* is located down-stream from the integron In16 in place of the integron 3′-CS (Liebert *et al.* 1999; Partridge *et al.* 2001).

Integrons from different isolates are highly variable with regards to the type, number and order of gene cassettes harboured (Recchia and Hall 1995). Gene cassettes of resistance integrons have been described that confer resistance to many important classes of antimicrobials including aminoglycosides, β-lactams, cephalosporins, carbapenems, trimethoprim, rifampicin, erythromycin, chloramphenicol and quaternary ammonium compounds (Fluit and Schmitz 1999). Environmental integrons differ from integrons isolated from clinical sources as they do not generally contain gene cassettes encoding antibiotic resistance genes (Holmes *et al.* 2003; Nield *et al.* 2001; Stokes *et al.* 2001). In addition to the characterised gene cassettes, many studies have identified class 1 integrons where the gene cassette regions cannot be amplified by standard PCR methods using primers that target the 5′- and 3′-CS (Gallego and Towner 2001; Maguire *et al.* 2001; Schmitz *et al.* 2001; Thungapathra *et al.* 2002; White *et al.* 2001). In order to gain

reliable antibiotic resistance surveillance data to inform management and prevention strategies, it is imperative to accurately characterise the complete antibiotic resistance gene-pool.

E. coli is a prime candidate species to study antibiotic resistance as it is an enteric commensal with a wide natural distribution and is a common cause of infection in both animals and humans. *E. coli* are routinely exposed to various antibiotic agents allowing for the selection of resistant strains, which may act as a reservoir of antibiotic resistance genes. *E. coli* may act as traffickers of integron-related antibiotic resistance genes, acquiring resistance genes from, or transferring resistance to, pathogenic bacteria in transit through the colon (Salyers *et al.* 2004). In this Chapter, class 1 integron-related antibiotic resistance gene carriage in *E. coli* isolated from animal and human sources was examined. The characterisation of gene cassette carriage in all class 1 integrons, including those in which gene cassettes could not be amplified using standard PCR methods will be presented.

4.2 – Results

4.2.1 – PCR detection of the *intI1* gene

PCR screening for the class 1 integrase gene revealed by inference that 81/514 (15.8%) of the *E. coli* strains contained class 1 integrons. The *intI1* gene was detected in 32/304 (10.5%) of strains sourced from animals that exhibited symptoms of disease, specifically strains from bovine, canine and porcine sources. The *intI1* gene was not detected in any of the 59 *E. coli* strains sourced from Australian native animals. Of the 204 human *E. coli* strains screened, 49 (24.0%) contained the *intI1* gene. The *intI1* gene was detected in strains sourced from both healthy and ill humans, including patients

suffering from UTI, diarrhoea, suspected diarrhoea, bloody diarrhoea, septicemia, enteritis and HUS.

4.2.2 – Characterisation of gene cassette arrays

Gene cassette arrays of class 1 integrons were PCR amplified using primers that target the integron 5- and 3′-CS. RFLP of the amplified gene cassette arrays revealed 7 distinct class 1 RFLP groups. DNA sequence analysis of the gene cassette arrays from a representative from each RFLP class revealed 100% identity to sequences previously deposited in GenBank. RFLP groups identified were designated RFLP A (*dfrA5*, AJ419169), RFLP B (*dfrA7*, EU250577), RFLP C (*aadA1*, AB188267), RFLP D (*aadA2,* DQ238100), RFLP E (*dfrA1/aadA1,* AJ884723), RFLP F (*dfrA17/aadA5*, AY748452) and RFLP G (*dfrA12/orfF/aadA2*, AB297450) (Figure 4.1). The gene cassette arrays *dfrA5*, *aadA1* and *dfrA17/aadA5,* which were detected in *E. coli* strains of different serotypes sourced from both human and animal hosts, provide evidence of integron-related horizontal gene transfer between animals and humans (Table 4.1). Inability to amplify the gene cassette region, using standard PCR conditions occurred in 33/81 (40%) of the isolates that contained the *intI1* gene. These strains were sourced from 27 diagnostic cattle specimens and 6 human patients suffering from UTI (n=3), bloody diarrhoea (n=2) and diarrhoea (n=1).

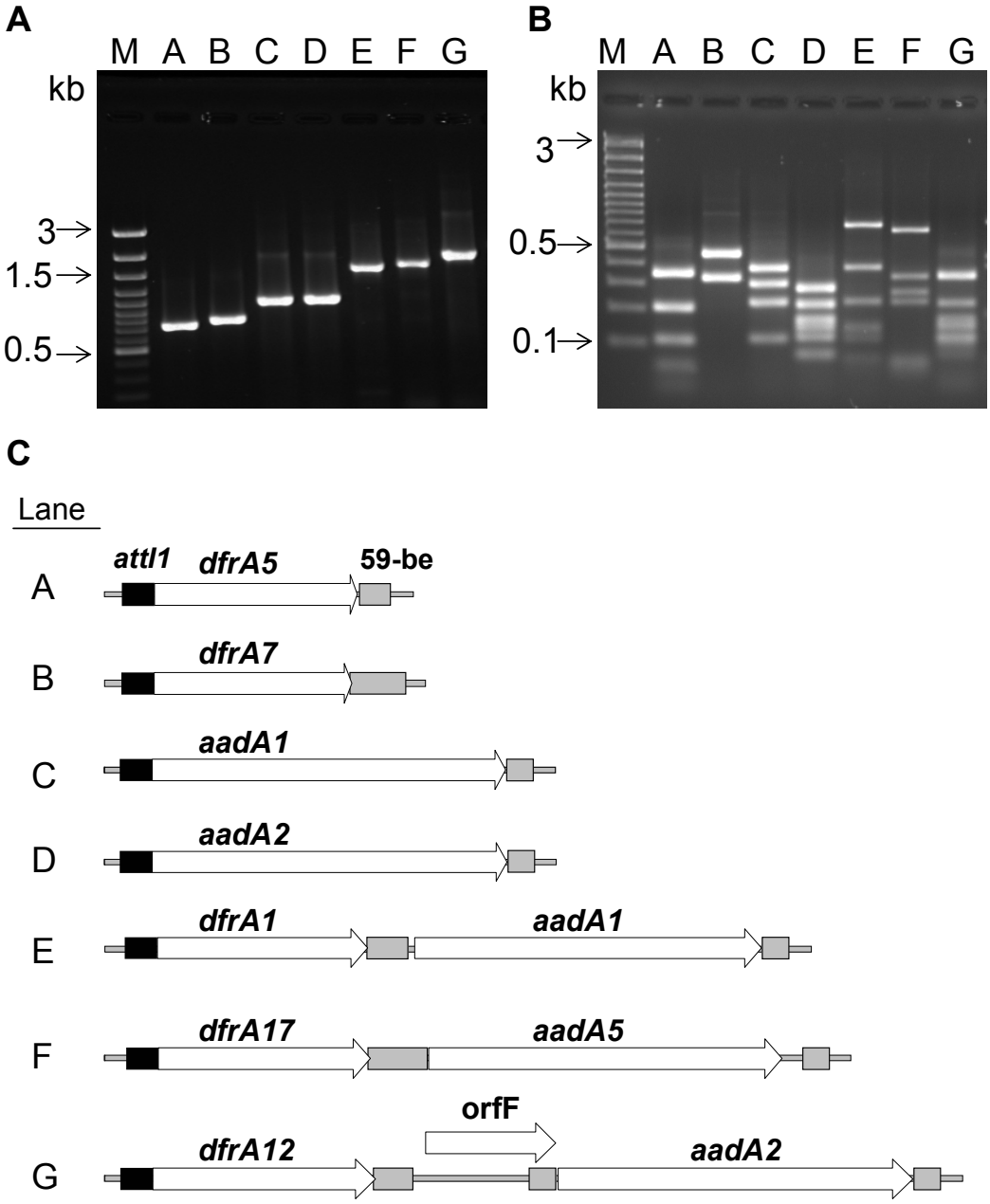

Figure 4.1. RFLP analysis of class 1 integron gene cassette arrays. (**A**) Lanes A-G show the cassette array amplicons produced using the standard PCR primers L1 and R1. The 100 bp DNA ladder is shown in lane M. (**B**) Corresponding gene cassette array amplicon RFLP following digestion with *Alu*I. (**C**) The genetic structure of the gene cassette arrays RFLP A (*dfrA5*); RFLP B (*dfrA7*); RFLP C (*aadA1*); RFLP D (*aadA2*); RFLP E (*dfrA1/aadA1*); RFLP F (*dfrA/aadA5*) and RFLP G (*dfrA12/orfF/aadA2*). The direction of cassette gene transcription is indicated by the arrows. The following features are indicated: cassette genes (unfilled arrows); recombination sites *attI1* (filled black box) and 59-bes, which are part of the gene cassette (grey filled box). All diagrams are drawn to scale.

Table 4.1. Gene cassette arrays detected in class 1 integrons.

RFLP class	Cassette array	Serotype	No. of isolates	Resistance to:											Source
				A	S	T	C	Su	Tm	K	Na	G	Cp	Sp	
A	*dfrA5*	Ont:H4	1	-	-	-	-	+	+	-	-	-	-	-	Human, UTI
A	*dfrA5*	O4:HR	1	+	-	-	-	+	+	-	-	-	-	-	Human, UTI
A	*dfrA5*	O4:H1	1	+	-	+	-	+	+	-	-	-	-	-	Healthy infant
A	*dfrA5*	O6:H1	1	+	*+	+	-	-	+	-	-	-	-	-	Human, UTI
A	*dfrA5*	O20:H-	1	-	-	-	-	+	+	-	-	-	-	-	Human, UTI
A	*dfrA5*	O21:H-	1	+	-	-	-	-	+	-	-	-	-	-	Healthy infant
A	*dfrA5*	O48:H8	1	-	-	+	-	-	[A]-	-	-	-	-	-	Canine, diagnostic specimen
A	*dfrA5*	O141:H4	1	-	*+	+	-	+	+	-	-	-	-	+	Porcine, diagnostic specimen
A	*dfrA5*	[B]O116:H15	1	+	-	-	-	+	+	-	-	-	-	-	Canine, diagnostic specimen
B	*dfrA7*	O17/106:H18	1	+	+	+	+	+	+	-	-	-	-	-	Human, UTI

RFLP class	Cassette array	Serotype	No. of isolates	Resistance to:											Source
				A	S	T	C	Su	Tm	K	Na	G	Cp	Sp	
B	*dfrA7*	O81:H-	1	+	+	+	-	+	+	-	+	-	-	-	Human, diarrhoea
C	*aadA1*	Ont:H1	2	+	*+	-	-	+	-	-	-	-	-	+	Human, UTI
C	*aadA1*	Ont:H1	1	+	[C]-	-	-	+	-	-	-	-	-	[C]-	Healthy infant
C	*aadA1*	Ont:H-	1	+	[C]-	-	-	+	-	-	-	-	-	[C]-	Human, UTI
C	*aadA1*	OR:H1	1	+	*+	-	-	+	-	-	-	-	-	+	Human, UTI
C	*aadA1*	OR:H-	1	+	[C]-	-	-	+	-	-	-	-	-	+	Human, UTI
C	*aadA1*	O2:H7	1	+	*+	-	-	+	-	-	-	-	-	+	Human, UTI
C	*aadA1*	O2:H1/12	1	+	[C]-	-	-	+	-	-	-	-	-	[C]-	Healthy infant
C	*aadA1*	O6:H1	1	+	+	-	-	+	-	-	-	-	-	[C]-	Human, UTI
C	*aadA1*	O6:H1	3	+	*+	-	-	+	-	-	-	-	-	+	Human, UTI
C	*aadA1*	O6:H1	1	+	[C]-	-	-	+	-	-	-	-	-	+	Human, HUS

RFLP class	Cassette array	Serotype	No. of isolates	Resistance to:											Source
				A	S	T	C	Su	Tm	K	Na	G	Cp	Sp	
C	*aadA1*	O6:H-	2	+	*+	-	-	+	-	-	-	-	-	+	Human, UTI
C	*aadA1*	O6:H-	2	+	[C]-	-	-	+	-	-	-	-	-	[C]-	Healthy infant
C	*aadA1*	O25:H1	1	+	*+	-	-	+	-	-	-	-	-	+	Human, UTI
C	*aadA1*	O25:H1	1	+	[C]-	-	-	+	-	-	-	-	-	[C]-	Human, UTI
C	*aadA1*	O25:H1	2	+	[C]-	-	-	+	-	-	-	-	-	[C]-	Healthy infant
C	*aadA1*	O75:H7	1	+	*+	+	-	+	-	-	-	-	-	+	Human, bloody diarrhoea
C	*aadA1*	O75:H7	2	+	*+	-	-	+	-	-	-	-	-	[C]-	Human, septicemia
C	*aadA1*	O75:H7	1	+	[C]-	-	-	+	-	-	-	-	-	[C]-	Human, suspected diarrhoea
C	*aadA1*	O75:H-	1	+	*+	-	-	+	-	-	-	-	-	+	Human, UTI
C	*aadA1*	O128:H-	2	+	*+	-	-	+	-	-	-	-	-	+	Human, UTI
C	*aadA1*	O165:H-	1	+	+	+	+	+	+	-	-	-	-	+	Human, enteritis

RFLP class	Cassette array	Serotype	No. of isolates	Resistance to:											Source
				A	S	T	C	Su	Tm	K	Na	G	Cp	Sp	
D	*aadA2*	Ont:H4	1	-	*+	-	-	+	-	-	-	-	-	+	Porcine, diagnostic specimen
E	*dfrA1/aadA1*	OR:H6	1	+	+	-	-	+	+	-	-	-	-	[C]-	Human, UTI
E	*dfrA1/aadA1*	O25:H-	1	+	[C]-	+	-	+	+	-	-	-	-	[C]-	Human, UTI
F	*dfrA17/aadA5*	O17:H18	1	-	*+	-	+	+	+	-	-	-	-	[C]-	Human, diarrhoea
F	*dfrA17/aadA5*	O45:H-	1	+	+	+	-	+	+	-	-	+	-	[C]-	Human, diarrhoea
F	*dfrA17/aadA5*	O169:H41	1	-	[C]-	+	-	+	+	-	-	-	-	+	Human, UTI
G	*dfrA12*/*orfF*/*aadA2*	O7:H-	1	+	+	-	+	+	+	-	-	+	-	+	Human, UTI
C & F	*aadA1 & dfrA17/aadA5*	O149:H10	1	+	+	+	-	+	+	+	-	-	-	[C]-	Porcine, diagnostic specimen

Abbreviations: A, ampicillin (32 µg /ml); S, streptomycin (25 µg /ml); T, tetracycline (20 µg /ml); C, chloramphenicol (10 µg /ml); Su, sulfathiazole (550 µg /ml); Tm, trimethoprim (50 µg /ml); K, kanamycin (10 µg /ml); Nal, nalidixic acid (50 µg /ml); G, gentamicin (2.5 µg /ml); Cp, ciprofloxacin (2 µg /ml); Sp, spectinomycin (50 µg /ml). Symbols: + resistant; - sensitive; and *moderate level of resistance; defined as significant resistance although not fully resistant. [A]Resistance to trimethoprim was not detected even though this strain harboured a *dfrA5* gene cassette. [B]O116:H15 related serotype. [C]Resistance to spectinomycin and/or streptomycin was not detected even though an *aadA* gene cassette was identified in this strain.

4.2.3 – Sequence of a class 1 integron containing a "non-amplifiable" gene cassette array

A subset of 7 *E. coli* isolates harbouring class 1 integrons containing "non-amplifiable" gene cassette arrays were screened for the presence of the *intI1* gene on a plasmid (results to Southern hybridisation are not shown). The bovine *E. coli* strain D22 contained an integron located on a 22 kb plasmid, which was subjected to DNA sequence analysis. This analysis revealed a class 1 integron which contained the typical 5′-CS and the complete *dfrA5* gene cassette, but lacked the usual class 1 integron-associated 3′-CS due to the insertion of IS*26* (Figure 4.2 and Figure 4.3). IS*26* was found to be inserted beyond the 59-be of the *dfrA5* gene cassette and after the first 24 bp of the class 1 integron 3′-CS (Figure 4.3). The Tn*21* transposition genes *tnpM, tnpR* and *tnpA* and the entry exclusion protein genes *exc1* and *exc2* were located downstream of *intI1*. The insertion element IS*1* transposase genes *insA* and *insB* and the kanamycin resistance gene *aphA1* were located upstream of the IS*26 tnpA* gene. Nucleotide sequence of the class 1 integron and flanking regions of the bovine-derived plasmid is deposited in GenBank (no. EU914098).

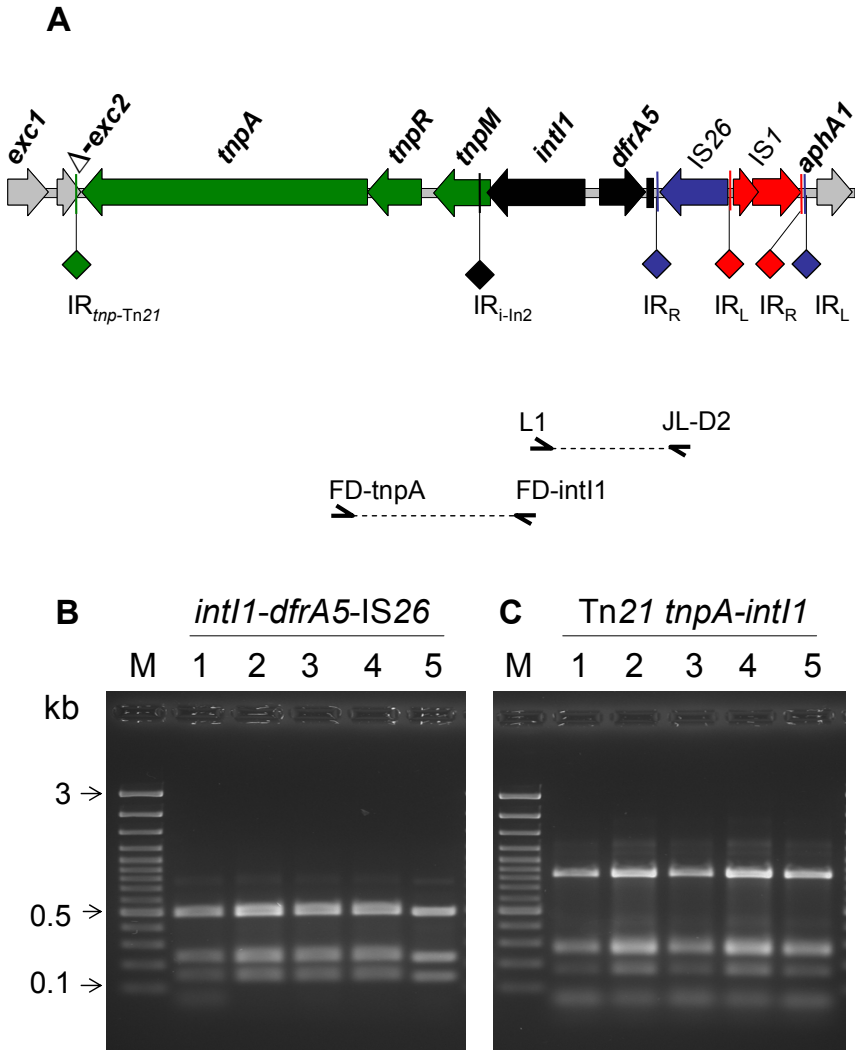

Figure 4.2. (**A**) Genetic structure of an atypical class 1 integron located on a plasmid isolated from the bovine-derived *E. coli* strain, D22 (GenBank no. EU914098). The genetic map represents 8756 bp of nucleotide sequence. The class 1 integron, In2, located on a Tn*21*-like transposon is shown. Features shown include the integrase gene, *intI1* and the *dfrA5* cassette gene (black arrows), the 59-be (black box) and the 25 bp terminal imperfect, IRi located at the 5' end of the integron ($IR_{i\text{-}In2}$). Entry exclusion protein genes *exc1* and *exc2* (grey arrows; Δ- denotes gene truncation); the kanamycin resistance gene, *aphA1* (grey arrow; partial sequence); and the Tn*21* transposition genes *tnpM*, *tnpR* and *tnpA* (green arrows). The IS*26* transposase gene, *tnpA* (blue arrow) and IS*1* genes *insA* and *insB* (red arrows) are indicated. Colour coded diamonds indicate the position of the following inverted repeats (IRs): 38 bp terminal imperfect inverted repeat of Tn*21* ($IR_{tnp\text{-}Tn21}$); 25 bp terminal imperfect IRi of integron In2 ($IR_{i\text{-}In2}$) and the left (IR_L) and right (IR_R) inverted repeats of IS*26* and IS*1*. The position of PCR primers used to screen all the strains for this atypical class 1 integron is shown. RFLP analysis of PCR amplicons from representative bovine-derived *E. coli* strains (D55, D79, D81, D111 and D22) include the following (**B**) integrase-IS*26*-*dfrA5* amplicons digested with the restriction enzyme *Rsa*I and (**C**) *tnpA*-integrase amplicons digested with the restriction enzyme *Eco*RI.

