

University of Wollongong - Research Online

Thesis Collection

Title: Detailed molecular analysis of antibiotic resistance regions within a collection of multi-drug resistant *Salmonella* spp. from Australian sources

Author: Renee S Levings

Year: 2008

Repository DOI:

Copyright Warning

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site.

You are reminded of the following: This work is copyright. Apart from any use permitted under the Copyright Act 1968, no part of this work may be reproduced by any process, nor may any other exclusive right be exercised, without the permission of the author. Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material.

Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

Unless otherwise indicated, the views expressed in this thesis are those of the author and do not necessarily represent the views of the University of Wollongong.

Research Online is the open access repository for the University of Wollongong. For further information contact the UOW Library: research-pubs@uow.edu.au

University of Wollongong Theses Collection

University of Wollongong Theses Collection

University of Wollongong

Year 2008

Detailed molecular analysis of antibiotic
resistance regions within a collection of
multi-drug resistant *Salmonella* spp.
from Australian sources

Renee S. Levings
University of Wollongong

Levings, Renee S, Detailed molecular analysis of antibiotic resistance regions within a collection of multi-drug resistant *Salmonella* spp. from Australian sources, PhD thesis, Department of Biological Sciences, University of Wollongong, 2008. <http://ro.uow.edu.au/theses/119>

This paper is posted at Research Online.

<http://ro.uow.edu.au/theses/119>

NOTE

This online version of the thesis may have different page formatting and pagination from the paper copy held in the University of Wollongong Library.

UNIVERSITY OF WOLLONGONG

COPYRIGHT WARNING

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site. You are reminded of the following:

Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material. Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

Detailed molecular analysis of antibiotic resistance
regions within a collection of multi-drug resistant
Salmonella spp. from Australian sources

A thesis submitted in fulfilment of the requirements for the award of the degree

Doctor of Philosophy

From

The University of Wollongong

By

Renee S. Levings

Department of Biological Sciences

2008

Thesis Declaration

I, Renee S. Levings, declare that this thesis is submitted in accordance with the regulations required of the University of Wollongong in fulfilment of the degree of Doctor of Philosophy, in the Department of Biological Sciences. This thesis does not include work previously published by another person unless appropriate reference is stated in the text. This document has not been submitted for qualifications at any other academic institution.

Renee S. Levings

May 2008

Acknowledgements

There are many people that I would like to thank for helping me along the way of my PhD journey. Firstly, Dr Steven Djordjevic and Professor Mark Walker for giving me the opportunity and the necessary laboratory tools and space to undertake my further studies. I am sincerely grateful to Mark Walker for taking the time to proof read and comment on the draft of my thesis. I would like to thank Steve for his constant enthusiasm, it did prove to be a successful data generating technique.

A very special and personal thanks to Professor Ruth Hall. Your knowledge and commitment to this area is remarkable. Particularly, the way in which you selflessly share your wisdom with others. I will always be appreciative of our day long discussions and the insight you gave me into this area. I would also like to thank Dr Sally Partridge for all the Bioinformatics advice and guidance. I don't know what I would have done without the detailed notes on annotation among other things that you so patiently wrote out for me. To Dr Diane Lightfoot and the staff down at MDU, I am extremely grateful to you, for not only giving me access to this diverse collection of strains, but also for the serotype and antibiotic susceptibility testing information provided.

To Dr Cheryl Jenkins, you have been a truly supportive friend, throughout this experience. Thanks for all the laughs and hour plus conversations we shared along the dreaded M5 motorway (often in the form of a car park). I am also particularly grateful for the fact that you read through a draft of my literature review and provided very useful comments. Big thanks to Linda Falconer for not only technical help but for being a dear friend and a smiley face to go to. To Dr Tracey Kuit, who was there at

the very beginning, thankyou also for the laughs, the wonderful friendship that we have shared and your ongoing support. To my other lab confined friends that I have met and shared some very memorable and enjoyable in and out of work experiences with, Jenny Liu, Carola Venturini and Angela Reeves, thanks for all your friendships and the wonderful memories you have given me. To the remaining members of Team Base camp (Bodgy, Ania and new recruit Connor), thankyou for your friendships and putting up with my somewhat annoying habit of singing nursery rhymes among other things.

Finally to my long suffering family, I made it. Mum and Dad, thanks for your belief in me from a very young age and giving me all the opportunities in life to get to the place I am now. Thankyou Rod, for a loving and supportive partnership and your sound advice to keep pushing on when I sometimes didn't have the momentum and energy to keep going.

Abstract

Salmonella spp., in particular *Salmonella* Typhimurium is an important zoonotic pathogen both here in Australia and internationally. Over the past few decades the use of antimicrobials in human, agricultural and aquacultural settings has created significant selection pressures, giving rise to multiply antibiotic resistant bacteria, including *Salmonella*. The acquisition and dissemination of the genes responsible for antimicrobial resistance has been largely attributed to mobile genetic elements, including Class 1 integrons and the gene cassettes they contain. The initial aim of this study was to examine a collection of 136 multiply resistant *Salmonella* of different serovars from varying Australian sources (predominately animal and to a lesser extent human) for the presence of Class 1 integrons and to identify the gene cassettes present. Using PCR to amplify up specific regions within the Class 1 integron structure, 51.4% of the isolates examined were found to contain the Class 1 integron associated *intI1* integrase. All of these, apart from 11 isolates, contained cassette arrays which were characterised using restriction enzyme analysis and DNA sequencing. The gene cassettes identified among the collection were almost solely responsible for resistance to trimethoprim and aminoglycosides. The *dfrA5* gene cassette (responsible for resistance to trimethoprim) was the most prevalent cassette, particularly among the bovine isolates. Three new gene cassettes responsible for resistance to aminoglycosides, trimethoprim and lincosamides (*aacCA5*, *dfrB6* and *linG*, respectively) were identified. SGI1 (*Salmonella* Genomic Island 1), a 43 kb chromosomal island known to contain a large multi-drug resistance integron, In104 was found to be present in 10 experimental isolates and associated with 4 new serovars, namely Kiambu, Dusseldorf, Cerro and Emek. The integron in the *Salmonella* Emek strain was found to have inserted via transposition at a unique site

within the island backbone and this structure has been named SGI2. This unique insertion site suggests that SGI2 has evolved independently of SGI1.

A second collection of multi-drug resistant *Salmonella* Paratyphi BdT⁺ isolates sourced from human infections in Melbourne and the corresponding home aquaria of infected patients were examined for clonality and the presence of SGI1. All *S. Paratyphi* BdT⁺ from infected individuals were indistinguishable from the isolates from their respective fish tanks, using IS200 profiling techniques and pulse field gel electrophoresis of *Xba*I digested chromosomal DNA. SGI1 (containing the *aadA2* and *blaP1* gene cassettes) was found to be present in all the *S. Paratyphi* BdT⁺ isolates examined. This is the first definitive molecular study showing that ornamental fish tanks are a reservoir for multiply resistant *Salmonella* Paratyphi BdT⁺. Studies examining the molecular mechanisms involved in antimicrobial resistance, and the way in which mobile elements are incorporated and clustered into large multi-drug resistance regions such as SGI1, provide useful information needed for the ongoing surveillance of multiply resistant *Salmonella* and other bacterial pathogens involved in outbreaks domestically and internationally.

Table of Contents

Declaration	i
Acknowledgements	ii
Abstract	iv
Table of contents	vi
List of Figures	xii
List of Tables	xvi
Abbreviations	xviii
Publications arising from this thesis	xx

Chapter 1: Review of the Literature

1.1	The Genus: <i>Salmonella</i>	1
1.2	<i>Salmonella</i> infections	3
1.3	Antibiotics and the emergence of resistance	6
1.3.1	Antibiotics and their usage	6
1.3.2	Major classes of antibiotics	7
1.3.3	Emergence of multiply antibiotic resistant bacteria	7
1.3.4	Global clones of MR <i>Salmonella</i> Typhi and MR <i>Salmonella</i> Typhimurium DT104	10
1.4	Mobile genetic elements	13
1.4.1	Mobile genetic elements and their role in the acquisition and dissemination of resistance genes	13
1.4.2	Plasmids	14
1.4.3	Transposons and IS elements	15
1.4.4	Integrans: a natural cloning and expression system	17
1.4.4.1	Class 1 integrans: basic structure	19