Figure 4.3. DNA sequence of the class 1 integron-*dfrA5*-IS*26* element. The 848 bp PCR amplicon was produced using the primer pair L1 and JL-D2. The 3′-CS of this element has been truncated by the insertion of IS*26*. The following features are indicated 5′-CS (boxed); *attI1* (bold font with grey background) (Partridge *et al.* 2000); *dfrA5* gene cassette array (green); 59-be, which includes the last 6 nt (GGTTAA) of the *dfrA5* gene cassette (bold boxed); truncated 3′-CS (red, boxed); IS26 right terminal inverted repeat (IR_R; bold, blue, boxed) (Chandler and Mahillon 2002) and IS*26* (blue). Arrows indicated the direction of genes. Start and stop codons are shown in bold underlined and stop codons are additionally indicated by (*).

4.2.4 – Distribution of class 1 integron-*dfrA5*-IS*26* elements and Tn*21* transposition genes

Identification of the unique class 1 integron-IS*26* element in the bovine strain D22 prompted the PCR interrogation of all strains with "non-amplifiable" gene cassettes in class 1 integrons using primers that target *intI1* and IS*26*. The integron-IS*26* element was detected using primers L1 and JL-D2. A single 0.8 kb amplification product was detected in 27/27 bovine isolates and 4/6 human isolates. Subsequent RFLP analysis using *Rsa*I revealed the integron-*dfrA5*-IS*26* element in each instance (RFLP H; Table 4.2 and Figure 4.2B). Nucleotide sequencing and ClustalW alignment of the 848 bp amplicons in 2 human-derived and 4 bovine-derived *E. coli* strains confirmed that in each instance IS*26* was inserted in an identical position as that described for *E. coli* strain D22 which was beyond the 59-be of the *dfrA5* gene cassette and after the first 24 bp of the class 1 integron 3′-CS. The distribution of the integron-*dfrA5*-IS*26* element was widespread with regard to geographical and temporal location (Table 4.2). The integron-*dfrA5*-IS*26* element was detected in *E. coli* strains sourced from cattle located at 9 NSW properties separated by up to 900 km from 2002 to 2003. The integron-*dfrA5*-IS*26* element was detected in *E. coli* collected between 1998 and 2003 from human hosts exhibiting symptoms of disease. Tn*21* transposition genes linked to *intI1* were detected using the primers FD-tnpA and FD-intI1. A single 1.5 kb PCR amplification product was obtained from 18/27 bovine isolates and 2/6 human isolates (Table 4.2). RFLP analysis indicated these PCR amplification products were identical and nucleotide sequencing of the 1490 bp amplicon identified the integrase gene, *intI1* and the Tn*21* transposition genes, *tnpM, tnpR* and *tnpA* (Figure 4.2C).

Table 4.2. Structure of atypical class 1 integrons.

RFLP class	Integron-IS*26* element	Tn*21*; *tnpM, tnpR, & tnpA*	Serotype	No. isolates	Resistant to:											Year	Location	Source
					A	S	T	C	Su	Tm	K	Na	G	Cp	Sp			
H	*dfrA5*-IS*26*	+	Ont:H9	1	+	+	+	-	+	+	+	-	-	-	-	2002	Kameruka-Property 1	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	+	Ont:H32	1	+	+	+	-	+	+	+	-	-	-	-	2003	Cowra-Property 2	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	-	Ont:H32	1	+	-	-	-	-	+	-	-	-	-	-	1999	MDU, Melbourne	Human, UTI
H	*dfrA5*-IS*26*	-	O11:H-	1	-	-	-	-	-	+	-	-	-	-	-	1999	MDU, Melbourne	Human, UTI
H	*dfrA5*-IS*26*[A]	+	O26:H11	1	+	+	+	-	+	+	+	-	-	-	-	2002	Eden - Property 3	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	+	O26:H-	2	+	+	+	-	+	+	+	-	-	-	-	1998	MDU, Melbourne	Human, bloody diarrhoea
H	*dfrA5*-IS*26*	-	O111:H11	1	+	+	+	-	+	[B]-	-	-	-	-	-	2002	Dungog-Property 4	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	-	O111:H11	1	+	+	+	-	+	+	+	-	-	-	-	2002	Cowra-Property 2	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	-	O111:H11	1	+	+	+	-	+	+	+	-	-	-	-	2002	Finley-Property 5	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	-	O111:H11	1	+	+	-	-	+	+	+	-	-	-	-	2003	Finley-Property 5	Bovine, diagnostic sample

RFLP class	Integron-IS*26* element	Tn*21*; *tnpM, tnpR, & tnpA*	Serotype	No. isolates	Resistant to:											Year	Location	Source
					A	S	T	C	Su	Tm	K	Na	G	Cp	Sp			
H	*dfrA5*-IS*26*	+	O111:H11	1	+	+	-	-	+	+	+	-	-	-	-	2003	Finley-Property 5	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	+	O111:H-	2	+	+	+	-	+	+	+	-	-	-	-	2002	Gerringong-Property 6	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	+	O111:H-	3	+	+	+	-	+	+	+	-	-	-	-	2002	Kameruka-Property 1	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	+	O111:H-	4	+	+	+	-	+	+	+	-	-	-	-	2003	Kameruka-Property 1	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	-	O111:H-	2	+	*+	+	-	+	+	+	-	-	-	-	2002	Cowra-Property 2	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	-	O111:H-	1	+	*+	-	-	+	+	+	-	-	-	-	2002	Cowra-Property 2	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	+	O123:H11	2	+	+	+	-	+	+	+	-	-	-	-	2002	Bega-Property 7	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	+	O123:H11	1	+	+	+	-	+	+	+	-	-	-	-	2002	Canowindra-Property 8	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	-	O162:H9	1	+	+	+	-	+	+	+	-	-	-	-	2003	Kameruka-Property 1	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	+	O177:H11	1	-	+	+	-	+	+	+	-	-	-	-	2002	Kameruka-Property 1	Bovine, diagnostic sample
H	*dfrA5*-IS*26*	-	O177:H11	1	-	+	+	-	+	+	+	-	-	-	-	2003	Kameruka-Property 1	Bovine, diagnostic sample

RFLP class	Integron-IS*26* element	Tn*21*; *tnpM, tnpR, & tnpA*	Serotype	No. isolates	Resistant to:											Year	Location	Source
					A	S	T	C	Su	Tm	K	Na	G	Cp	Sp			
H	*dfrA5*-IS*26*	+	O180:H-	1	+	+	+	-	+	+	+	-	-	-	-	2003	Richmond-Property 9	Bovine, diagnostic sample
H & F	*dfrA5*-IS*26*[C] *dfrA17/aadA5*[D]	[E]+	O25:H-	1	+	+	+	+	+	+	-	-	-	-	[F]-	2003	MDU, Melbourne	Human, symptoms unknown
I	*dfrA15*-IS*26*[G]	-	OR:H-	1	-	-	+	-	+	+	-	+	-	-	-	1999	MDU, Melbourne	Human, UTI

Abbreviations as outlined in Table 4.1. Strain identification, GenBank accession numbers and Figures showing the genetic structure of atypical integrons is given: [A]strain D22, EU914098, Figure 4.2 A; [C]*E. coli* strain 59, EU914100 and [D]*E. coli* strain 59, EU914101, Figure 4.4 B; and [G]strain 24, EU914099, Figure 4.4 A. [E]An atypical Tn*21* element identified in strain 59 with IS*26* inserted into *tnpR* (accession number EU914102, Figure 4.4 C). [B]Resistance to trimethoprim was not detected even though this strain harboured a *dfrA5* gene cassette. [F]Resistance to spectinomycin and/or streptomycin was not detected even though an *aadA5* gene cassette was identified in this strain.

4.2.5 – Analysis of other class 1 integrons containing "non-amplifiable" gene cassette arrays

The two *E. coli* isolates sourced from human hosts (strain 24 and strain 59) were further examined as they harboured different arrangements to the class 1 integron-IS*26* elements described above. Strain 24, which was negative for the initial integron-IS*26* screening, was examined for the presence of IS*26* in the reverse orientation compared to the elements described above (Figure 4.2) using the primer pair JL-D7 and L1. Successful PCR amplification indicated IS*26* was inserted in the reverse direction. Nucleotide sequencing of the resulting 1488 bp amplicon revealed the presence of an integron-*dfrA15*-IS*26* element (accession EU914099; RFLP I, Figure 4.4A). Two atypical integrons *intI1-dfrA5*-IS*26* (accession EU914100; Figure 4.2) and *intI1-dfrA17-aadA5-qacEΔ1-sul1-orf5-chrA*-IS*26* (accession EU914101; Figure 4.4B) were detected in *E. coli* strain 59. This strain contained a second copy of IS*26* inserted into the Tn*21* resolvase gene, *tnpR*. This was revealed by PCR, using the primers FD-tnpA and FD-intI1, and subsequent sequencing of the resulting 2318 bp amplicon (accession EU914102; Figure 4.4C).

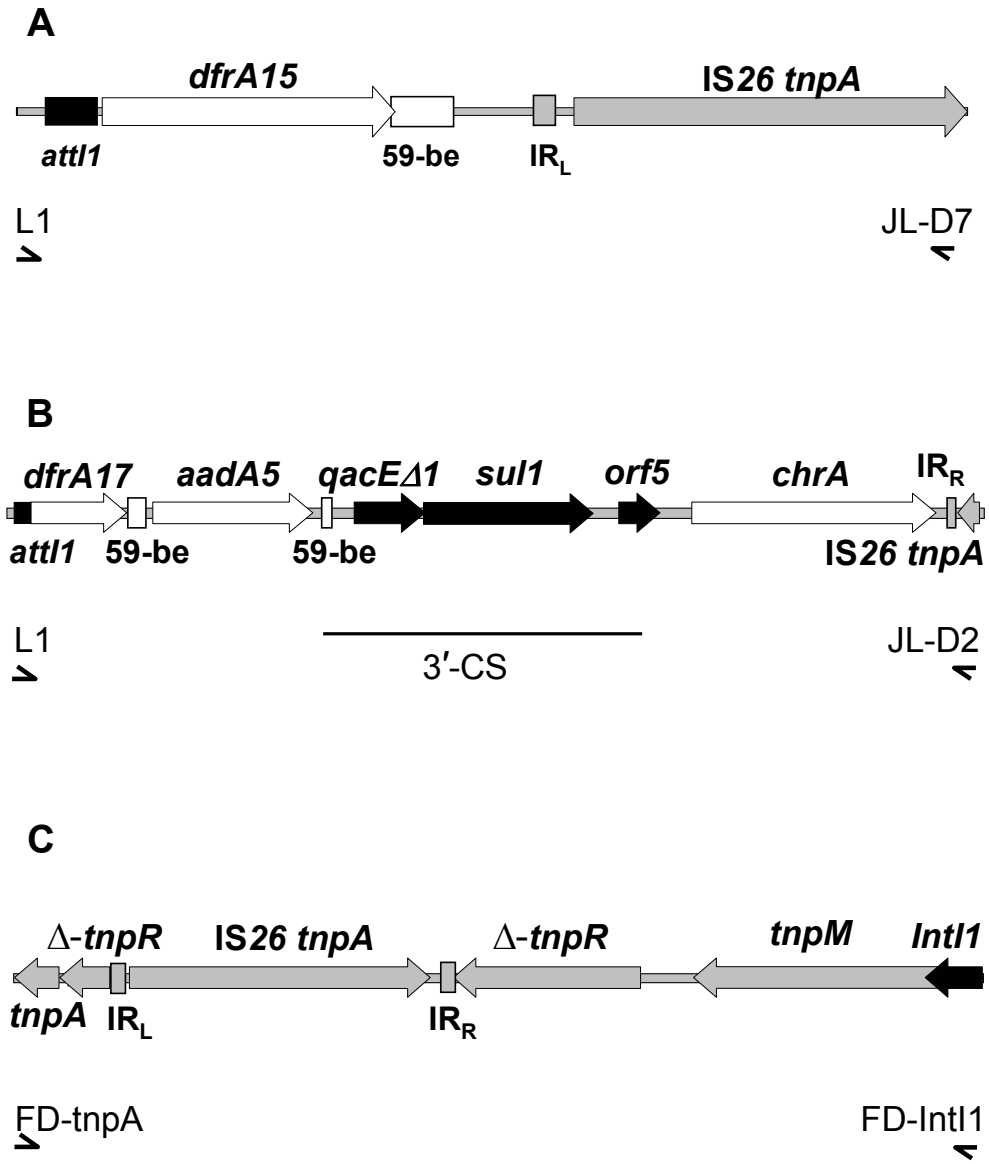

Figure 4.4. Genetic structure of atypical class 1 integrons. PCR primer binding sites, the recombination site *attI1* (filled black boxes), 59-be (open boxes) and the right (IR_R) and left (IR_L) terminal inverted repeats of IS*26* (grey filled boxes) are indicated. **(A)** 1488 bp PCR amplicon from strain 24 showing IS*26* inserted in the reverse orientation to other *E. coli* strains that contain integron-IS*26* elements (GenBank no. EU914099). The *dfrA15* cassette gene (open arrow) and IS*26 tnpA* gene (grey arrow) are shown. **(B)** 4828 bp PCR amplicon from *E. coli* strain 59 showing the *dfrA17* and *aadA5* cassette genes (open arrows); the chromate transport protein gene, *chrA* (open arrow) and the classical class 1 integron 3′-CS consisting of the *qacEΔ1* and *sul1* genes and *orf5* (black arrows) (GenBank no. EU914101). **(C)** 2318 bp PCR amplicon from *E. coli* strain 59 showing IS*26* inserted into the Tn*21* resolvase gene, *tnpR* (truncated or partially deleted *tnpR* gene is indicated by Δ-*tnpR*) (GenBank no. EU914102).

4.2.6 – Serotypes and resistance phenotypes

Class 1 integron gene cassette arrays detected in this study and their corresponding strain serotypes, resistance profiles and the source of strains are summarised in Table 4.1 and Table 4.2. Antibiotic susceptibility testing showed resistance phenotypes generally correlated with the antibiotic resistance conferred by the gene cassettes identified, although some discrepancies between the antimicrobial resistance genes detected and susceptibility patterns were evident. The *E. coli* serotypes were variable within each distinct cassette array class, indicating that horizontal transfer of integrons had occurred. Two *E. coli* strains had the serotypes O4:H1 and O25:H-, which are known to be associated with extra-intestinal infections (Johnson and Russo 2002). These strains with different serotypes harboured cassette arrays that were identified in both human and animal hosts, provide evidence of integron-associated resistance gene transfer. One *E. coli* strain isolated from a healthy infant with the serotype O4:H1 carried the *dfrA5* gene cassette; this cassette was also detected in *E. coli* strains from animal hosts exhibiting symptoms of disease (Table 4.1). A second *E. coli* strain isolated from a human host with the serotype O25:H- carried an integron-*dfrA5*-IS*26* element, which was also found in *E. coli* sourced from cattle (Table 4.1). Multiple strains which have identical cassettes arrays, serotypes and resistance profiles are suggestive of the clonal spread of integron-containing *E. coli* between hosts (Table 4.1 and 4.2). The most prominent example of this phenomenon is the 9 strains of O111:H- harbouring the *dfrA5*-IS*26* element, which were isolated between 2002 and 2003 and geographically separated by up to 319 km (Table 4.2).

4.3 – Discussion

In this study we have characterised antibiotic resistance gene cassettes in class 1 integrons, which could not be amplified using standard PCR primers, by using primer pairs that target the *intI1* gene and IS*26*. The amplification of these gene cassettes has allowed the identification of a unique integron-*dfrA5*-IS*26* element, which is identical in *E. coli* strains isolated from both animal and human sources. The location of IS*26,* beyond the 59-be of the *dfrA5* gene cassette and after the first 24 bp of the class 1 integron 3′-CS, is identical in each strain. The exact IS*26* insertion site was confirmed by sequence analysis in 6 of the strains and RFLP analysis for the remainder of the strains. The widespread distribution of the integron-*dfrA5*-IS*26* element described in this study is unlikely to be due to IS*26* insertion into a "hot spot" in the integron 3′-CS, as no marked target site specificity has been previously observed for IS*26* (Mollet *et al.* 1985). It is improbable that this structure is the result of separate IS*26* insertion events targeting exactly the same position in the integron on multiple occasions and is more likely that the *dfrA5*-IS*26* element has arisen only once and then spread to other *E. coli* strains.

Variation in *E. coli* serotypes containing the integron-*dfrA5*-IS*26* element supports the contention that horizontal transfer of this element has occurred. Identification of this novel integron-*dfrA5*-IS*26* element in *E. coli* with different serotypes sourced from animals and humans suggests that the horizontal transfer of integron-related antibiotic resistance genes has occurred between these hosts. The detection of this integron-*dfrA5*-IS*26* element in *Salmonella enterica* serovar Typhimurium (R. S. Levings, J. Liu, D. Lightfoot, M. J. Walker, R. M. Hall, and S. P. Djordjevic, unpublished data) showing an identical insertion point of IS*26* as that described in

E. coli suggests that inter-species horizontal transfer of this element has occurred. This novel integron-*dfrA5*-IS*26* element, which was widespread in *E. coli* isolates of bovine origin and also found in *E. coli* of human origin, may act as a conduit for the transfer of integron-related resistance genes to human pathogens. Epidemiological evidence is needed to confirm the horizontal transfer of the integron-*dfrA5*-IS*26* element in *E. coli* between animal and human hosts. Further studies could also be performed to determine if the unit of horizontal transfer of the integron-*dfrA5*-IS*26* element is via a conjugative transposon or plasmid.

IS*26* has been implicated in the dissemination of resistance genes and is widely distributed among plasmids (Lee *et al.* 1990; Miriagou *et al.* 2005; Naas *et al.* 2001). Many composite transposons flanked by IS*26* have been described including Tn*5091* and Tn*2000,* which harbours the integron In53 (Naas *et al.* 2001; Sundström *et al.* 1995). IS*26* is commonly associated with integrons and has been found inserted into the 3′ end of *intI1* in the integrons In-t3, In-111 and In-e541 (Miriagou *et al.* 2005) and the 5′ end of *intI1* (Segal *et al.* 2003).

Investigation of the significance and mode of integron-associated antibiotic resistance gene transfer in *E. coli* isolated from animal and human hosts in Australia has not previously been performed. This study suggests integron-related horizontal gene transfer of the gene cassettes *dfrA5*, *aadA1*, and *dfrA17/aadA5* and the *dfrA5*-IS*26* element have occurred, as they were detected in both human and animal *E. coli* strains of different serotypes. Inference of integron-related resistance gene transfer in *E. coli* of different serotypes sourced from humans and animals underlies the importance of developing surveillance systems and research to further define food-chain, veterinary

and environmental factors involved in the spread of antibiotic-resistant bacteria to humans. If a causal link is found between antibiotic use in animals and resistance in humans it will provide impetus to limit antibiotic use in animal husbandry in order to preserve the efficacy of antibiotics important to human health.

Only 7 different gene cassette arrays of *dfrA5, dfrA7, aadA1, aadA2, dfrA1/aadA1, dfrA17/aadA5, dfrA12/orfF/aadA2,* and 2 atypical integrons *dfrA5*-IS*26* and *dfrA15*-IS*26* were detected in this study, despite being examined from a variable strain set. The *E. coli* strains were sourced from healthy and ill animals (Australian native animals, cattle and swine), humans (healthy infants and humans showing symptoms of various diseases) and from the Tasmanian environment. The predominance of *dfrA* or *aadA* gene cassettes reflects the stability and persistence of class 1 integron-related antibiotic resistance genes. This finding, in conjunction with the absence of gene cassette rearrangements, is suggestive of the entire integron structure being transferred rather than the mobilisation and integration of individual gene cassettes. Our data is in agreement with reports worldwide that have detected a predominance of *dfrA* and *aadA* gene cassettes in *Enterobacteriaceae* isolated from animal and human hosts (Antunes *et al.* 2006; Cocchi *et al.* 2007; Martinez-Freijo *et al.* 1999; Nogrady *et al.* 2007; Srinivasan *et al.* 2007).

The two families of gene cassettes identified, *dfrA* and *aadA,* confer resistance to trimethoprim, and spectinomycin and streptomycin, respectively. The high prevalence of *dfrA* gene cassettes correlates with the high use of trimethoprim as first line therapy for UTI in humans (EAGAR 2006), and in the treatment of various infections in food-producing animals (APVMA 2008). The high incidence of class 1 integrons

harbouring *aadA* gene cassettes in *E. coli* isolated from humans cannot be explained as a direct response to selective pressure, as spectinomycin and streptomycin are rarely used as therapeutic agents in humans (EAGAR 2006). Selective pressure for the *aadA* genes still exists in bacteria isolated from food-producing animals, as even though streptomycin has been banned from use in animal husbandry since 1999, spectinomycin is still used to treat animal infections (APVMA 2008; NRA 1999).

Possible explanations for the high incidence of the *aadA* gene cassettes in *E. coli* strains sourced from humans include genetic stability due to co-selection or the horizontal transfer of resistance genes. Co-selection for other resistance genes within class 1 integrons such as *sul1* or *dfrA* may have occurred. The likelihood of co-selection is increased by the use of trimethoprim and sulfonamide combination therapy in animals and humans, which is given for their synergistic effect (APVMA 2008; Huovinen *et al.* 1995). Alternatively, the high prevalence of the *aadA* gene cassettes detected in *E. coli* strains isolated from human sources may be due to the administration of streptomycin to food-producing animals and the subsequent class 1 integron-associated horizontal gene transfer to bacteria in human hosts.

In this study, the resistance profiles of some *E. coli* strains do not correlate with the antibiotic resistance gene cassettes detected in the integrons. The level of expression of integron-borne gene cassettes is variable and dependent on several factors including promoter strength (Lévesque *et al.* 1994; Papagiannitsis *et al.* 2009), the position of the gene cassette in relation to the promoter (Collis and Hall 1995) and whether the integron is located on the chromosome or on a high/low copy number plasmid. A trimethoprim-susceptible *E. coli* isolate harbouring the *dfrA5* gene cassette and (13/37)

streptomycin-susceptible and (17/37) spectinomycin-susceptible *E. coli* isolates harbouring an *aadA* gene cassette were detected. Low-level streptomycin resistance conferred by the *aadA* gene cassette in *E. coli* was previously observed at MIC_{50} 16 μg/ml and as low as 8 μg/ml and 4 μg/ml in some *E. coli* strains. Low-level streptomycin resistance has been previously recognised as an obstacle to classifying *E. coli* as susceptible or resistant to streptomycin (Sunde and Norström 2005). The low-level of resistance conferred by the integron-borne *aadA* gene cassette is below the detection of the epidemiological breakpoints for streptomycin used for this study (25 μg /ml), as outlined by the Clinical and Laboratory Standards Institute. Three of the *E. coli* strains harbouring the classical class 1 integron were susceptible to sulfonamide suggesting they lacked the *sul1* gene in the integron 3′-CS, a variation commonly in encountered class 1 integrons (Sunde 2005).

Information derived from antibiotic resistance surveillance studies is crucial when assessing the risks to public health and determining the impact of regulatory guidelines concerning the responsible use of antibiotics in human and veterinary medicine. Advances outlined in this study involving the application of primer pairs that target IS*26* and class 1 integron CSs could be used to identify previously uncharacterised resistance genes and improve the accuracy of antibiotic resistance surveillance data.

4.4 – Conclusion

A largely undiscovered pool of resistance genes exists in integrons, where the gene cassette regions can not be characterised using standard PCR methods. This study characterised all gene cassettes using a two step process. Initially, PCR amplification was performed using primers that target class 1 integron conserved regions. If this was

unsuccessful a second PCR was performed using an IS*26* primer. A unique class 1 integron, with most of the 3′-CS deleted by the insertion of IS*26,* was detected in 31/514 *E. coli* strains isolated from cattle and humans. The novel integron-*dfrA5*-IS*26* element which was widespread in *E. coli* isolates of bovine origin and also found in *E. coli* of human origin, may act as a conduit for the transfer of integron-related resistance genes to human pathogens. The utilisation of primers that target IS*26* in combination with integron specific primers in PCR screening studies will allow a thorough characterisation of the antibiotic resistance gene pool. Improvements in detection of integron-associated antibiotic resistance genes outlined in this study will facilitate the generation of more accurate surveillance data to inform regulatory bodies of potential risks to human health.

CHAPTER 5 – MOLECULAR CHARACTERISATION OF CLASS 2 INTEGRONS ISOLATED FROM *ESCHERICHIA COLI*

5.1 – Introduction

The classification system for integrons has been described based primarily on the phylogenetic relationships between integrases. Class 2 integrons are a unique class of integron as they contain an integrase gene (*intI2*) with a premature in-frame stop codon, which is likely to encode a defective IntI2 protein (Hansson *et al.* 2002). Recently, an *intI2* gene was identified in pathogenic *E. coli* strains in which the stop codon was replaced with a glutamine codon, encoding a functional class 2 integrase (Márquez *et al.* 2008).

Most class 2 integron screening studies have revealed the Tn*7*-type cassette array of *dfrA1-sat2-aadA1,* which confers resistance to trimethoprim, streptothricin, and streptomycin and spectinomycin respectively (Barlow *et al.* 2004; DeLappe *et al.* 2003; DeLappe *et al.* 2005; Jones *et al.* 2003; Miko *et al.* 2003; Sáenz *et al.* 2004; White *et al.* 2001; Yu *et al.* 2003). Class 2 integrons are typically located at a unique site near the left end of the non-replicative transposon Tn*7* or related transposons including Tn*1825,* Tn*1826* and Tn*4132*, which provide a means for their mobilisation (Sundström *et al.* 1991; Young *et al.* 1994).

Gene cassette variability in class 2 integrons must involve other mobile elements or integrases as this class of integron expresses an inactive integrase. In addition to the classical Tn*7*-like gene cassette array *dfrA1-sat2-aadA1*, class 2 integrons have been identified with variation in the gene cassettes they harbour. Gene cassettes conferring resistance to the following antibiotics have been identified in class 2 integrons: erythromycin (*ereA*) (Biskri and Mazel 2003); chloramphenicol (*catB2)*, gentamicin, tobramycin and kanamycin (*aadB*) (Ramírez *et al.* 2005); and trimethoprim (*dfrA14* alternatively known as *dfrA1b*) (Young *et al.* 1994).

Tn*7*-like transposons that show class 2 integron gene cassette diversity include Tn*4132,* which contains the *dfrA14* gene cassette (Young *et al.* 1994); Tn*1825*, which contains *sat1,* an *estX-sat2* fusion (Partridge and Hall 2005) and no *dfrA1* gene cassette; and Tn*1826*, with a *sat2-aadA1* cassette array (Tietze *et al.* 1987). The class 2 integron Tn*7*::IS*1ereA*, which contains the gene cassette *ereA,* the insertion sequence IS*1* was found in close proximity to the genes *intI2* and *sat2* (Biskri and Mazel 2003) (GenBank no. AY183453). Another class 2 integron Tn*7*::In2-8, which contains a duplication of the *sat2* cassette and the gene cassettes *aadB* and *catB2* (Δ*attC*), is embedded in a Tn*7*-like transposon. It was proposed that the acquisition of novel resistance gene cassettes in Tn*7*::In2-8 occurred via an illegitimate integrase intermolecular recombination event (Ramírez *et al.* 2005). Similarly, variations in class 2 integron gene cassette carriage described above may have arisen via intermolecular recombination events catalysed by illegitimate integrases (Hansson *et al.* 2002; Ramírez *et al.* 2005).

Two distinct Tn*7* target-site selection pathways work in unison to ensure the efficient dissemination of Tn*7* between diverse bacterial hosts and to minimise the negative effects of Tn*7* insertion. Tn*7* encodes five transposition proteins including the core proteins TnsA, TnsB and TnsC and target site selecting proteins TnsD or TnsE (Rogers *et al.* 1986; Waddell and Craig 1988). TnsD-directed transposition involves Tn*7* recognition and insertion at a unique site in the *E. coli* chromosome designated *attTn7* (Lichtenstein and Brenner 1981; Lichtenstein and Brenner 1982), which is located within the transcriptional termination signal of the *glmS* gene (Gay *et al.* 1986; McKown *et al.* 1988). In TnsE-directed transposition, Tn*7* recognises a structure or complex involved in DNA replication and preferentially inserts into non-specific sites in conjugating plasmids (Wolkow *et al.* 1996) or chromosomal DNA (Peters and Craig 2000).

In this Chapter, class 2 integron-related antibiotic resistance gene carriage in *E. coli* isolated from animal and human sources was examined. Gene cassette carriage in class 2 integrons will be described, including one strain in which the gene cassette region could not be amplified using standard PCR methods. In this strain the *intI2* gene was found to be located on a plasmid, the nucleotide sequence of which was determined.

5.2 – Results

5.2.1 – PCR detection of class 2 integrons

PCR screening for the *intI2* gene revealed by inference that 7/514 of the *E. coli* strains contained class 2 integrons (Figure 5.1 and Table 5.1). All class 2 integron positive *E. coli* strains were isolated from human hosts; 6 of the strains were from human

diagnostic specimens and 1 strain was sourced from a healthy infant. Diagnostic specimens were from patients exhibiting symptoms of the following conditions: UTI (n=3), diarrhoea (n=2), and suspected diarrhoea (n=1). Class 2 integrons were not detected in the *E. coli* strains sourced from animals, including specimens from animals exhibiting symptoms of disease (cattle, dogs and swine) and Australian native animals.

5.2.2 – Serotypes and resistance phenotypes

Class 2 integron cassette arrays and the corresponding serotypes, resistance profiles and the origin of the strains are summarised in Table 5.1. Antibiotic susceptibility testing generally showed resistance phenotypes correlated with the resistance conferred by the gene cassettes identified, except in regard to spectinomycin resistance, which was only detected in one strain harbouring the *aadA1* gene cassette. The low-level of spectinomycin resistance conferred by the integron-borne *aadA1* gene cassette array may be below the detection level of the epidemiological breakpoints for streptomycin used for this study (25 μg/ml; as outlined by the Clinical and Laboratory Standards Institute). This finding has been previously observed in a study analysing the level of spectinomycin resistance conferred by integron-borne *aadA1a* and other closely related *aadA* gene cassettes (excluding *aadA4 and aadA5)* (Sunde and Norström 2005). The *E. coli* serotypes were variable in class 2 integron positive strains, which is suggestive of horizontal transfer of the integron.

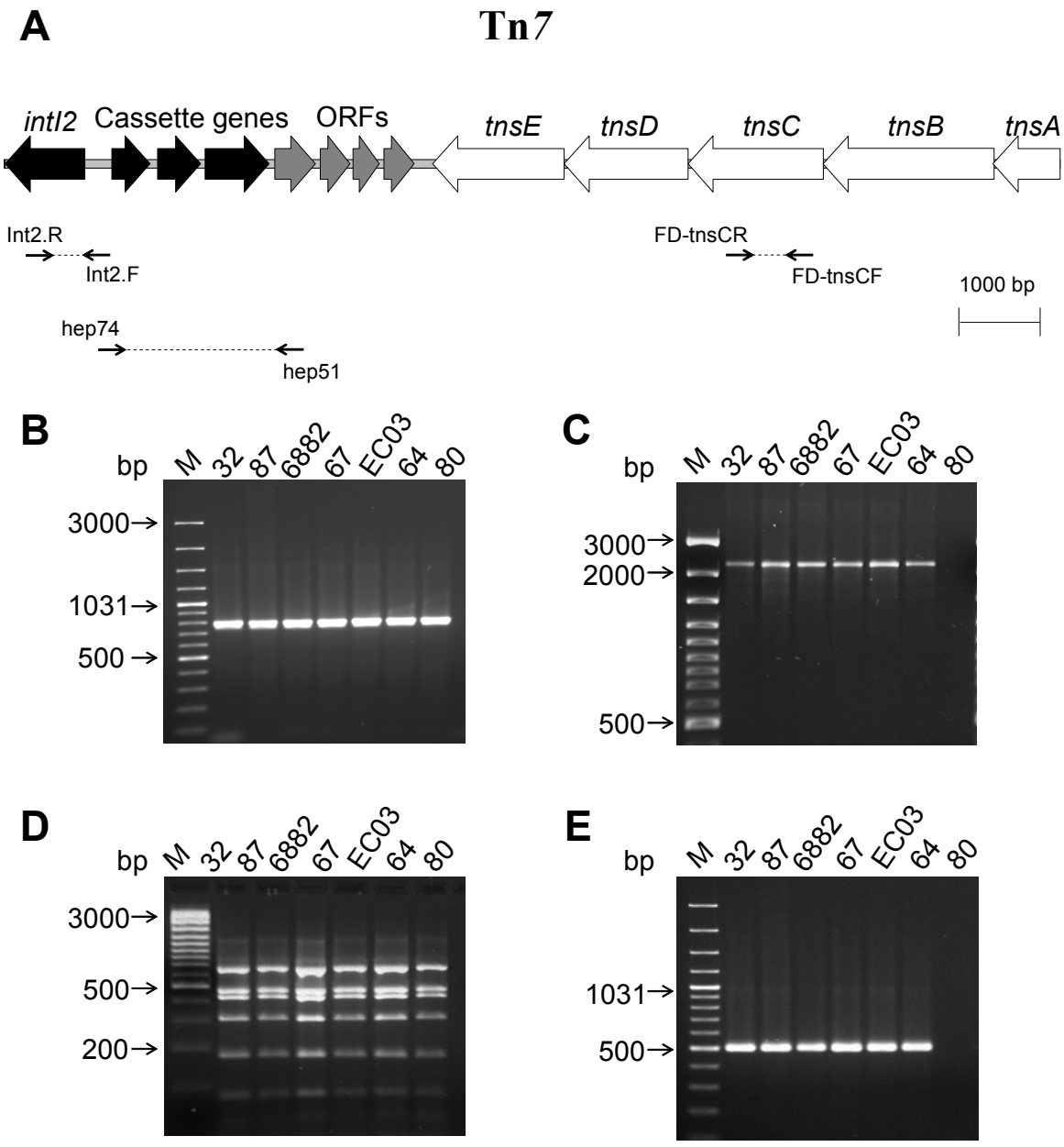

Figure 5.1. **(A)** The genetic structure of Tn*7* representing 12998 bp of nucleotide sequence (GenBank no. DQ275532). The following features are indicated: PCR primer binding sites (small arrows); class 2 integron consisting of the *intI2* gene and cassette genes (black arrows); ORFs *ybeA* (*orfX*)*, ybfA, ybfB* and *ybgA* (grey arrows); and the Tn*7* transposition genes *tnsA*, *tnsB*, *tnsC*, *tnsD*, and *tnsE* (open arrows). **(B)** Detection of class 2 integrons by PCR screening for the *intI2* gene using the primers Int2.R and Int2.F. **(C)** 2500 bp gene cassette array PCR amplicons produced using the primers hep51 and hep74. (**D**) RFLP of gene cassette array amplicons digested with *Alu*I. (**E**) 450 bp PCR amplicon of the *tnsC* gene using the primers FD-tnsCF and FD-tnsCR. The 100 bp DNA ladder is shown in lane M.

Table 5.1. Description of class 2 integron positive *E. coli* strains.

Strain identity	Cassette array	Year	Source	Serotype	Resistance to:										
					A	S	T	C	Su	Tm	K	Na	G	Cp	Sp
80	unamplifiable	2001	Human, suspected diarrhoea	O108:H-	-	*+	+	-	+	+	-	+	-	-	-
EC03	*dfrA1/sat2/aadA1*	1989-1992	Healthy infant	O6:H1	-	*+	-	-	+	+	-	-	-	-	-
06882	*dfrA1/sat2/aadA1*	1998	Human, UTI	O6:H-	+	*+	+		-	+	-	-	-	-	-
32	*dfrA1/sat2/aadA1*	1999	Human, UTI	O22:H-	-	*+	+	-	+	+	-	-	-	-	-
64	*dfrA1/sat2/aadA1*	2002	Human, UTI	O17/106 :H18	+	*+	+	-	+	+	-	+	+	+	+
87	*dfrA1/sat2/aadA1*	2001	Human, diarrhoea	O151:H2	-	*+	+	-	+	+	-	+	-	+	-
67	*dfrA1/sat2/aadA1*	2002	Human, diarrhoea	O3:H2	+	*+	-	+	+	+	+	-	-	-	-

Abbreviations: UTI, urinary tract infection; A, ampicillin; S, streptomycin; T, tetracycline; C, chloramphenicol; Su, sulfathiazole; Tm, trimethoprim; K, kanamycin; Nal, nalidixic acid; G, gentamicin; Cp, ciprofloxacin; Sp, spectinomycin. Symbols: + resistant, – sensitive, *moderate level of resistance is defined as significant resistance but not fully resistant.

5.2.3 – Molecular characterisation of class 2 integrons

Nucleotide sequence analysis of the *intI2* gene in *E. coli* strain 80 confirmed the presence of a putative defective class 2 integrase, characterised by an internal stop codon in the *intI2* gene at amino acid position 179 (Hansson *et al.* 2002). PCR amplification of gene cassette regions using the primer pair hep51 and hep74 produced a single 2.2 kb product in 6/7 strains. RFLP and DNA sequence analysis of the PCR amplified integron variable regions (in a representative *E. coli* strain), revealed that each of these strains carried the gene cassette array *dfrA1-sat2-aadA1* (100% identity to GenBank no. AB188272.1; Figure 5.1C). The gene cassette region could not be amplified using standard PCR methods for the class 2 integron positive *E. coli* strain 80, which was sourced from a human with suspected diarrhoea.

All class 2 integron positive isolates, except *E. coli* strain 80, were identified as carrying Tn*7* by PCR screening for the Tn*7* transposition gene *tnsC* using the primer pair FD-tnsCF and FD-tnsCR. A single 0.5 kb amplification product was detected in 6/7 class 2 integron positive isolates with the gene cassettes *dfrA1-sat2-aadA1* described above (Figure 5.1D and Table 3). DNA sequence of the *tnsC* PCR amplification product from a representative strain showed 100% identity to sequence previously deposited in GenBank (X17693.1). Southern blot analysis using PCR-amplified *intI2* as a probe against plasmid and total genomic DNA showed 6/7 class 2 integrons with the classical gene cassette array *dfrA1/sat2/aadA1* were located on the *E. coli* chromosome. In *E. coli* strain 80, the *intI2* gene was found to be plasmid-located (Southern hybridisation results not shown).