1.4.4.2 The 5'CS	20
1.4.4.3 The 3'CS	20
1.4.4.4 Gene Cassettes	22
<i>1.4.4.4.1 Gene cassette integration</i>	23
1.4.4.5 Class 2, Class 3 and Class 4 integrons	24
1.4.5 Genomic Islands	26
1.4.5.1 <i>Vibrio cholerae</i> SXT element and <i>Salmonella</i> Genomic Island 1 (SGI1)	26

Chapter 2: General Methods and Materials

2.1 Bacterial strains and culture conditions	29
2.2 DNA preparation	32
2.2.1 Crude DNA extraction procedure	32
2.2.2 Phenol/chloroform DNA extraction procedure	33
2.3 PCR analysis and conditions for initial screening experiments	34
2.3.1 Multiplex PCR for identification of Class 1 integrons	34
2.3.2 Gene cassette variable region PCR	35
2.4 Restriction enzyme analysis of L1/R1 PCR products	36
2.5 Agarose gel electrophoresis	37
2.6 Southern hybridisation of digested chromosomal DNA probed with digoxigenin (DIG) labelled DNA probes	37
2.6.1 Nitrocellulose membrane preparation and DNA transfer	37
2.6.2 DIG-labelled DNA probe preparation and hybridisation	38
2.6.3 Development of southern membranes	39
2.7 DNA sequencing	40
2.8 Cloning of novel gene cassettes	41
2.8.1 <i>Pwo</i> I PCR amplification of L2/R1 PCR products for cloning	41

2.8.2	Ligation of L2/R1 PCR products into pPCR-script™ Amp SK(+) cloning vector	41
2.8.3	Transformation of pPCR-script™ Amp SK(+) vector containing L2/R1 inserts into <i>E. coli</i> 294 and <i>E. coli</i> DH5α cells	42
2.9	Antimicrobial susceptibility testing of clones and controls	43

Chapter 3: Molecular analysis of Class 1 integrons in a collection of multi-drug resistant Australian *Salmonella* spp. from human, animal and environmental sources

3.1	Introduction	46
3.2	Materials and Methods	51
3.2.1	PCR amplification and restriction enzyme analysis	51
3.2.2	Southern hybridisation of <i>Bgl</i> II digested DNA with <i>sul</i> 1 DIG-labelled probe and <i>Pst</i> I digested <i>S. Infantis</i> DNA with IS200 DIG-labelled DNA probe	51
3.2.2.1	<i>sul</i> 1 digoxigenin DNA probe preparation	51
3.2.2.2	IS200 digoxigenin DNA probe preparation	52
3.2.2.3	<i>Bgl</i> II and <i>Pst</i> I chromosomal DNA digestions	52
3.2.2.4	Nitrocellulose membrane preparation, transfer and hybridisation	53
3.2.3	Cloning of novel gene cassettes and antimicrobial susceptibility testing	53
3.3	Results	53
3.3.1	L2/L3 multiplex PCR screening of <i>Salmonella</i> isolates	53
3.3.2	Southern hybridisation of <i>Bgl</i> II digested chromosomal DNA with the DIG-labelled <i>sul</i> 1 DNA probe	55
3.3.3	L1/R1 gene cassette PCR	56
3.3.4	<i>Rsa</i> I restriction enzyme digestion analysis of gene cassette amplicons	57
3.3.5	DNA sequence analysis of gene cassette amplicons with different <i>Rsa</i> I profiles	57