5.2.4 – Determination of class 2 integron (Tn*7*) insertion sites

E. coli strains were analysed by Southern hybridisation to examine the chromosomal site of class 2 integron insertion using *intI2* and *phoS/glmS* probes. Initial results found 6/7 class 2 integrons, with classical gene cassette array *dfrA1-sat2-aadA1*, were inserted into the *E. coli* chromosome near the *phoS/glmS* gene junction. In *E. coli* strain 87, a 6.6 kb DNA fragment hybridised with the *phoS/glmS* gene probe. This additional hybridisation fragment could be due to cross-hybridisation with genomic DNA showing identity to the *phoS/glmS* gene sequence in this particular strain. For *E. coli* strain 80, a larger sized DNA fragment hybridised with the *intI2* gene probe when compared to all other strains, indicating a different site of Tn*7* insertion (Figure 5.2).

PCR analysis of Tn7 insertion in the *E. coli* chromosome is shown in Figure 5.2 and the exact Tn*7* insertion sites are illustrated in Figure 5.3. PCR of the left-end of Tn*7* and the *phoS* gene produced a 2 kb amplicon in *E. coli* strains 32, 6882 and 67. Subsequent RFLP with the restriction enzyme *Alu*I revealed an identical profile for these *E. coli* strains (Figure 5.2C). Nucleotide sequence analysis of the 2 kb PCR product from strain 67 showed Tn*7* was inserted into the *attTn7* site located downstream of the *phoS* gene within the transcriptional termination signal of the *glmS* gene (GenBank no. FJ914211). PCR targeting the right-end of Tn*7* and the *glmS* gene, followed by DNA sequence analysis of the resulting 0.9 kb product confirmed Tn*7* insertion at *attTn7* in these strains.

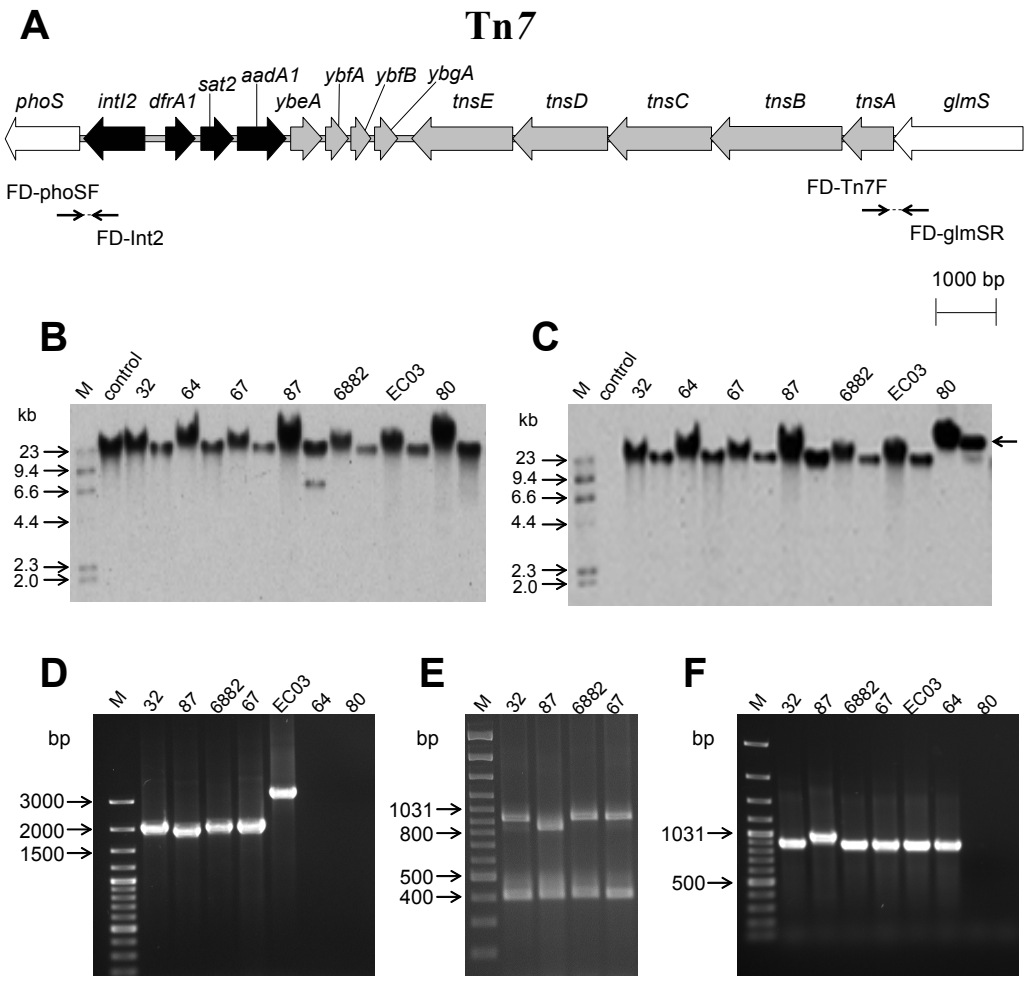

Figure 5.2. **(A)** A genetic map showing Tn*7* insertion into the *E. coli* chromosome between the *phoS* and *glmS* genes within the transcriptional termination signal of the *glmS* gene. 16181 bp of nucleotide sequence is represented in the genetic map (GenBank no. DQ275532 and NC000913). The following features are indicated: class 2 integron consisting of the *intI2* gene and cassette genes (black arrows); ORFs *ybeA* (*orfX*), *ybfA, ybfB* and *ybgA* (grey arrows); Tn*7* transposition genes *tnsA*, *tnsB*, *tnsC*, *tnsD*, and *tnsE* (grey arrows); and the *phoS* and *glmS* genes (open arrows). Southern hybridisations showing the different location of the class 2 integron in *E. coli* strain 80. **(B)** Southern hybridisation performed with a DIG-labelled PCR amplified *phoS/glmS* gene probe against genomic DNA and **(C)** a second hybridisation using a DIG-labelled PCR amplified *intI2* gene probe against the same genomic DNA. A larger sized DNA fragment for *E. coli* strain 80 is indicated by an arrow. Lambda DNA markers are shown in lane M. Only uncut DNA was used for the negative control *E. coli* strain. Uncut genomic DNA samples from the *E. coli* strains indicated are followed by DNA digested with *Sal*I in the subsequent lane. **(D)** PCR amplification of the left end of Tn*7* and the *phoS* gene produced using the PCR primers FD-phoSF and FD-Int2. **(E)** RFLP of PCR amplification products of the left end of Tn*7* and the *phoS* gene following digestion with *Alu*I. **(F)** PCR amplification of the right end of Tn*7* and the *glmS* gene produced using the PCR primers FD-Tn7F and FD-glmSR. PCR amplicons for class 2 integron positive *E. coli* strains are indicated at the top of the lanes. The 100 bp DNA ladder is shown in lane M.

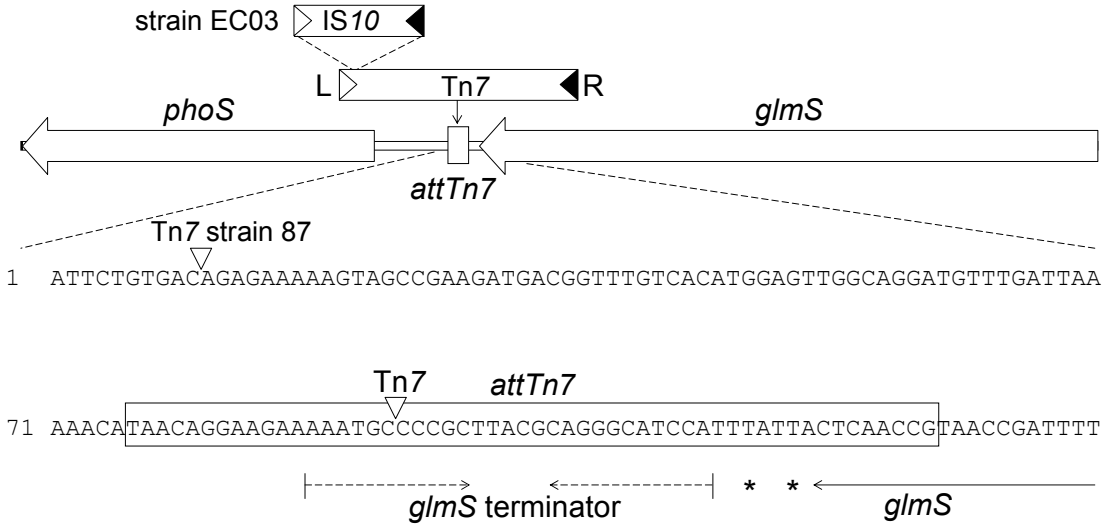

Figure 5.3. Diagram showing variation in Tn*7* insertion in the *E. coli* chromosome. Nucleotide sequence of the 3′ end of the *glmS* gene and the non-coding region between *glmS* and *phoS* genes in the *E. coli* strain K-12, without Tn*7* inserted is shown (GenBank no. NC000913). The usual Tn*7* insertion site in the *glmS* transcriptional terminator is indicated for *E. coli* strains 32, 6882, 67, EC03 and 64. The Tn*7* insertion site for *E. coli* strain 87, which is 83 bp away from the usual insertion site in *attTn7* is also shown. In strain EC03, IS*10* is inserted into the left end of Tn*7*. The following features are indicated: the Tn*7* insertion sites (open triangle); the *glmS* transcriptional terminator (opposing broken arrows); the *attTn7* site (open box); the location and direction of transcription of the *glmS* gene; the tandem stop codons of the *glmS* gene (*); and inverted repeats of IS*10* and Tn*7* (inward facing open/closed triangles).

In *E. coli* strain 64, no amplicon was detected by PCR amplification screening of the left-end of Tn*7* and the *phoS* gene. Inability to amplify the left-end of Tn*7* was probably due to the loss of PCR primer binding sites, which may have been caused by various DNA rearrangement events. PCR of the right-end of Tn*7* and the *glmS* gene followed by nucleotide sequence analysis in this strain revealed Tn*7* insertion at *attTn7*.

In *E. coli* strain EC03, PCR of the left-end of Tn*7* and the *phoS* gene produced a 3.3 kb amplification product. Nucleotide sequence analysis revealed the insertion of IS*10* into

Tn*7* and Tn*7* insertion at the *attTn7* site (GenBank no. FJ914212). PCR of the right-end of Tn*7* and the *glmS* gene followed by nucleotide sequence analysis revealed Tn*7* insertion at *attTn7*. ClustalW alignment of sequence from PCR amplicons of the right-end of Tn*7* in *E. coli* strains 67, 32, 6882, 64 and EC03 confirmed Tn*7* was inserted at the *attTn7* site at an identical position in all these strains (GenBank nos. FJ914213 to FJ914217). BLASTn analysis showed the site of Tn*7* insertion was 100% identical to sequence previously deposited in GenBank (X03474).

In *E. coli* strain 87, PCR of the left-end of Tn*7* and the *phoS* gene followed by RFLP with the restriction enzyme *Alu*I gave a different profile to strains 32, 6882 and 67. Nucleotide sequence determination of the 1.9 kb PCR amplicon from strain 87, revealed an intact *attTn7* site and Tn*7* insertion 83 bp away from the usual insertion site (GenBank no. FJ914218). PCR of the right-end of Tn*7* and the *glmS* gene followed by nucleotide sequence analysis of the resulting 1 kb amplicon, provided further evidence of an 83 bp shift in Tn*7* insertion towards *phoS* gene (GenBank no. FJ914219).

5.2.5 – DNA Sequence analysis of pECTm80 containing a unique class 2 integron gene cassette array

E. coli strain 80, in which the gene cassette region could not be PCR-amplified by standard methods, was subjected to further analysis. A mating-out assay, performed with and without the helper plasmid pRK600, confirmed the integron-containing plasmid was mobilisable. The plasmid, designated pECTm80, was subsequently transformed into *E. coli* JM109, and the nucleotide sequence was determined (GenBank no. FJ914220). The CDS identified are illustrated in Figure 5.4 and described in Table 5.2.

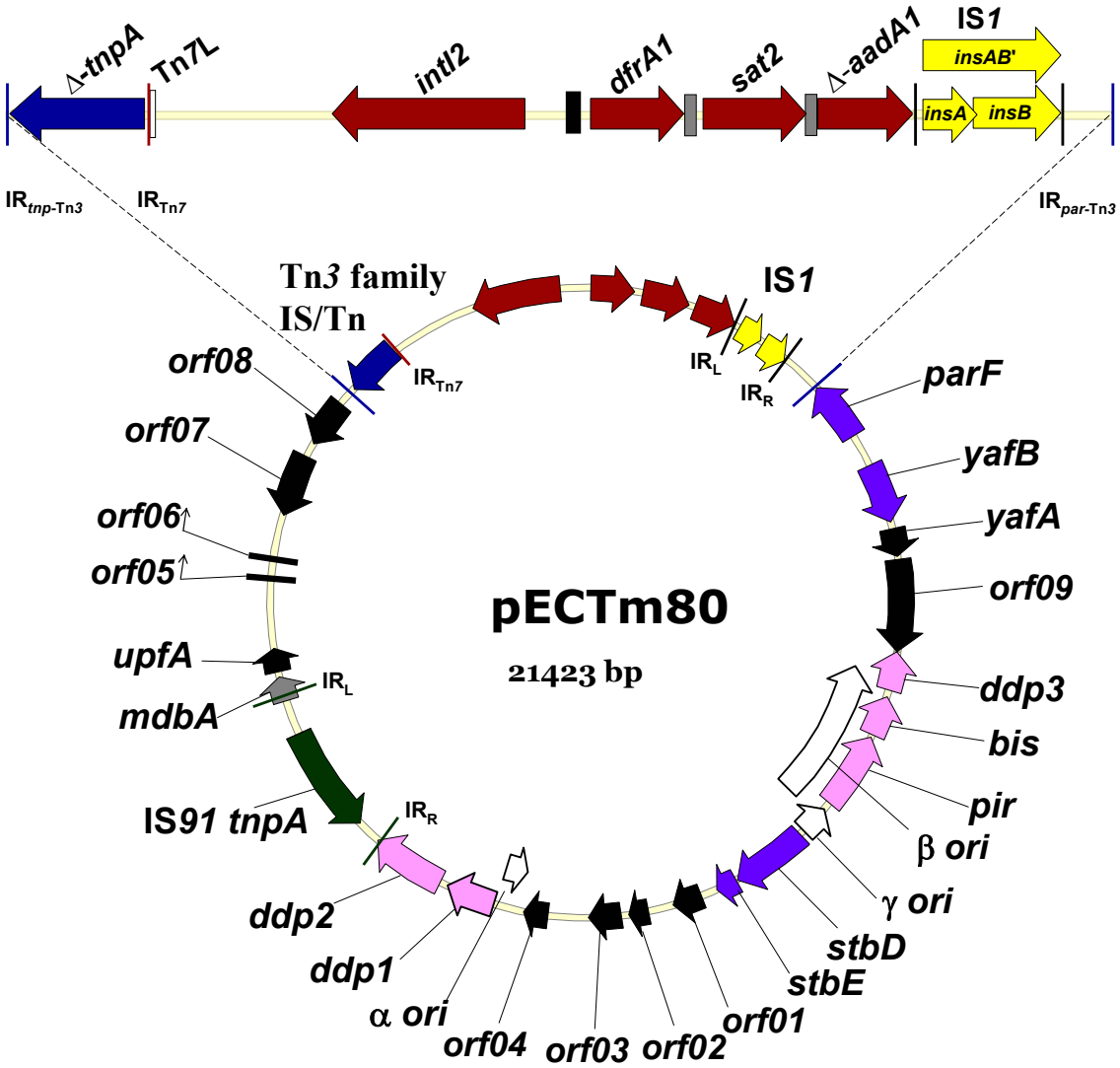

Figure 5.4. Illustration of plasmid pECTm80 isolated from *E. coli* strain 80 (GenBank no. FJ914220). Arrows indicate the direction of transcription. Δ- denotes partial deletion or truncation in a gene. Terminal inverted repeats (IR) of transposons and insertion sequences (IS) (left IR, IR_L and right IR, IR_R) are indicated by colour-coded bars: 38 bp IR of a Tn*3* family transposon/IS remnant adjacent to the Δ-*tnpA* gene ($IR_{tnp\text{-}Tn3}$) and the *parF* gene ($IR_{par\text{-}Tn3}$) (blue), 25 bp IR IS*1* (black), 8 bp IR IS*91* (green), 8 bp IR at the left end of Tn*7* (IR_{Tn7}, red). Tn*7*L denotes 150 bp at the left end of Tn*7* containing multiple TnsB binding sites (open box) (Craig *et al.* 2002). α, β and γ origins of replication are indicated by unfilled arrows. Functional categories of predicted CDS include *ori* regulatory genes *ddp1*, *ddp2* and *ddp3*, and replication initiation proteins *bis* and *pir* (pink); IS*91 tnpA* (green); IS*1 insA*, IS*1 insB* and IS*1 insAB*' (yellow); Δ-*tnpA* gene from a Tn*3* family transposon/IS remnant (blue); plasmid partition genes, *parF* and *yafB* and toxin anti-toxin system genes, *stbD* and *stbE* (mauve); putative DNA-binding protein gene *mdbA* (grey arrow); and ORFs encoding hypothetical proteins (black). Integron features indicated include *intI2* and cassette genes (red arrows), *attI2* (closed box) and 59-bes (grey boxes).

Table 5.2. Identification of CDS in the nucleotide sequence of pECTm80.

Gene/ORF	Function/name of protein	Accession no.	% Identity
ddp3	DNA distortion protein 3	DQ115387.2	93
bis	Replication initiation protein	EU370913.1	98
pir	Replication initiation protein P1	M65025.1	45
stbD	Stability protein StbD	AF072126.1	100
stbE	Stability protein StbE	AF072126.1	100
orf01	Conserved hypothetical protein	EU370913.1	100
orf02	Conserved hypothetical protein	EU370913.1	100
orf03	Conserved hypothetical protein	EU370913.1	99
orf04	Conserved hypothetical protein	EU370913.1	100
ddp1	DNA distortion protein 1	FJ666132.1	99
ddp2	DNA distortion protein 2	FJ666132.1	98
IS*91 tnpA*	IS*91* transposase	AE005674.1	97
mdbA	Putative DNA-binding protein H-NS histone family	U47048.1	88
upfA	UpfA conserved hypothetical protein	FJ666132.1	100
orf05	Conserved hypothetical protein SCH_083	AY509004.1	84
orf06	Conserved hypothetical protein	DQ115387.2	100
orf07	Conserved hypothetical protein	CU928145.2	47

Gene/ORF	Function/name of protein	Accession no.	% Identity
orf08	Conserved hypothetical protein	EF536825.1	47
Tn*3* family transposon/IS Δ-*tnpA*[A]	Transposase truncated at the N-terminus by the insertion of Tn*7*	EU330199	99
intI2	Class 2 integrase	AB188272	100
dfrA1	Dihydrofolate reductase	AAT36680	100
sat2	Streptothricin acetyltransferase	AB188272	100
Δ-*aadA1*	Aminoglycoside adenylyltransferase, truncated at the C-terminus by the insertion of IS*1*	AB188272	100
IS*1 insA*	InsA transcription repressor Inhibits transposition	AF250878.1	100
IS*1 insB*	None	CP000946.1	100
IS*1 insAB'*	IS*1* transposase InsAB' (frame shift product)	AY309066.1	100
parF	Plasmid partition protein ParF	AF204292	99
yafB	Plasmid partition protein A	AP002527.1	75
yafA	Conserved hypothetical protein YafA	DQ115387.2	96
orf09	Conserved hypothetical protein	DQ115387.2	100

[A]Δ-*tnpA* gene showed 64% to 76% identity to transposase genes of the Tn*3* family of transposons (subgroups Tn*501* and Tn*3*) and the IS elements IS*Sba14*, IS*Sod9*, and IS*Bmu14*, of the Tn*3* family.

DNA sequence analysis revealed the class 2 integron did not contain the ORFs *ybeA* (*orfX*)*, ybfA, ybfB,* and Tn*7* transposition genes *TnsABCDE*, which are normally found at the 3′ end of class 2 integrons. This class 2 integron harboured intact gene cassettes *dfrA1* and *sat2*, and an *aadA1* cassette (Δ-*aadA1*) truncated by IS*1* insertion. Significant features of the plasmid sequence include plasmid partition genes, three origins of replication, a toxin-antitoxin system, a truncated transposase (Δ-*tnpA*) gene and the insertion sequences IS*1* and IS*91*.

BLASTp of Δ-TnpA identified the multi-domain transposase_7 family (pfam01526), which includes the transposases of Tn*3* family transposons (Fennewaldt *et al.* 1981; Yurieva and Nikiforov 1996). BLAST searches of the Δ-*tnpA* gene (NCBI and IS Blast server available at http://www-is.biotoul.fr/) revealed 64% to 76% identity to transposase genes of various transposons in the Tn*3* family (subgroups Tn*501* and Tn*3*) and IS elements IS*Sba14*, IS*Sod9*, and IS*Bmu14* also of the Tn*3* family. The highest identity was displayed to IS*Sba14* and Tn*2501* (76% identity and 87% query coverage). BLAST results indicate the Δ-*tnpA* gene may be a remnant from a transposon or IS element of the Tn*3* family. The complete ΔTn*3*-ΔTn*7*-IS*1* gene configuration is flanked by 38 bp IRs and 5-bp target-site direct repeats (TATAT). The generation short directly repeated sequences of the target site flanking the IS element is a general feature of IS element insertion (Craig *et al.* 2002).

The *ori* region spanned 7.6 kb and contained α, β and γ origins of replication, three ori regulatory genes (*ddp1*, *ddp2*, *ddp3*) and the initiation transfer genes *bis* and *pir* (Flashner *et al.* 1996). Nucleotide sequence in pECTm80 showed identity with a 34.5 kb *Salmonella enterica* plasmid pOU1114 (Chu and Chiu 2006).

Comparative analysis of the DNA sequence of the plasmids pECTm80 and pOU1114 (GenBank no. NC_010421), performed using the Artemis Comparison Tool program (Carver *et al.* 2005) revealed extensive regions of identity. A region of 95% identity between pECTm80 and pOU1114 was found which spanned the plasmid partition genes *parF* and *yafB*, *yafA* gene*, orf09*, *ddp3* and *bis* genes of pECTm80. A second region of 98% identity containing the genes *stbD* and *stbE*, *orf01*, *orf02*, *orf03*, and the *ori* regulatory genes *ddp1* and *ddp2* was also identified in both pECTm80 and pOU1114. Many (18/29) CDS in pECTm80 were also found in pOU1114, indicating these plasmids share a common backbone and may have arisen from a common ancestral plasmid (Figure 5.5).

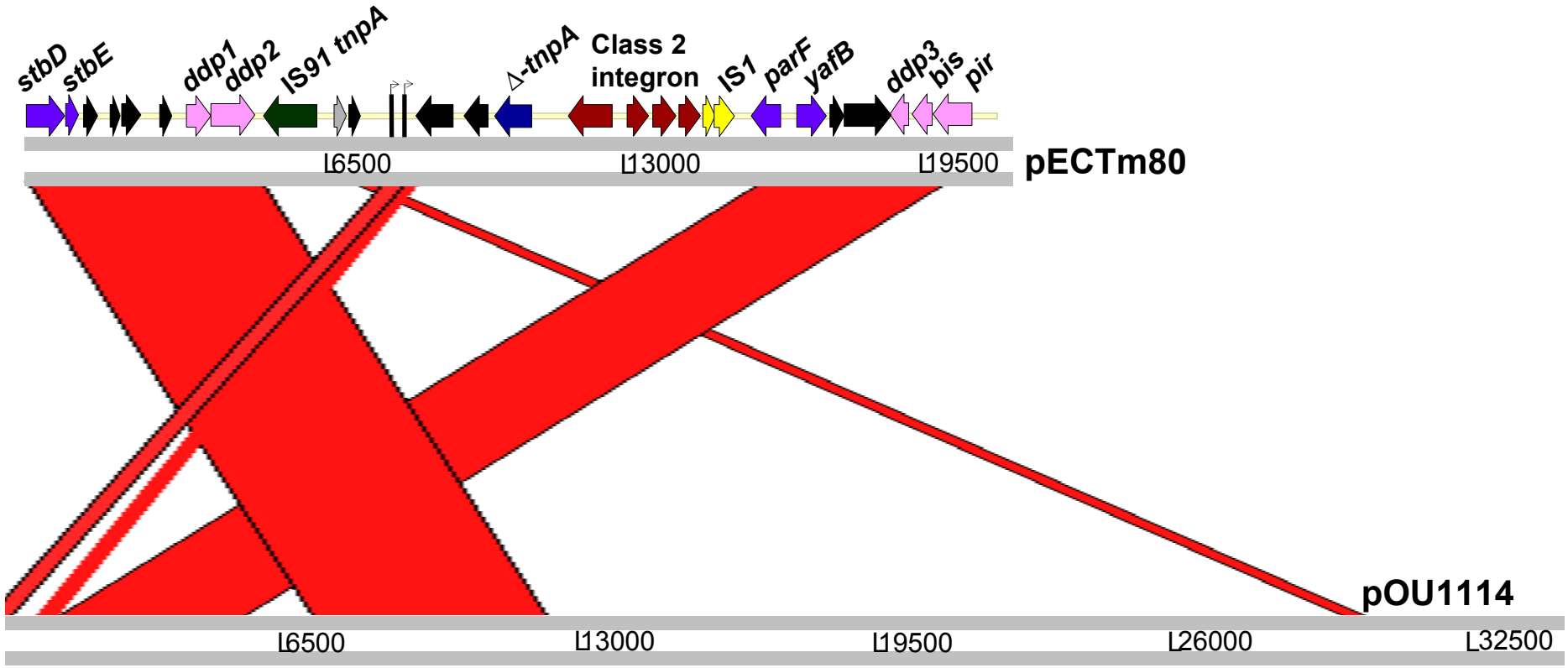

Figure 5.5. DNA sequence comparison of the plasmids pECTm80 and pOU1114. Regions of with greater than 92% identity are connected by vertical red blocks and lines. The following CDS identified in pECTm80 and are indicated: *stbD*, *stbE*, *ddp1*, IS*91 tnpA*, Δ-*tnpA* a Tn*3* family transposon/IS remnant, class 2 integron (consisting of the *intI2* gene, the cassette genes *dfrA1* and *sat2,* and the Δ-*aadA1* gene), IS*1 insA*, IS*1 insB*, *parF*, *yafB*, *ddp3*, *bis* and *pir* genes. The position of the DNA sequence (bp) is shown between horizontal grey bars that indicate forward and reverse DNA strands. The colour coding of functional categories of predicted CDS identified in pECTm80 is the same as that used in Figure 5.4. This diagram was adapted from a graphical output generated using the Artemis Comparison Tool program.

5.3 – Discussion

The efficient dissemination of class 2 integrons is evident in their global distribution among Gram-negative bacteria of the *Enterobacteriaceae* family. Class 2 integrons have been detected in Australia (Barlow *et al.* 2004), USA (Goldstein *et al.* 2001), South America (Márquez *et al.* 2008), Germany (Miko *et al.* 2003), Ireland (DeLappe *et al.* 2003), Spain (Vinué *et al.* 2008), China (Pan *et al.* 2006) and Africa (Gassama Sow *et al.* 2008). Class 2 integrons are a unique class of integron; because of their inactive integrase they must rely on other mobile elements or illegitimate integrase recombination events for gene cassette insertion. Tn*7* insertion, which predominately occurs in chromosomal DNA at a unique site (Peters and Craig 2000), may also occur at low frequency at non-specific sites in conjugating plasmids (Wolkow *et al.* 1996). Insight into the role of class 2 integrons in antibiotic resistance gene carriage and their interaction with other mobile DNA elements was gained in this study by examining *E. coli* strains isolated from animal and human sources.

This investigation revealed 7/514 of *E. coli* isolates harboured class 2 integrons. Six of seven class 2 integrons contained the complete gene cassette array of *dfrA1-sat2-aadA1*. In the remaining integron positive *E. coli* strain the integron was found to be located on a mobilisable plasmid (pECTm80). DNA sequence analysis of pECTm80 revealed complete *dfrA1* and *sat2* gene cassettes and an IS*1*-mediated deletion resulting in truncation of the *aadA1* gene cassette and absence of the ORFs *ybeA* (*orfX*)*, ybfA* and *ybfB,* and Tn*7* transposition genes *TnsABCDE*, which are normally found at the 3′ end of class 2 integrons.

The plasmid pECTm80 has the potential to facilitate the horizontal transfer of the tightly-linked antibiotic resistance genes *dfrA1, sat2* and *aadA1*. Several key features of the integron-containing plasmid pECTm80 attest to its clinical relevance. These features include (i) the ability of the plasmid to be mobilised, (ii) the presence of plasmid partitioning and segregational stability genes to ensure the stable maintenance of the plasmid through successive bacterial cell divisions and (iii) a highly-regulated DNA replication system consisting of three distinct origins of replication α, β and γ, that are controlled and activated by plasmid- and host-encoded genes.

The plasmid pECTm80 has evolved to ensure its vertical transfer via the acquisition of multiple genes including the plasmid partition (segregational) genes and toxin-antitoxin system genes. Proteins of active plasmid partition systems are encoded by the genes *parF* (a distinct subgroup of the ParA superfamily) (Hayes 2000) and *parA*. These proteins direct the intracellular movement of plasmids during cell division to ensure the distribution of low-copy number plasmids to daughter bacterial cells (Schumacher 2007). The toxin-antitoxin system, encoded by *stbD* and *stbE* genes, promotes segregational stability by compromising the survival of plasmid-free daughter cells that may arise during cell division (Hayes 1998). The toxin-antitoxin system consists of a toxin gene that encodes a stable protein while the antitoxin is either untranslated antisense RNA or a labile protein. When the plasmid is maintained in the cell the toxin is neutralised by the antitoxin. If a plasmid-free cell is produced the antitoxin is degraded more quickly than the toxin, and since the antitoxin can not be replenished the toxin causes cell death or growth restriction in the cell (Hayes 2003).

The complex replication system in pECTm80, which consists of three distinct replicons α, β and γ, is a plasmid strategy for achieving broad-host-range (Toukdarian 2004). The plasmid pECTm80 belongs to the incompatibility (Inc) group X (showing 96% identity to the IncX1 plasmid R485 replication origin region, accession no. M11688.1 and 72% ID to the IncX2 plasmid R6K α origin region, accession no. X05644.1) (Jones *et al.* 1993). A study characterising the host-range of an IncX plasmid (γ origin plasmid of R6K lineage) found the plasmid was established in 9 of 16 species tested including all of the *Enterobacteriales* (*Enterobacter nimipressuralis*, *Erwinia amylovora, Erwinia carotovora, Escherichia coli*, *Plesiomonas shigelloides, Salmonella enterica* and *Serratia marcescens*), *Zoogloea ramigera* and *Vibrio cholerae* (Wild et al. 2004).

The close association of class 2 integrons with other mobile DNA elements including a mobilisable plasmid, transposons and IS elements is reported in this study. The complex and dynamic interaction of mobilisable elements described, act to enhance their collective ability for horizontal gene transfer and increase their potential for gene shuffling (Toussaint and Merlin 2002). In this study 6/7 class 2 integrons with the gene cassette array *dfrA1-sat2-aadA1* were located near the left end of the non-replicative transposon Tn*7*. The integron from *E. coli* strain 80 was in proximity to insertion sequences IS*1* and a Tn*3* family transposon/IS remnant and was carried on a mobilisable plasmid containing IS*91*.

The association of the truncated class 2 integron with IS*1,* IS*91* and a Tn*3* family transposon/IS remnant implicates these DNA elements in the evolution of this unique integron. The structure of this integron and its association with ISs suggests its formation involved multiple rearrangement events. The plasmid pECTm80 carries the

mdbA gene that encodes for a putative H-NS, which is a nucleotide-associated DNA-binding protein required for the transposition of IS*1* (Shiga et al. 1999). The insertion of IS*1* has presumably caused truncation of the *aadA1* gene cassette of pECTm80. IS*1*-mediated deletions have been previously described (Reif and Saedler 1975). Furthermore, the retention of IS*1* in a DNA sequence can promote further rounds of deletions (Reif and Saedler 1977).

It is of interest that the DNA sequence of the Tn*3* family remnant detected in this study displays 68% identity to IS*Sod9*; as the IR sequence of IS*Sod9* is highly similar to that of a proposed integron mobilisation unit (IMU). An IMU has been described as identical 288 bp DNA elements with 39 bp imperfect IRs flanking a class 1 integron remnant. Transposition assays providing IS*Sod9* transposase activity in *trans,* have demonstrated transposition of the entire integron-IMU element (Poirel *et al.* 2009).

Comparative DNA sequence analysis of pECTm80 with pOU1114, a non-virulence plasmid isolated from *Salmonella enterica* serovar Dublin, provide insight into how this potentially clinically relevant plasmid and its resident class 2 integron evolved. It is of significance that IS*91*, the Tn*3* family transposon/IS remnant, IS*1* and the class 2 integron are located in the pECTm80 sequence and are absent from pOU1114 (Figure 5.5). This observation suggests either the integration of IS*91*, the Tn*3* family transposon/IS remnant, IS*1* and the class 2 integron into a common ancestral plasmid occurred to create pECTm80, or alternatively the deletion of these elements from a common ancestral plasmid may have occurred to create pOU1114. Insertion sequences IS*91* and IS*1* are intact in pECTm80 and may represent a recent insertion into the plasmid. While the exact mechanism of evolution of this unique plasmid and its resident

class 2 integron remains unknown, multiple insertion, deletion and rearrangement events are likely, as suggested by its proximity to several insertion sequences.

The target selection bias for insertion of Tn*7*-related class 2 integrons in the 7 clinical *E. coli* strains is described. The two pathways of Tn*7* transposition, which were both observed in this study, are known to act collectively to ensure the safe insertion and propagation of the transposon in diverse hosts (Waddell and Craig 1988). Target site specificity of Tn*7* promotes its propagation by ensuring insertion is not deleterious to the host. Tn*7* recognises the *glmS* gene sequence, an essential and highly conserved gene, and transposition occurs down stream of the gene avoiding mutation (Peters and Craig 2001a). High target specificity, characteristic of the TnsD-dependent pathway was evident in *E. coli* strains 32, 67, 6882, EC03 and 64. In these stains Tn*7* inserted into the *E. coli* chromosome in an identical position in the *attTn7* site within the transcriptional termination signal of the *glmS* gene. Tn*7* insertion was identified 83 bp away from the usual insertion site at *attTn7* in strain 87, which was collected from a patient who had recently travelled to Nepal. The preference of Tn*7* insertion for pseudo *attTn7* sites with sequence similarity to *attTn7* (Kubo and Craig 1990) and sequences with the potential to form secondary structures have been described (Lichtenstein and Brenner 1982). No significant similarity between the target sequence of Tn*7* in *E. coli* strain 87 and the *attTn7* sequence was observed (NCBI; blast 2).

The target preference of Tn*7* for conjugating plasmids, which are generally broad-host-range, facilitates the dispersal of Tn*7* among diverse bacterial hosts (Peters and Craig 2001b). The TnsE-dependent pathway of Tn*7* insertion was expected in the plasmid-located class 2 integron in *E. coli* strain 80, as the preferential insertion of Tn*7*

into non-specific sites in conjugating plasmids has been well established (Wolkow *et al.* 1996). Tn*7* transposition in plasmids has been described at many different sites unrelated to *attTn7* DNA sequence (Barth *et al.* 1978; McKown *et al.* 1988). *E. coli* strain 80, contained only the left end of Tn*7* as the right end had been deleted by the insertion of IS*1*. Consequently, only comparison of the Tn*7* target sequence adjacent to the left end of Tn*7* could be determined. A Blast 2 search (NCBI) showed no significant similarity between Tn*7* target sequence at the left end of Tn*7* and the *attTn7* sequence in *E. coli* strain 80. This low target site specificity is typical of the TnsE-dependent transposition pathway. The capacity of the plasmid from *E. coli* strain 80 to be transferred to a recipient *E. coli* strain is also consistent with the TnsE-dependent pathway, in which Tn*7* recognises a structure or complex involved with DNA replication and transposes preferentially to conjugating plasmids as they enter the bacterial cell.

Although class 2 integrons were only detected in *E. coli* sourced from humans in this study, the potential for animal-to-human or human-to-animal transmission of integrons via vertical transfer of specific *E. coli* serotypes exists. Class 2 integrons harbouring the gene cassette array *dfrA1-sat2-aadA1* were detected in serotypes O6:H- and O17:H18 and serogroup O22 isolated from humans with UTIs. These serotypes have been associated with Verotoxin-producing *Escherichia coli* (VTEC) in cattle, the main reservoir of VTEC that cause human VTEC infections (Nataro and Kaper 1998). VTEC cause diarrhoea in humans and also more serious complications of hemorrhagic colitis and HUS (Blanco *et al.* 2008; Nataro and Kaper 1998).

DNA sequence determination of plasmid pECTm80 revealed a class 2 integron in which the ORFs *ybeA* (*orfX*), *ybfA* and *ybfB,* and Tn*7* transposition genes *TnsABCDE*, which are normally found at the 3′ end of class 2 integrons had been deleted by the insertion of IS*1*. Comparative DNA sequence analysis of pECTm80 with pOU1114 provide insight into how this potentially clinically relevant plasmid and its resident class 2 integron evolved. A role for IS elements in generating diversity in class 2 gene cassette arrays is suggested by the close proximity of IS elements to class 2 integrons with variation in their gene cassette carriage in this and other studies.

5.4 - Conclusion

Integrons are genetic elements that facilitate the clustering and mobilisation of resistance genes. In this study, a collection of 514 *E. coli* strains from animal and human sources was examined for the presence of class 2 integrons using a PCR based screening method. Six of seven class 2 integrons contained the complete classical gene cassette array *dfrA1-sat2-aadA1* analogous to that found in Tn*7*. In the remaining *intI2* positive strain, the *intI2* gene was found to be located on a plasmid, the nucleotide sequence of which was determined. The plasmid was found to encode intact *dfrA1* and *sat2* gene cassettes and a truncated *aadA1* gene cassette. The ORFs *ybeA* (*orfX*), *ybfA* and *ybfB,* and the Tn*7* transposition genes *TnsABCDE*, normally conserved at the 3′ end of Tn*7*-like class 2 integrons, were not detected. This class 2 integron was flanked by IS*1* and a Tn*3* family transposon/IS remnant and IS*91* was also found within the integron-containing plasmid sequence. Two other class 2 integron variants were characterised including one where IS*10* was inserted into Tn*7* and second where Tn*7* was inserted 83 bp away from the usual site in the *E. coli* chromosome at *attTn7*.