3.3.6	Analysis of new cassette arrays	58
3.3.6.1	Analysis of cassette array amplicon from SRC54	58
3.3.6.1.1	<i>Protein alignment of cassette array and 59 base element from SRC54 with cassettes with similar identities</i>	58
3.3.6.1.2	<i>Gradient plate antibiotic susceptibility results for E. coli DH5α strain containing cloned aadA2-linG cassette array</i>	60
3.3.6.2	Analysis of cassette array amplicon from SRC73	60
3.3.6.2.1	<i>Protein alignment of cassette array and 59 base element from SRC73 with cassettes with similar identities</i>	60
3.3.6.2.2	<i>Antibiotic susceptibility disc results for E. coli 294 strain containing the cloned aacCA5-aadA7 cassette array</i>	63
3.3.6.3	Analysis of cassette array amplicon from SRC70	63
3.3.6.3.1	<i>Protein alignment of cassette array and 59 base element from SRC70 with cassettes with similar identities</i>	63
3.3.6.3.2	<i>Gradient plate antibiotic susceptibility results for E. coli DH5α strain containing cloned dfrB6-aadA1 cassette array</i>	65
3.3.6.4	SRC22 Δ orfF-aadA2 cassette array	65
3.3.7	Potential SGI1 containing strains	67
3.3.8	IS200 profiles and the genetic relationship between the dfrB6-aadA1 containing S. Infantis strains	67
3.4	Discussion	68
<u>Chapter 4: A detailed analysis of the <i>Salmonella</i> Genomic Island SGI1 containing a multiple antibiotic resistance region and variants thereof, from a collection of different <i>Salmonella</i> spp.</u>		
4.1	Introduction	83
4.2	Materials and Methods	88
4.2.1	SGI1 specific screening PCRs	88

4.2.2	Antimicrobial resistance testing for florfenicol	91
4.2.3	Southern hybridisation of <i>Bsa</i> I digested chromosomal DNA probed with <i>tetA</i> (G) and CR3 (orf2) DIG-labelled DNA probes	91
4.2.4	DNA sequencing	91
4.3	Results	92
4.3.1	SGI1 major junction PCRs	92
4.3.2	Cassette PCRs and <i>Rsa</i> I digestions of SGI1 containing strains	94
4.3.3	Location of the gene cassettes within In104	96
4.3.4	Southern hybridisations of <i>Bsa</i> I digested chromosomal DNA probed with <i>tetA</i> (G) and CR3 DIG-labelled probes	97
4.3.5	Summary of resistance genes and other gene linkages identified in SGI1 containing <i>Salmonella</i> spp.	99
4.3.6	Further linkage analysis of trimethoprim resistant SGI1 strains not containing the <i>dfrA</i> 1-orfC cassette array	100
4.4	Discussion	103

Chapter 5: A molecular and epidemiological study of multiply antibiotic resistant *Salmonella* Paratyphi BdT⁺ containing SGI1 sourced from ornamental fish tanks and human infections in Australia

5.1	Introduction	108
5.2	Materials and Methods	111
5.2.1	Bacterial collection	111
5.2.2	SGI1 PCR amplification and In104 cassette analysis	111
5.2.3	Pulse field gel electrophoresis (PFGE) of <i>Xba</i> I digested <i>S. Paratyphi</i> BdT ⁺ isolates	113
5.2.4	Southern hybridisation of <i>Pst</i> I digested whole cell <i>S. Paratyphi</i> DNA probed with DIG-labelled IS200	115
5.2.4.1	Whole cell DNA digestion with <i>Pst</i> I and membrane preparation/transfer	115
5.2.4.2	IS200 DIG-labelled DNA probe preparation	115

5.2.4.3	Hybridisation and development of southern membrane	115
5.3	Results	116
5.3.1	PCR identification of SGI1 containing strains and the cassettes they contain	116
5.3.2	PFGE analysis of <i>Xba</i> I digested chromosomal DNA for <i>S. Paratyphi</i> BdT ⁺ isolates	119
5.3.3	IS200 profiles of <i>Pst</i> I digested chromosomal DNA for <i>S. Paratyphi</i> BdT ⁺ isolates	120
5.4	Discussion	120
 <u>Chapter 6: General Discussion and future studies</u>		
6	General Discussion	124
References		130
Appendix		151

List of Figures

Figure No.	Page No.
-------------------	-----------------

Chapter 1: Review of the Literature

1.1	Likely routes of transmission of <i>Salmonella</i> spp. between animals and humans.	4
1.2	Worldwide distribution of antibiotic resistant strains of <i>Salmonella enterica</i> serotype Typhi 1990-2002.	11
1.3	The range of gene transfer among bacteria and other taxa.	14
1.4A	Insertion sequence (IS) – the simplest transposable element.	16
1.4B	Transposon Tn21.	16
1.5	Plasmid NR1 (R100).	18
1.6	General structure of commonly described Class 1 integrons.	19
1.7	Tn402, the ancestral model for Class 1 integrons.	21
1.8	The boundaries of gene cassettes.	24
1.9	SGI1 and its major genetic components.	28