CHAPTER 6 – CONCLUSIONS

6.1 – Introduction

In this study antibiotic resistant genes located on integrons in *E. coli* sourced from humans and animals were identified. The importance of monitoring antibiotic resistance genes and the role of integrons in the emergence of antibiotic resistant pathogens has been reviewed. As part of the integron-associated gene cassette array characterisation, this thesis examines evidence regarding the dissemination of integrons in *E. coli* between animal and human hosts. Considerable attention has been given to discover the identity of cassette arrays harboured by all integron positive isolates, including those that could not be amplified using standard PCR conditions. Techniques have been developed in this study to assist in the detection of integrons and the gene cassettes they harbour. These techniques include the development of a m-PCR to simultaneously screen for *intI1*, *intI2, intI3* and *uspA* genes and the recommendation of the use of integrase and IS*26*-specific PCR primers to amplify cassette arrays that were previously unamplifiable. Improvements in the detection of integron-related antibiotic resistance genes will allow the generation of more accurate surveillance data.

6.2 – Main results and future directions

The gene cassette arrays *dfrA5, dfrA7, aadA1, aadA2, dfrA1/aadA1, dfrA17/aadA5* and *dfrA12/orfF/aadA2,* were detected in class 1 integrons. The molecular characterisation of previously unamplifiable class 1 cassette arrays lead to the identification of novel integron-IS*26* elements *dfrA5*-IS*26* and *dfrA15*-IS*26.* The *dfrA5*-IS*26* element was

detected in *E. coli* isolated from both human and bovine sources. The location of IS*26*, beyond the 59-be of the *dfrA5* gene cassette and after the first 24 bp of the class 1 integron 3′-CS, is identical in each strain. It is unlikely that this structure is the result of separate IS*26* insertion events targeting exactly the same position in the integron on multiple occasions. It is more likely that the *dfrA5*-IS*26* element has arisen only once and then spread to other *E. coli* strains.

Variation in *E. coli* serotypes containing the integron-*dfrA5*-IS*26* gene cassette supports the contention that horizontal transfer of this element has occurred. Further studies are needed to determine the exact mechanism of horizontal transfer of the integron-*dfrA5*-IS*26* element, between animal and human hosts. These studies would include epidemiological evidence to confirm horizontal gene transfer of the integron-*dfrA5*-IS*26* element and determination of whether the unit of transfer is a conjugative transposon or plasmid.

Seven of the 514 *E. coli* strains were found to contain class 2 integrons. Six of these class 2 integrons harboured the complete classical gene cassette array *dfrA1-sat2-aadA1* analogous to that found in Tn*7*. In the remaining *intI2* positive strain, the *intI2* gene was found to be located on a plasmid and the nucleotide sequence of the entire plasmid designated pECTm80 was determined. The plasmid was found to carry intact *dfrA1* and *sat2* gene cassettes, and an IS*1*-mediated deletion resulting in truncation of the *aadA1* gene cassette. The ORFs typically found in the 3′ region of class 2 integrons were not present. This truncated class 2 integron was flanked by IS*1* and a Tn*3* family transposon/IS remnant and IS*91* was also found within the integron-containing plasmid sequence.

This study described an IncX plasmid, pECTm80 that has the potential to facilitate the horizontal transfer of tightly-linked antibiotic resistance genes to diverse microbial species. Features which contribute to the clinical relevance of the plasmid include its ability to be mobilised, the presence of genes to ensure the stable maintenance of the plasmid through successive bacterial cell divisions and the presence of a highly-regulated DNA replication system consisting of α, β and γ origins of replication. It would be interesting to conduct further studies to determine the prevalence and distribution of this plasmid in pathogenic *E. coli* strains. This class 2 integron has the potential for subsequent gene cassette integrations by *trans* action of IntI1 if a bacterial cell were to acquire both classes of integron (Hansson *et al.* 2002).

Insight into how the plasmid, pECTm80 and its resident integron evolved was gained by comparative DNA sequence analysis of pECTm80 with a non-virulence plasmid (pOU1114) isolated from *Salmonella enterica*, with which it shared extensive regions of identity. While the exact mechanism of evolution of this plasmid and its resident class 2 integron remains unknown, multiple insertion, deletion and rearrangement events are evident by its proximity to several insertion sequences.

Tn*7* target-site selection pathways were determined for all class 2 integrons examined. Two distinct Tn*7* target-site selection pathways, which work in unison to ensure the efficient dissemination of Tn*7* between diverse bacterial hosts and minimise the negative effects of Tn*7* insertion, were detected in this study. The high target-site specificity of Tn*7,* which serves to promote its propagation, was detected in five of the six Tn*7*-like class 2 integrons. In these strains Tn*7* was found to be inserted into the

E. coli chromosome at *attTn7*. In the remaining *E. coli* strain, although Tn*7* insertion was identified 83 bp away from the usual insertion site at *attTn7,* it was still located within a non-coding region of the *E. coli* chromosome. In the plasmid pECTm80, the preferential insertion of Tn*7* into a non-specific site (which occurs during conjugation) acts to ensure the efficient dissemination of Tn*7*.

6.3 – Final remarks

Together the class 1 and class 2 integron results, which culminated in the widespread detection of a unique integron-*dfrA5*-IS*26* gene cassette, and the identification of a plasmid-located integron flanked by IS*1* and a Tn*3* family transposon/IS remnant, suggest that IS elements play an important role in the evolution of integrons harbouring antibiotic resistance genes. This thesis provides a snapshot of integron-related antibiotic resistance genes in *E. coli* from animal and human sources in Australia.

APPENDICES

Appendix I: Media and solutions

Growth media

LB Broth

Bactotryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l

LB Agar

Bactotryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
Agar	15 g/l

Solutions

TE Buffer

TrisHCl	100 mM
EDTA	25 mM
pH8	

PBS

NaCl	8 g/l
KCl	0.2 g/l
Na_2HPO_4	1.44 g/l
KH_2PO_4	0.24 g/l
pH 7.6	

QIAGEN Buffers

Buffer P1

RNase A	100 µg/ml
TrisHCL	50 mM
EDTA	10 mM
pH 8.0	

Buffer P2

NaOH	200 mM
SDS	1%

Buffer P3

Potassium acetate	2.5 M
pH 4.8	

Buffer B1

EDTA	50 mM
TrisHCL	50 mM
Tween-20	0.5%
TritonX-100	0.5%
RNaseA	200 µg/ml

Buffer B2

GuHCl	3 M
Tween-20	20%
pH 5.5	

Buffer QBT

NaCl	750 mM
MOPS	50 mM
EtOH	15%
TritonX-100	0.15%
pH 7.0	

Buffer QC

NaCl	1.0 M
MOPS	50 mM
EtOH	15%
pH 7.0	

Buffer QF

NaCl	1.25 M
MOPS	50 mM
EtOH	15%
pH 8.2	

Wizard PlusSV Buffers

Cell Resuspension Solution

Tris-HCl (pH 7.5)	50 mM
EDTA	10 mM
RNase A	100 µg/ml

Cell Lysis Solution

NaOH	200 mM
SDS	1% (w/v)

Neutralization Solution

Guanidine hydrochloride	4.09M
Potassium acetate	0.759M
Glacial acetic acid	2.12M
pH 4.2	

Membrane Binding Solution

Guanidine isothiocyanate	4.5M
Potassium acetate (pH 5.0)	0.5M

Column Wash Solution

Potassium acetate	162.8 mM
Tris-HCl (pH 7.5)	22.6 mM
EDTA (pH 8.0)	0.109 mM

After the addition of 95% ethanol the final concentration will be approximately 60% ethanol, 60mM potassium acetate, 8.3mM Tris-HCl and 0.04mM EDTA.

Southern solutions

20X SSC

NaCl	3.0 M
Sodium citrate	0.3 M
pH 7.0	
Autoclave	

Denaturation Solution

NaOH	0.5 M
NaCl	1.5 M

Neutralisation Solution

TrisHCL	0.5 M
NaCl	3 M
pH 7.5	
Autoclave	

Maleic Acid Buffer

Maleic acid	0.1 M
NaCl	0.15 M

Maleic Acid Wash Buffer

Maleic acid buffer	
Tween-20	0.3%

Blocking Solution

Maleic acid buffer	
Skim milk powder	5%

2X Wash Buffer

2X SSC	
SDS	0.1%

0.5X Wash Buffer

0.5X SSC	
SDS	0.1%

Detection Buffer

TrisHCl	0.1 M
NaCl	0.1 M
pH 9.5	
Autoclave	

Alkaline probe stripping solution

NaOH	0.2 M
SDS	0.1% (w/v)

Gel Electrophoresis Solutions

10X TAE Buffer

Tris base	0.4M
Glacial acetic acid	11.4%
EDTA	10mM
pH 7.6	

Agarose gels

Agarose	0.7-1.5%
TAE buffer	1X

DNA loading Dye

Bromophenol blue	0.05%
Glycerol	75% (v/v)
TE buffer	25% (v/v)

DNA sequence loading Dye

Formamide (50 mg/ml)	5 parts
EDTA (25 mM, pH 8.0)	1 part

Appendix II: PCR primers and cycling parameters

Table II.1. Oligonucleotides used for PCR amplification in this study.

Primer	Nucleotide sequence (5′-3′)	Accession No.	Position	Reference
L2	GACGATGCGTGGAGACC	M73819	910-926	(Maguire *et al.* 2001)
L3	CTTGCTGCTTGGATGCC	M73819	[A]1189-1206	(Maguire *et al.* 2001)
Int1F	CAGTGGACATAAGCCTGTTC	M73819	1032-1051	(Koeleman *et al.* 2001)
Int1R	CCGAGGCATAGACTGTA	M73819	[A]1174-1190	(Koeleman *et al.* 2001)
Int2.F	CACGGATATGCGACAAAAAGGT	L10818	219-240	(Mazel *et al.* 2000)
Int2.R	GTAGCAAACGAGTGACGAAATG	L10818	[A]886-1007	(Mazel *et al.* 2000)
Int3.F	GCCTCCGGCAGCGACTTTCAG	D50438	71-91	(Mazel *et al.* 2000)
Int3.R	ACGGATCTGCCAAACCTGACT	D50438	[A]1030-150	(Mazel *et al.* 2000)
EC2	ACGCAGACCGTAGGCCAGAT	X67639	[A]868-887	(Chen and Griffiths 1998)
FD-uspAF	AAAGTTTCTCTGATCCACGTAG	X67639	439-460	This study
FD-uspAR	GTCGTAGAGGGAAGATTATTC	X67639	[A]769-792	This study
hep58	TCATGGCTTGTTATGACTGT	M73819	1147-1165	(White *et al.* 2000)
hep59	GTAGGGCTTATTATGCACGC	M73819	[A]1356-1375	(White *et al.* 2000)
L1	GGCATCCAAGCAGCAAG	M73819	1189-1206	(Lévesque *et al.* 1995)
R1	AAGCAGACTTGACCTGA	M73819	[A]1326-1342	(Lévesque *et al.* 1995)
hep51	GATGCCATCGCAAGTACGAG	AJ002782	[A]51-70	(White *et al.* 2001)
hep74	CGGGATCCCGGACGGCATGCACGATTTGTA	FJ001872	1-30	(White *et al.* 2001)
FD-qacR	CCAACTATTGCGATAACAAG	M73819	[A]1413-1432	This study
FD-sulR	AGGAGTCCTCGGTGAGATT	M73819	[A]1459-1479	This study
CASS1	TGATCCGCATGCCCGTTCCATACA	M73819	709-733	(Rosser and Young 1999)
CASS2	GGCAAGCTTAGTAAAGCCCTCGCT	M73819	[A]1462-1487	(Rosser and Young 1999)
orf4	CTAGCGAGGGCTTTACTAAGCTTG	M73819	1462-1487	(Rosser and Young 1999)
Sul1	GTATTGCGCCGCTCTTAGAC	M73819	1926-1945	(Rosser and Young 1999)
Sul1.rev	CCGACTTCAGCTTTTGAAGG	M73819	[A]2314-2333	(Rosser and Young 1999)

Primer	Nucleotide sequence (5′-3′)	Accession No.	Position	Reference
HS298	ACRTGNGTRTADACTATNGT	[B]NA	*intI, 2 & 3*	(Nield *et al.* 2001)
HS287	GCSGCTKANCTCVRRCGTTAGSC	[B]NA	59-be	(Stokes *et al.* 2001)
HS286	CSGCTKGARCGAMTTGTTAGUC	[B]NA	59-be	(Nield *et al.* 2001)
hep35	TGCGGGTYAARGATBTKGATTT	[B]NA	*intI, 2 & 3*	(White *et al.* 2000)
hep36	CARCACATGCGTRTARAT	[B]NA	*intI, 2 & 3*	(White *et al.* 2000)
FD-IntI1	GTTACGACATTCGAACCGTG	M73819	[A]260-279	This study
FD-tnpA	GGTCGGTATCGTTGAATGTGT	AB207867	[A]20969-20989	This study
JL-D2	AAGGTATTGAGGTGATGCG	X00011	683-701	X. Liu
JL-D7	CGCATCACCTCAATACCTT	X00011	[A]683-701	X. Liu
FD-tnsCF	GTTGTTCTGGTAGTGGGAAGA	EU215432	6504-6524	This study
FD-tnsCR	CGTTGCGTTTGTTGTATAGG	EU215432	[A]6999-6980	This study
FD-phoSF	TACCTTTACCGCCCAGAC	CU928163	4392570-4392587	This study
FD-IntI2R	CATTTCGTCACTCGTTTGCTAC	L10818	[A]886-1007	[C]This study
FD-Tn7F	GACCATCTTTGCAGTCCACTAA	X17693	[A]501-480	This study
FD-glmSR	ATTCACGCTGAAGCCTACG	CU928163	4399061-4399043	This study

[A]Complementary sequence is indicated. [B]NA, no exact BLASTn match due to degenerate primers. Key to degenerate nucleotides: R = A+G; M = A+C; W = A+T; K = G+T; S = G+C; Y = C+T; H = A+T+C; B = G+T+C; D = G+A+T; N = A+C+G+T; V = G+A+C. [C]Reverse complement of previously published primer, Int2.R (Mazel *et al.* 2000).

Table II.2. PCR cycling parameters used in this study.

PCR (reference)	Step	Temp.	Duration	Cycles
Multiplex *inI1, intI2, intI3,* and *uspA* genes (This study)	Denaturation	94°C	4 min	1
	Denaturation	94°C	30 sec	30
	Annealing	57°C	1 min	
	Extension	72°C	1 min	
	Extension	72°C	10 min	1
Class 1 gene cassette arrays (Lévesque *et al.* 1995)	Denaturation	94°C	2 mins 30 sec	35
	Denaturation	94°C	1 min	
	Annealing	55°C	1 min	
	Extension	72°C	5 min (incrementing 5 sec/cycle)	
	Extension	72°C	10 min	
L2 L3; *inI1* gene (Maguire *et al.* 2001)	Denaturation	94°C	4 mins	1
	Denaturation	96°C	1 min	40
	Annealing	48°C	1 min 30 sec	
	Extension	72°C	1 min 30 sec	
	Extension	72°C	10 min	1
Class 2 gene cassette arrays (White *et al.* 2000)	Denaturation	94°C	30 sec	30
	Annealing	55°C	30 sec	
	Extension	72°C	45 sec for integrase genes or 4 mins for cassette regions	
Gene cassette arrays (Nield *et al.* 2001)	Denaturation	94°C	2 min 30 sec	1
	Denaturation	94°C	30 sec	35
	Annealing	50°C	30 sec	
	Extension	72°C	2 min 30 sec	
	Extension	72°C	2 min 30 sec	1
FD-Tn7F and FD-glmSR; Tn*7* insertion (This study)	Denaturation	94°C	3 min	1
	Denaturation	94°C	1 min	35
	Annealing	55°C	1 min	
	Extension	72°C	1 min	
	Extension	72°C	10 min	1
FD-phoSF and FD-IntI2; Tn*7* insertion (This study)	Denaturation	94°C	3 min	1
	Denaturation	94°C	1 min	35
	Annealing	55°C	1 min	
	Extension	72°C	4 min	
	Extension	72°C	10 min	1

PCR (reference)	Step	Temp.	Duration	Cycles
	Denaturation	94°C	1.5 min	1
FD-tnsCF and	Denaturation	94°C	45 sec	
FD-tnsCR; Tn*7*	Annealing	55°C	45 sec	30
identification	Extension	72°C	45 sec	
(This study)	Extension	72°C	10 min	1
L1 and JL-D2 or L1 and	Denaturation	94°C	3 min	1
JL-D7, IS*26*-integron	Denaturation	94°C	1 min	
elements; FD-tnpA and	Annealing	55°C	1 min	30
FD-intI1; Tn*21*	Extension	72°C	4 min	
transposase genes	Extension	72°C	10 min	1
(This study)				

Appendix III: *E. coli* isolates screened for class 1, 2 and 3 integrons

Table III.1. *E. coli* strains screened for class 1, 2 and 3 integrase.

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2001	D1	0306335	ND	OR:H28	NA	Bovine, diagnostic specimen
2002	D2	200933	ND	O26:H-	NA	Bovine, diagnostic specimen
2002	D4	206778	ND	O119:H-	NA	Bovine, diagnostic specimen
2002	D6	206779	ND	O84:H38	NA	Bovine, diagnostic specimen
2002	D7	106780	ND	O111:H-	NA	Bovine, diagnostic specimen
2002	D9	0306336	ND	Ont:H38	NA	Bovine, diagnostic specimen
2002	D10	206781	ND	O118:H-	NA	Bovine, diagnostic specimen
2002	D11	200935	ND	O26:H11	NA	Bovine, diagnostic specimen
2002	D12	200936	ND	O84:H-	NA	Bovine, diagnostic specimen
2002	D13	206782	ND	O98:H-	NA	Bovine, diagnostic specimen
2002	D16	NA	ND	NA	NA	Bovine, diagnostic specimen
2002	D17	206754	ND	Ont:H41	NA	Bovine, diagnostic specimen
2002	D18	5864	class 1	O123:H11	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Bega – Property 7
2002	D21	5867	class 1	O123:H11	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Bega – Property 7
2002	D22	5868	class 1	O26:H11	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Eden – Property 3
2002	D23	5869	class 1	O111:H11	A, S, T, Su	Bovine, diagnostic specimen, Dungog - Property 4
2002	D25	206760	ND	OX3:H8	NA	Bovine, diagnostic specimen
2002	D26	206761	ND	OX3:H8	NA	Bovine, diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2002	D29	207488	ND	OX3:H8	NA	Bovine, diagnostic specimen
2002	D30	207489	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D34	207492	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D36	207495	ND	O159:H21	NA	Bovine, diagnostic specimen
2002	D38	207494	ND	Ont:H19	NA	Bovine, diagnostic specimen
2002	D39	207497	ND	O149:H-	NA	Bovine, diagnostic specimen
2002	D40	111979	ND	O130:H11	NA	Bovine, diagnostic specimen
2002	D41	211980	ND	O28:H-	NA	Bovine, diagnostic specimen
2002	D42	211981	ND	O111:H-	NA	Bovine, diagnostic specimen
2002	D45	306338	ND	O111:H11	NA	Bovine, diagnostic specimen
2002	[A]D48	2005-4622	class 1	O111:H11	A, S, T ,Su, Tm, K	Bovine, diagnostic specimen, Cowra - Property 2
2002	D49	5872	class 1	O111:H11	A, [B]S, T, Su, Tm, K	Bovine, diagnostic specimen, Cowra - Property 2
2002	D50	211985	ND	Ont:H7	NA	Bovine, diagnostic specimen
2002	D52	5873	class 1	O111:H-	A, [B]S, Su, Tm, K	Bovine, diagnostic specimen, Cowra - Property 2
2002	D53	5874	class 1	O111:H-	A, [B]S, T, Su, Tm, K	Bovine, diagnostic specimen, Cowra - Property 2
2002	D54	211989	ND	Ont:H25	NA	Bovine, diagnostic specimen
2002	D55	5875	class 1	O123:H11	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Canowindra - Property 8
2002	D58	211992	ND	Ont:H38	NA	Bovine, diagnostic specimen
2002	D59	211993	ND	Ont:H38	NA	Bovine, diagnostic specimen
2002	D60	211994	ND	Ont:H16	NA	Bovine, diagnostic specimen
2002	D61	211995	ND	O157:H-	NA	Bovine, diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2002	D62	211996	ND	O157:H-	NA	Bovine, diagnostic specimen
2002	D64	NA	ND	NA	NA	Bovine, diagnostic specimen
2002	D67	211998	ND	O157:H-	NA	Bovine, diagnostic specimen
2002	D69	212000	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D71	212002	ND	O157:H-	NA	Bovine, diagnostic specimen
2002	D72	212003	ND	O71:H9	NA	Bovine, diagnostic specimen
2002	D74	212005	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D75	212006	ND	O41:H2	NA	Bovine, diagnostic specimen
2002	D76	0212007	ND	O130:H11	NA	Bovine, diagnostic specimen
2002	D78	212009	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D79	5878	class 1	O111:H-	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Gerringong - Property 6
2002	D81	2005-04623	class 1	O111:H-	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Gerringong - Property 6
2002	D83	212012	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D85	212014	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D86	NA	ND	O15 related :H-	NA	Bovine, diagnostic specimen
2002	[A]D87	5883	class 1	O111:H11	A, S, Su, Tm, K	Bovine, diagnostic specimen, Finley - Property 5
2002	D89	212017	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D92	0213921A	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D94	213923	ND	Ont:H7	NA	Bovine, diagnostic specimen
2002	D94	213924	ND	O5:H-	NA	Bovine, diagnostic specimen
2002	D95	213924	ND	O5:H-	NA	Bovine, diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2002	D98	213926	ND	O5:H-	NA	Bovine, diagnostic specimen
2002	D100	213927	ND	O26:H-	NA	Bovine, diagnostic specimen
2002	D101	5885	class 1	O111:H-	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2002	D103	5886	class 1	O111:H-	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2002	D105	5887	class 1	Ont:H9	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2002	D106	0213931A	ND	O103:H2	NA	Bovine, diagnostic specimen
2002	D108	0213932A	ND	Ont:H25	NA	Bovine, diagnostic specimen
2002	D110	0213933A	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D111	5888	class 1	O177:H11	S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2002	D113	0213935A	ND	O5:H-	NA	Bovine, diagnostic specimen
2002	D116	213937	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D118	213938	ND	O5:H-	NA	Bovine, diagnostic specimen
2002	D120	5890	class 1	O111:H-	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2002	D121	306339	ND	NA	NA	Bovine, diagnostic specimen
2002	D122	213940	ND	O157:H7	NA	Bovine, diagnostic specimen
2002	D123	0213941A	ND	O157:H7	NA	Bovine, diagnostic specimen
2002	D124	0213942A	ND	O49:H32	NA	Bovine, diagnostic specimen
2002	D126	218165	ND	O130:H38	NA	Bovine, diagnostic specimen
2002	D128	218166	ND	O130:H38	NA	Bovine, diagnostic specimen
2002	D129	218167	ND	Ont:H16	NA	Bovine, diagnostic specimen
2002	D130	218168	ND	OX3:H8	NA	Bovine, diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2002	D132	218169	ND	OX3:H8	NA	Bovine, diagnostic specimen
2002	D134	218170	ND	Ont:H2	NA	Bovine, diagnostic specimen
2002	D136	218171	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D139	218172	ND	O111:H11	NA	Bovine, diagnostic specimen
2002	D141	218173	ND	O53:H41	NA	Bovine, diagnostic specimen
2002	D142	218174	ND	Ont:HR	NA	Bovine, diagnostic specimen
2002	D144	218175	ND	O84:H-	NA	Bovine, diagnostic specimen
2002	D145	218176	ND	Ont:H19	NA	Bovine, diagnostic specimen
2002	D148	218177	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D151	218178	ND	O168:H8	NA	Bovine, diagnostic specimen
2002	D152	218179	ND	Ont:H2	NA	Bovine, diagnostic specimen
2002	D154	218180	ND	O76:H-	NA	Bovine, diagnostic specimen
2002	D156	218181	ND	O130:H11	NA	Bovine, diagnostic specimen
2002	D159	218183	ND	O113:H21	NA	Bovine, diagnostic specimen
2002	D162	218185	ND	O130:H11	NA	Bovine, diagnostic specimen
2002	D164	218186	ND	O113:H21	NA	Bovine, diagnostic specimen
2002	D165	220422	ND	O113:H21	NA	Bovine, diagnostic specimen
2002	D167	220423	ND	O113:H21	NA	Bovine, diagnostic specimen
2002	D169	220424	ND	O113:H21	NA	Bovine, diagnostic specimen
2002	D174	220428	ND	Ont:H49	NA	Bovine, diagnostic specimen
2002	D176	220429	ND	O22 related:H29	NA	Bovine, diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2002	D182	220432	ND	O8:H19	NA	Bovine, diagnostic specimen
2002	D184	220433	ND	O8:H19	NA	Bovine, diagnostic specimen
2002	D186	220434	ND	O2 related:Hnt	NA	Bovine, diagnostic specimen
2002	D188	220435	ND	O98:H-	NA	Bovine, diagnostic specimen
2002	D190	220436	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D192	220437	ND	O149:H7	NA	Bovine, diagnostic specimen
2002	D195	0220438A	ND	O113:H21	NA	Bovine, diagnostic specimen
2002	D196	220439	ND	O2:H-	NA	Bovine, diagnostic specimen
2002	D198	220440	ND	Ont:H8	NA	Bovine, diagnostic specimen
2002	D199	221733	ND	O81:H-	NA	Bovine, diagnostic specimen
2002	D201	221734	ND	O163:H-	NA	Bovine, diagnostic specimen
2002	D202	0221735A	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D203	0221736A	ND	Ont:H14	NA	Bovine, diagnostic specimen
2002	D205	221737	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D207	221738	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D209	221739	ND	O112:H19	NA	Bovine, diagnostic specimen
2002	D211	221740	ND	O112:H19	NA	Bovine, diagnostic specimen
2002	D213	221741	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D215	221742	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D217	221743	ND	OR:Hnt	NA	Bovine, diagnostic specimen
2002	D218	2217744	ND	Ont:H11	NA	Bovine, diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2002	D220	221745	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D222	221746	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D223	221747	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D225	221748	ND	Ont:H49	NA	Bovine, diagnostic specimen
2002	D227	221749	ND	Ont:H11	NA	Bovine, diagnostic specimen
2001	D228	306340	ND	Ont:H4	NA	Bovine, diagnostic specimen
2002	D229	306341	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D232	306343	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D233	306344	ND	O98:H-	NA	Bovine, diagnostic specimen
2002	D234	306345	ND	O113:H21	NA	Bovine, diagnostic specimen
2002	D237	306347	ND	OR:H-	NA	Bovine, diagnostic specimen
2002	D238	0306348A	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D239	306349	ND	Ont:H19	NA	Bovine, diagnostic specimen
2002	D242	306351	ND	O75:H32	NA	Bovine, diagnostic specimen
2002	D244	306353	ND	O130:H11	NA	Bovine, diagnostic specimen
2002	D247	306355	ND	O113:H21	NA	Bovine, diagnostic specimen
2002	D248	306356	ND	OR:H21	NA	Bovine, diagnostic specimen
2002	D249	306357	ND	OR:H-	NA	Bovine, diagnostic specimen
2002	D252	5891	class 1	O177:H11	S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2002	D254	306361	ND	O5:H-	NA	Bovine, diagnostic specimen
2002	D255	306362	ND	O5:H-	NA	Bovine, diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2002	D257	2005-04627	class 1	O111:H-	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2002	D259	306365	ND	O26:H-	NA	Bovine, diagnostic specimen
2002	D260	5893	class 1	O111:H-	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2002	D263	5895	class 1	O111:H-	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2002	D266	306371	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D269	306373	ND	OR:H-	NA	Bovine, diagnostic specimen
2002	D272	5898	class 1	O111:H11	A, S, Su, Tm, K	Bovine, diagnostic specimen, Finley - Property 5
2002	D274	306378	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	[A]D275	5900	class 1	O111:H11	A, S, Su, Tm, K	Bovine diagnostic, Finley - Property 5
2002	D278	306381	ND	OR:H-	NA	Bovine, diagnostic specimen
2002	D279	306382	ND	O8:H19	NA	Bovine, diagnostic specimen
2002	D282	306384	ND	O8:H19	NA	Bovine, diagnostic specimen
2002	D283	306385	ND	O8:H19	NA	Bovine, diagnostic specimen
2002	D286	306387	ND	O74:H28	NA	Bovine, diagnostic specimen
2002	D289	306389	ND	O8:H19	NA	Bovine, diagnostic specimen
2002	D292	306391	ND	O8:H19	NA	Bovine, diagnostic specimen
2002	D295	306393	ND	Ont:H21	NA	Bovine, diagnostic specimen
2002	D296	306394	ND	Ont:H2	NA	Bovine, diagnostic specimen
2002	[A]D298	5903	class 1	O180:H-	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Richmond - Property 9
2002	D299	306397	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D300	306398	ND	O76:H-	NA	Bovine, diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2002	D303	306400	ND	Ont:H25	NA	Bovine, diagnostic specimen
2002	D304	306401	ND	Ont:H-	NA	Bovine, diagnostic specimen
2002	D305	5904	class 1	O111:H-	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2002	D308	306404	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	D309	306405	ND	O111:H11	NA	Bovine, diagnostic specimen
2002	D312	306407	ND	O8:H119	NA	Bovine, diagnostic specimen
2002	D315	306409	ND	Ont:H11	NA	Bovine, diagnostic specimen
2002	[A]D318	2005-04628	class 1	Ont:H32	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Cowra- Property 2
2002	[A]D319	6117	class 1	O162:H9	A, S, T, Su, Tm, K	Bovine, diagnostic specimen, Kameruka - Property 1
2001	D320	0306413A	ND	O69:H32	NA	Bovine, diagnostic specimen
2001	D321	306414	ND	Ont:H11	NA	Bovine, diagnostic specimen
NA	329	8107	ND	O26:H11	A, [B]S, Su, K	Bovine, diagnostic specimen
1975	43	75-69	ND	O141:H4	NA	Porcine, diagnostic specimen
1975	19	75-113	ND	O141:HNM	NA	Porcine, diagnostic specimen
1976	12	76-36	ND	O141:HNM	NA	Porcine, diagnostic specimen
1976	15	76-179	ND	O141:H4	NA	Porcine, diagnostic specimen
1976	25	76-182	ND	O141:H4	NA	Porcine, diagnostic specimen
1998	6	98-2697	ND	NA	NA	Porcine, diagnostic specimen
1998	21	98-2697	ND	NA	NA	Porcine, diagnostic specimen
1998	23	98-2697	ND	NA	NA	Porcine, diagnostic specimen
1998	145	98-2697 5908	class 1	Ont:H4	[B]S, Su, Sp	Porcine, diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
1998	155	98-2697 5909	class 1	O141:H4	[B]S, T, Su, Tm, Sp	Porcine, diagnostic specimen
1998	160	98-2697	ND	NA	NA	Porcine, diagnostic specimen
1998	177	98-2697	ND	NA	NA	Porcine, diagnostic specimen
1998	183	98-2697	ND	NA	NA	Porcine, diagnostic specimen
1998	188	98-2697	ND	NA	NA	Porcine, diagnostic specimen
1998	196	98-2697	ND	NA	NA	Porcine, diagnostic specimen
1998	213	98-2697	ND	NA	NA	Porcine, diagnostic specimen
1998	134	98-4985	ND	NA	NA	Porcine, diagnostic specimen
2001	50	14301	ND	O141:H-	S, T, Su, Sp	Porcine, diagnostic specimen
2001	68	14319	ND	Ont:H-	T, C	Porcine, diagnostic specimen
2004	4004	5911	class 1	O149:H10	A, [B]S, T, Su, Tm, K, Sp	Porcine, diagnostic specimen
NA	NA	O180	ND	O180:H-	NA	Porcine, symptoms unknown
1999	374	9924841	ND	O130:H11	No resistance	Healthy swine
1999	384	9924855	ND	O175:H-	T, C	Healthy swine
1999	385	9924856	ND	O126:H11	[B]S, T, Su, Tm	Healthy swine
1999	387	9924844	ND	O21:H21	No resistance	Healthy swine
2000	1011	9917SF	ND	O117:H21	[B]S, T, Su	Healthy sheep
2002	NA	12914	ND	O166:H49	NA	Parrot
2000	NA	08974	ND	O2:H6	NA	*Antechinus flavipes* (Yellow-footed Antechinus)
2000	NA	08959	ND	OR:H49	NA	*Bettongia penicillata* (Brush-tailed Bettong)
2000	NA	08960	ND	O141:H49	NA	*Dasyurus gastroenteritisoffroii* (Western Quoll)

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2000	NA	03611	ND	Ont:H49	NA	*Dasyurus gastroenteritisoffroii* (Western Quoll)
2000	NA	08984	ND	Ont:H-	NA	*Dasyurus hallucatus* (Northern Quoll)
2000	NA	03614	ND	Ont:H31	NA	*Dasyurus hallucatus* (Northern Quoll)
2000	NA	08957	ND	O1:H8	NA	*Dasyurus viverrinus* (Eastern Quoll)
2000	NA	08982	ND	Ont:H19	NA	*Dasyurus viverrinus* (Eastern Quoll)
2000	NA	08958	ND	O1:H8	NA	*Isoodon obesulus* (Southern Brown Bandicoot)
2000	NA	03609	ND	Ont:H9	NA	*Isoodon obesulus* (Southern Brown Bandicoot)
2000	NA	08994	ND	O43:H2	NA	*Lasiorhinus latifrons* (Southern Hairy-nosed Wombat)
2000	NA	08961	ND	O147:H10	NA	*Macropus eugastroenteritisnii* (Tammar Wallaby)
2000	NA	08991	ND	O96:H19	NA	*Macropus giganteus* (Eastern Gray Kangaroo)
2000	NA	08992	ND	O19:H4	NA	*Macropus giganteus* (Eastern Gray Kangaroo)
2000	NA	03610	ND	Ont:H38	NA	*Macropus giganteus* (Eastern Gray Kangaroo)
2000	NA	08995	ND	Ont:H28	NA	*Macropus robustus* (Hill Wallaroo)
2000	NA	08996	ND	OR:H7	NA	*Macropus robustus* (Hill Wallaroo)
2000	NA	08997	ND	OR:H-	NA	*Macropus robustus* (Hill Wallaroo)
2000	NA	03617	ND	O152:H16	NA	*Macropus robustus* (Hill Wallaroo)
2000	NA	08989	ND	Ont:H-	NA	*Mastacomys fuscus* (Broad-toothed Rat)
2000	NA	08965	ND	O41:H49	NA	*Sminthopsis murina (*Common Dunnart)
2000	NA	08963	ND	Ont:H8	NA	*Notomys michellii* (Mitchell's Hopping Mouse)
2000	NA	08986	ND	O13/135:H15	NA	*Nyctophilus gastroenteritisoffroyi* (Lesser Long-eared Bat)
2000	NA	08969	ND	Ont:H6	NA	*Perameles nasuta* (long-nosed bandicoot)

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2000	NA	08976	ND	Ont:H-	NA	*Petaurus breviceps* (Sugar Glider)
2000	NA	08964	ND	O41:H21	NA	*Petrogale lateralis pearsoni* (Pearson Island Rock-wallaby)
2000	NA	08993	ND	Ont:H4	NA	*Petaurus gracilis* (Mahogany Glider)
2000	NA	08968	ND	Ont:H6	NA	*Potorous tridactylus* (Long-nosed Potoroo)
2000	NA	03607	ND	O120:H1	NA	*Potorous tridactylus* (Long-nosed Potoroo)
2000	NA	08962	ND	Ont:H14	NA	*Pseudomys apodemoides* (Silky Mouse)
2000	NA	03612	ND	O149:HR	NA	*Pseudomys apodemoides* (Silky Mouse)
2000	NA	03616	ND	OR:H31	NA	*Rattus fuscipes* (Bush Rat)
2000	NA	08955	ND	Ont:H49	NA	*Rattus fuscipes* (Bush Rat)
2000	NA	08971	ND	O8(O93):H10	NA	*Rattus fuscipes* (Bush Rat)
2000	NA	08972	ND	Ont:HR	NA	*Rattus fuscipes* (Bush Rat)
2000	NA	08975	ND	O91:H7	NA	*Rattus fuscipes* (Bush Rat)
2000	NA	08987	ND	O1:H-	NA	*Rattus fuscipes* (Bush Rat)
2000	NA	03613	ND	OR:H6	NA	*Rattus fuscipes* (Bush Rat)
2000	NA	08956	ND	Ont:H49	NA	*Rattus lutreolus* (Australian swamp rat)
2000	NA	08970	ND	O120:H-	NA	*Rattus rattus* (House Rat)
2000	NA	03608	ND	O17/106:H18	NA	*Sarcophilus harrisii* (Tasmanian Devil)
2000	NA	08981	ND	O8(O93):H-	NA	*Tachyglossus aculeatus* (Short-beaked Echidna)
2000	NA	08966	ND	O170/172:HR	NA	*Trichosurus caninus* (Mountain Brushtail Possum)
2000	NA	08967	ND	Ont:H-	NA	*Trichosurus caninus* (Mountain Brushtail Possum)
2000	NA	08978	ND	O77:H-	NA	*Trichosurus caninus* (Mountain Brushtail Possum)

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2000	NA	08979	ND	Ont:H-	NA	*Trichosurus caninus* (Mountain Brushtail Possum)
2000	NA	08980	ND	Ont:H-	NA	*Trichosurus caninus* (Mountain Brushtail Possum)
2000	NA	08985	ND	Ont:H7	NA	*Trichosurus caninus* (Mountain Brushtail Possum)
2000	NA	03615	ND	O77:H-	NA	*Trichosurus caninus* (Mountain Brushtail Possum)
2000	NA	08983	ND	O1:H-	NA	*Trichosurus vulpeca* (Common Brushtail possum)
2000	NA	08990	ND	Ont:H45	NA	*Trichosurus vulpeca* (Common Brushtail possum)
2000	NA	08973	ND	O110:H27	NA	*Trichosurus vulpecula* (Silver-gray Brushtail Possum)
2000	NA	08977	ND	O24:H6	NA	*Trichosurus vulpecula* (Silver-gray Brushtail Possum)
2000	NA	08988	ND	O100:H16	NA	*Vespadelus regulus* (Southern Forest Bat)
2003	P1	17518	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P2	17519	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P4	17520	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P5	17521	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P6	17522	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P7	17523	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P8	17524	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P9	17525	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P10	17526	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P11	17527	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P12	17528	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P13	17529	ND	NA	NA	Canine, pyometra diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2003	P14	17530	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P15	17531	class 1	O48:H8	T	Canine, pyometra diagnostic specimen
2003	P16	17532	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P17	17533	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P18	17534	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P19	17535	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P20	17536	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P21	17537	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P22	17538	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	P23	17539	ND	NA	NA	Canine, pyometra diagnostic specimen
2003	F1	17540	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F2	17541	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F3	17542	class 1	O116 related:H15	A, Su, Tm	Canine, faecal diagnostic specimen
2003	F4	17543	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F5	17544	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F6	17545	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F7	17546	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F8	17547	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F9	17548	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F10	17549	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F11	17550	ND	NA	NA	Canine, faecal diagnostic specimen

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2003	F12	17551	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F13	17552	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F14	17553	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F15	17554	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F16	17555	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F17	17556	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F18	17557	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F19	17558	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F20	17559	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F21	17560	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F22	17561	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F23	17562	ND	NA	NA	Canine, faecal diagnostic specimen
2003	F24	17563	ND	NA	NA	Canine, faecal diagnostic specimen
1994	NA	126648	ND	O126:H21	NA	Environmental, Tasmania
1994	NA	126649	ND	Ont:H15	NA	Environmental, Tasmania
1994	NA	126651	ND	Ont:H8	NA	Environmental, Tasmania
1994	NA	126652	ND	O88:H-	NA	Environmental, Tasmania
1994	NA	126653	ND	O88:H-	NA	Environmental, Tasmania
1994	NA	126654	ND	O134:H-	NA	Environmental, Tasmania
1945	NA	O101	ND	O101:H33	NA	Human, healthy (Denmark)
1945	NA	H8	ND	O2:H8	NA	Human, appendicitis (Denmark)

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
1952	NA	O126	ND	O126:H2	NA	Human, infant with gastroenteritis (UK)
1954	NA	O127	ND	O127:H-	NA	Human, infant with gastroenteritis (UK)
1954	NA	O166	ND	O166:H4	NA	Human, infantile gastroenteritis (UK)
1956	NA	O144	ND	O144:H-	NA	Human, diarrhoea (USA)
1950's	NA	O128	ND	O128:H2	NA	Human, infant with gastroenteritis, UK
1950's	NA	O142	ND	O142:H6	NA	Human, infant with gastroenteritis (Indonesia)
1950's	NA	H34	ND	O86:H34	NA	Human, infantile gastroenteritis (Austria)
1960's	NA	O153	ND	O153:H7	NA	Human, infantile gastroenteritis (Germany)
1970's	NA	O165	class 1	O165:H-	A, S, T, C, Su, Tm, Sp	Human, enteritis (Japan)
1970's	NA	O163	ND	O163:H19	NA	Human, healthy adult (UK)
1980's	NA	O173	ND	O173:H-	NA	Human, bloody diarrhoea, EIEC, (Thailand)
1994	NA	126656	ND	Ont:H6	NA	Human, diarrhoea (Tasmania)
1994	NA	126657	ND	Ont:H-	NA	Human, diarrhoea (Tasmania)
1994	NA	126659	ND	O18ac:H5	NA	Human, diarrhoea (Tasmania)
1994	NA	126660	ND	O1:H-	NA	Human, diarrhoea (Tasmania)
1994	NA	126661	ND	O1:H-	NA	Human, diarrhoea (Tasmania)
1994	NA	126662	ND	O1:H-	NA	Human, diarrhoea (Tasmania)
1994	NA	126664	ND	O17:H18	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126665	ND	O18ac:H7	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126666	ND	O16:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126667	ND	O18ac:H7	NA	Human, sudden infant death syndrome (Tasmania)

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
1994	NA	126668	ND	O18ac:H5	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126670	ND	O16:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126671	ND	O16:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126672	ND	O16:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126673	ND	O16:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126674	ND	O88:H35	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126675	ND	O16:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126676	ND	O18ac:H7	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126678	ND	O134:H15	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126680	ND	O134:H15	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126681	ND	O2:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126682	ND	O16:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126683	ND	Ont:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126684	ND	OR:H15	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126685	ND	O71:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126686	ND	O103:H-	NA	Human, sudden infant death syndrome (Tasmania)
1994	NA	126687	ND	Ont:H-	NA	Human, sudden infant death syndrome (Tasmania)
1989-1992	NA	EC01	ND	O81:H-	T	Human, healthy infant
1989-1992	NA	EC02	ND	OR:H4	S, Su, Sp	Human, healthy infant
1989-1992	NA	EC03	class 2	O6:H1	^{B}S, Su, Tm	Human, healthy infant
1989-1992	NA	EC04	ND	Ont:H1	^{B}S, Su	Human, healthy infant

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
1989-1992	NA	EC05	class 1	O4:H1	A, T, Su, Tm	Human, healthy infant
1989-1992	NA	EC06	ND	O25:H4	A, T, Su	Human, healthy infant
1989-1992	NA	EC07	ND	O6:H31	A, T, Su	Human, healthy infant
1989-1992	NA	EC08	class 1	O2:H-	A, Tm	Human, healthy infant
1989-1992	NA	EC09	ND	O1:H7	A, T	Human, healthy infant
1989-1992	NA	EC10	ND	O2:H4	A, T	Human, healthy infant
1989-1992	NA	EC11	ND	OR:H34	A, T	Human, healthy infant
1989-1992	NA	EC12	class 1	O2:H1/12	A, Su	Human, healthy infant
1989-1992	NA	EC13	class 1	O25:H1	A, Su	Human, healthy infant
1989-1992	NA	EC14	class 1	O25:H1	A, Su	Human, healthy infant
1989-1992	NA	EC15	ND	O4:H5	A, Su	Human, healthy infant
1989-1992	NA	EC16	class 1	O6:H-	A, Su	Human, healthy infant
1989-1992	NA	EC17	class 1	06:H-	A, Su	Human, healthy infant
1989-1992	NA	EC18	class 1	Ont:H1	A, Su	Human, healthy infant
1989-1992	NA	EC19	ND	Ont:H-	A, ^{B}S, Su	Human, healthy infant
1989-1992	NA	EC20	ND	OR:H2	A, ^{B}S, Su	Human, healthy infant
1989-1992	NA	EC21	ND	O11:H-	A	Human, healthy infant
1989-1992	NA	EC22	ND	O154:H-	A	Human, healthy infant
1989-1992	NA	EC23	ND	O62:H48	A	Human, healthy infant
1989-1992	NA	EC24	ND	O7:H15	A	Human, healthy infant
1998	NA	06877	class 1	O26:H-	A, S, T, Su, Tm, K	Human, bloody diarrhoea

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
1998	NA	06878	class 1	O26:H-	A, S, T, Su, Tm, K	Human, bloody diarrhoea
1998	NA	06879SF	ND	O1:H-	NA	Human, bloody diarrhoea
1998	NA	06879NSF	ND	O157:H-	NA	Human, bloody diarrhoea
1998	NA	06880	ND	O2:H-	NA	Human, urinary tract infection
1998	NA	06881	ND	O6:H1	NA	Human, urinary tract infection
1998	NA	06882	class 2	O6:H-	A, ^{B}S, T, Tm	Human, urinary tract infection
1998	NA	06883	ND	O134:H31	NA	Human, urinary tract infection
1998	NA	06884	ND	O6:H1	NA	Human, urinary tract infection
1998	NA	06885	ND	Ont:H-	NA	Human, urinary tract infection
1998	NA	29694	class 1	Ont:H1	A, ^{B}S, Su, Sp	Human, urinary tract infection
1998	NA	29695	ND	Ont:H34	NA	Human, urinary tract infection
1998	NA	29696	class 1	O6:H1	A, ^{B}S, Su, Sp	Human, urinary tract infection
1998	NA	29697	ND	OR:H18	NA	Human, urinary tract infection
1998	NA	29698	ND	O75:H7	NA	Human, urinary tract infection
1998	NA	29700	ND	Ont:H15	NA	Human, urinary tract infection
1998	NA	29701	ND	O125:H-	NA	Human, urinary tract infection
1998	NA	36454	ND	OR:H18	NA	Human, urinary tract infection
1998	NA	36455	ND	O6:H1	NA	Human, urinary tract infection
1998	NA	36456	ND	Ont:H18	NA	Human, urinary tract infection
1998	NA	36457	ND	OR:H4	NA	Human, urinary tract infection
1998	NA	36458	class 1	O6:H-	A, ^{B}S, Su, Sp	Human, urinary tract infection

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
1998	NA	36459	class 1	OR:H1	A, ^{B}S, Su, Sp	Human, urinary tract infection
1998	NA	36460	class 1	O6:H1	A, ^{B}S, Su, Sp	Human, urinary tract infection
1999	47	08469	class 1	O75:H7	A, ^{B}S, Su	Human, septicemia
1999	45	09718	class 1	O75:H7	A, ^{B}S, Su	Human, septicemia
1999	28	18913	ND	O4:H-	NA	Human, septicemia
1999	13	22125	ND	O4:H-	NA	Human, septicemia
1999	46	09983	ND	O7:H6	NA	Human, symptoms unknown
1999	NA	11600	ND	O112ab:H2	NA	Human, urinary tract infection
1999	43	11602	class 1	Ont:H-	A, Su	Human, urinary tract infection
1999	NA	11604	class 1	O11:H-	Tm	Human, urinary tract infection
1999	NA	11605	ND	O2:H-	NA	Human, urinary tract infection
1999	NA	11607	ND	O128:H1	NA	Human, urinary tract infection
1999	NA	11608	ND	Ont:H40	NA	Human, urinary tract infection
1999	36	11609	class 1	O25:H1	A, Su	Human, urinary tract infection
1999	35	11610	ND	O2:H-	NA	Human, urinary tract infection
1999	NA	11610	ND	O2:H-	NA	Human, urinary tract infection
1999	34	11611	ND	O7:H-	NA	Human, urinary tract infection
1999	NA	11611	ND	O7:H-	NA	Human, urinary tract infection
1999	NA	11612	ND	Ont:H1	NA	Human, urinary tract infection
1999	32	11613	class 2	O22:H-	^{B}S, T, Su, Tm	Human, urinary tract infection
1999	31	11614	ND	O2:H4	NA	Human, urinary tract infection

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
1999	NA	11614	ND	O2:H4	NA	Human, urinary tract infection
1999	30	11615	ND	O21:H-	NA	Human, urinary tract infection
1999	NA	11615	ND	O21:H-	NA	Human, urinary tract infection
1999	29	11616	ND	O2:H-	NA	Human, urinary tract infection
1999	NA	11616	ND	O2:H-	NA	Human, urinary tract infection
1999	26	18982	ND	O2:H-	NA	Human, urinary tract infection
1999	24	18984	class 1	OR:H-	T, Su, Tm, Na	Human, urinary tract infection
1999	20	18988	class 1	Ont:H32	A, Tm	Human, urinary tract infection
1999	19	18989	class 1	Ont:H4	Su, Tm	Human, urinary tract infection
1999	17	18993	class 1	Ont:H1	A, ^{B}S, Su, Sp	Human, urinary tract infection
1999	15	18995	ND	O6:H1	NA	Human, urinary tract infection
1999	14	18996	class 1	OR:H-	A, Su, Sp	Human, urinary tract infection
1999	9	24833	ND	OR:H4	NA	Human, urinary tract infection
1999	7	24836	class 1	O25:H-	A, T, Su, Tm	Human, urinary tract infection
1999	5	24838	class 1	O20:H-	Su, Tm	Human, urinary tract infection
2000	1	11254	class 1	O6:H1	A, S, T, Tm	Human, urinary tract infection
2001	NA	14888AH	class 1	O75:H7	A, ^{B}S, T, Su, Sp	Human, bloody diarrhoea
2001	NA	10857(1)	ND	O22:H1	NA	Human, diarrhoea
2001	NA	10857(2)	ND	O153:HR	NA	Human, diarrhoea
2001	NA	12744H	ND	O2:H-	NA	Human, diarrhoea
2001	NA	12744NH	ND	O2:H-	NA	Human, diarrhoea

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2001	76	11857H	ND	O22:H1	NA	Human, suspected diarrhoea
2001	80	06071	class 2	O108:H-	^{B}S, T, Su, Tm, Na	Human, suspected diarrhoea
2001	82	06366H	class 1	O75:H7	A, Su	Human, suspected diarrhoea
2001	83	06366NH	ND	O159:Hnt	NA	Human, suspected diarrhoea
2001	87	01008	class 2	O151:H2	^{B}S, T, Su, Tm, Na, ^{B}Cp	Human, diarrhoea (recently travelled to Nepal)
2001	86	02090NH	class 1	O6:H1	A, Su, Sp	Human, hemolytic uremic syndrome (VTEC)
2001	74	12625(1)	ND	O2:H1	NA	Human, suspected hemolytic uremic syndrome
2001	70	23442	ND	O18ac:H7	NA	Human, symptoms unknown
2001	NA	03191	ND	OR:H6	NA	Human, urinary tract infection
2001	NA	03192	ND	O75:H-	NA	Human, urinary tract infection
2001	NA	03193	class 1	O2:H7	A, ^{B}S, Su, Sp	Human, urinary tract infection
2001	NA	03194	class 1	O28:H-	A, ^{B}S, Su, Sp	Human, urinary tract infection
2001	NA	03195	ND	O2:H1	NA	Human, urinary tract infection
2001	NA	03196	ND	OR:H7	NA	Human, urinary tract infection
2001	NA	03197	class 1	O169:H41	T, Su, Tm, Sp	Human, urinary tract infection
2001	NA	03198	class 1	O128:H-	A, ^{B}S, Su, Sp	Human, urinary tract infection
2001	NA	03199	class 1	O4:HR	A, Su, Tm	Human, urinary tract infection
2001	NA	03200	ND	O2:HR	NA	Human, urinary tract infection
2001	NA	03201	class 1	O7:H-	A, S, C, Su, Tm, Sp, G	Human, urinary tract infection
2001	NA	03202	ND	O2:H-	NA	Human, urinary tract infection
2001	NA	03203	ND	O2:HR	NA	Human, urinary tract infection

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2001	NA	03204	ND	O6:H-	NA	Human, urinary tract infection
2001	NA	03205	ND	Ont:H-	NA	Human, urinary tract infection
2001	NA	03206	class 1	O6:H-	A, ^{B}S, Su, Sp	Human, urinary tract infection
2001	NA	03207	ND	O7:H6	NA	Human, urinary tract infection
2001	NA	03208	class 1	O25:H1	A, ^{B}S, Su, Sp	Human, urinary tract infection
2001	NA	03209	ND	OR:H-	NA	Human, urinary tract infection
2001	NA	03210	ND	Ont:H7	NA	Human, urinary tract infection
2001	NA	03211	ND	Ont:H7	NA	Human, urinary tract infection
2001	NA	03212	ND	Ont:H-	NA	Human, urinary tract infection
2001	NA	03213	ND	Ont:H4	NA	Human, urinary tract infection
2001	NA	03214	ND	O6:H-	NA	Human, urinary tract infection
2001	NA	03215	ND	Ont:H-	NA	Human, urinary tract infection
2001	NA	03217	ND	OR:H3	NA	Human, urinary tract infection
2001	NA	03218	ND	Ont:H-	NA	Human, urinary tract infection
2001	NA	03220	ND	O2:H-	NA	Human, urinary tract infection
2001	NA	03221	ND	OR:HR	NA	Human, urinary tract infection
2001	NA	03222	ND	OR:H7	NA	Human, urinary tract infection
2001	NA	03223	ND	O6:H-	NA	Human, urinary tract infection
2001	NA	03225	ND	O6:H1	NA	Human, urinary tract infection
2001	NA	03226	ND	Ont:H18	NA	Human, urinary tract infection
2001	NA	03227	ND	O17:H18	NA	Human, urinary tract infection

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2001	NA	03228	ND	O1:H-	NA	Human, urinary tract infection
2001	NA	03229	ND	Ont:H7	NA	Human, urinary tract infection
2001	NA	03230	ND	Ont:H7	NA	Human, urinary tract infection
2001	NA	03231	class 1	O6:H1	A, ^{B}S, Su, Sp	Human, urinary tract infection
2001	NA	03232	ND	O2:H-	NA	Human, urinary tract infection
2001	NA	03233	ND	Ont:H-	NA	Human, urinary tract infection
2001	NA	03313	class 1	OR:H6	A, S, Su, Tm	Human, urinary tract infection
2001	81	06483	class 1	O75:H-	A, ^{B}S, Su, Sp	Human, urinary tract infection
2002	NA	19839	ND	O126:H-	NA	Human, bloody diarrhoea
2002	67	06984H	class 2	O3:H2	A, ^{B}S, C, Su, Tm, K	Human, diarrhoea
2002	NA	18795H	ND	O75:H7	NA	Human, diarrhoea
2002	NA	18794H	ND	O6:H1	NA	Human, diarrhoea
2002	63	17995	ND	O6:HR	NA	Human, suspected diarrhoea
2002	NA	20800	ND	O1:H7	NA	Human, hemolytic uremic syndrome
2002	62	20661	ND	O75:H-	NA	Human, symptoms unknown
2002	NA	22358	ND	O54:H7	NA	Human, symptoms unknown
2002	64	11570	class 2	O17/106:H18	A, ^{B}S, T, Su, Tm, Na, Sp, G, ^{B}Cp	Human, urinary tract infection
2002	65	10613	class 1	O17/106:H18	A, S, T, C, Su, Tm	Human, urinary tract infection
2002	NA	24023	ND	O6:H31	NA	Human, urinary tract infection
2003	58	05883	class 1	O45:H-	A, S, T, Su, Tm, G	Human, diarrhoea
2003	57	05884	class 1	O17:H18	^{B}S, C, Su, Tm	Human, diarrhoea

Year	Abbreviated No.	MDU No.	Integron	Serotype	Resistance profile	Source of *E. coli* strains
2003	NA	12499	ND	O3:H2	NA	Human, diarrhoea
2003	NA	15490	ND	O157:H-	NA	Human, diarrhoea
2003	NA	15633	ND	O1:H-	NA	Human, diarrhoea
2003	NA	16675	ND	O28:H-	NA	Human, diarrhoea
2003	NA	04275A	class 1	O81:H-	A, S, T, Su, Tm, Na	Human, diarrhoea
2003	NA	17754	ND	OR:H-	NA	Human, neonatal meningitis
2003	NA	12928	ND	O1:H7	NA	Human, Septicemia
2003	59	06546	class 1	NA	A, S, T, C, Su, Tm	Human, symptoms unknown
2003	NA	12587	ND	O25:H1	NA	Human, symptoms unknown
2003	56	08821	class 1	O6:H1	A, [B]S, Su	Human, urinary tract infection
NA	NA	O167	ND	O167:H5	NA	Human, suspected travellers diarrhoea/infantile diarrhoea (ETEC)
NA	NA	O135	ND	O135:H-	NA	Human, symptoms unknown
NA	NA	H22	ND	O8:H22	NA	Human, symptoms unknown

[A]*E. coli* strain was subcultured from the original strain. [B]Moderate level of resistance. The abbreviated number is referred to in the body of the text when available. Abbreviations: ND, an integron was not detected in the *E. coli* strain; NA, data not available; A, ampicillin (32 μg/ml); S, streptomycin (25 μg/ml); T, tetracycline (20 μg/ml); C, chloramphenicol (10 μg/ml); Su, sulfathiazole (550 μg/ml); Tm, trimethoprim (50 μg/ml); K, kanamycin (10 μg/ml); Nal, nalidixic acid (50μg/ml); G, gentamicin (2.5 μg/ml); Cp, ciprofloxacin (2 μg/ml); Sp, spectinomycin (50 μg/ml).

Appendix IV: Oral presentation and manuscripts based on this thesis

An oral presentation based on this thesis was given at the annual scientific meeting of The Australian Society for Microbiology (ASM) held at the Adelaide Convention and Exhibition Centre, Adelaide on the 9-13 July 2007:

"The role of insertion sequences in the evolution of a unique class 2 integron isolated from *Escherichia coli"* (Fay E. Dawes, Dieter M. Bulach, Alexander Kuzevski, Karl A. Bettelheim, Steven P. Djordjevic, and Mark J. Walker).

The manuscript "Class 1 integrons with IS*26*-mediated deletions in their 3′ -conserved segments are widespread in clinical *Escherichia coli* of human and animal origin" (Fay E. Dawes, Alexander Kuzevski, Karl A. Bettelheim, Michael A. Hornitzky, Steven P. Djordjevic, and Mark J. Walker) has been submitted for publication.

The manuscript "Molecular characterisation of a unique class 2 integron*"* (Fay E. Dawes, Dieter M. Bulach, Alexander Kuzevski, Karl A. Bettelheim, Steven P. Djordjevic, and Mark J. Walker) is in preparation for submission.

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