Chapter 2: General Methods and Materials

2.1	A diagrammatic representation of Class 1 integron primers and their amplification targets.	36
2.2	Diagram of pPCR-Script TM Amp SK (+) cloning vector used in this study.	42
2.3A	Gradient plate method for testing antibiotic susceptibility.	44
2.3B	Antibiotic disc susceptibility testing method.	44

Chapter 3: Molecular analysis of Class 1 integrons in a collection of multi-drug resistant Australian *Salmonella* spp. from human, animal and environmental sources

3.1	Genetic variability of the 3'CS of Class 1 integrons.	48
3.2	Agarose gel electrophoresis results for the L2/L3 and Salm3/Salm4 multiplex PCR.	54

3.3	Southern blot of <i>Bgl</i> III digested DNA for representative <i>Salmonella</i> strains using a DIG-labelled <i>sul</i> I PCR probe.	55
3.4	An agarose gel of all the representative L1/R1 PCR amplicons.	56
3.5	An agarose gel of the 14 different L1/R1 amplicons digested with <i>Rsa</i> I.	57
3.6	Alignment of the Lin proteins.	59
3.7	Alignment of <i>linF</i> and <i>linG</i> 59-be.	60
3.8	Alignment of AacC-A proteins in the AAC(3)-I family.	61
3.9	Alignment of the 59-be of the six known <i>aacC-A</i> gene cassettes.	62
3.10	Alignment of DfrB proteins in the DfrB family.	64
3.11	Alignment of the 59-be of the six known <i>dfrB</i> gene cassettes.	65
3.12	A diagrammatic representation of the putative deletion event in the <i>dfrA12</i> -orfF- <i>aadA2</i> cassette array.	66
3.13	Crossover region leading to the formation of 5'-CS-orfF boundary in SRC22.	66
3.14	<i>Pst</i> I digested DNA of a representative of all the <i>S. Infantis</i> strains after hybridisation with the dig-labelled IS200 probe.	68

Chapter 4: A detailed analysis of the *Salmonella* Genomic Island SGI1 containing a multiple antibiotic resistance region and variants thereof, from a collection of different *Salmonella* spp.

4.1	A theoretic model of SGI1 excision from and integration into the chromosome of <i>S. enterica</i> and <i>E. coli</i> .	86
4.2A	Diagrammatic representation of all major junction PCR primer sites used to characterise the genetic content of In104 in SGI1 containing isolates.	90
4.2B	Diagrammatic representation of all major linkage PCR primer sites used to characterise the genetic content of In104 in SGI1 containing isolates.	90
4.3	Diagram of primer amplification products for the CR1 (orf513)- <i>dfrA10</i> insertion found in variants of SGI1.	90

4.4A	A representative agarose gel of SGI1 left junction PCR results.	92
4.4B	A representative agarose gel of SGI1 right junction PCR results.	92
4.5	DNA sequence obtained for the SGI1 backbone and In104 junctions for SRC19 (Emek) and DT104.	94
4.6	Diagrammatic representation of new insertion site of In104Emek into S023 in the SGI1 backbone.	94
4.7A	L1/R1 products for isolates that were positive for the left (SGI1 LJ) and right arm (SGI1 RJ) PCRs for SGI1.	95
4.7B	<i>RsaI</i> digestion of L1/R1 PCR products for isolates that were positive for the left (SGI1 LJ) and right arm (SGI1 RJ) PCRs for SGI1.	95
4.8A	S026-FW/aadA2-R2 linkage PCRs on SGI1 containing strains.	97
4.8B	groEL(F)/pse-R2 linkage PCRs on SGI1 containing strains.	97
4.9	Diagrammatic representation of the 4.1 kb <i>BsaI</i> fragment that hybridised with the <i>tetA</i> (G) CR3 (orf2) DIG labelled DNA probes.	98
4.10	Southern hybridisation blot of CR3 (orf2) DIG-labelled probe to <i>BsaI</i> digested chromosomal DNA for all SGI1 containing strains.	98
4.11	Southern hybridisation of <i>tetA</i> (G) DIG-labelled probe to <i>BsaI</i> digested chromosomal DNA for all experimental SGI1 containing strains.	98
4.12A	Agarose gel of <i>floR</i> PCR products.	99
4.12B	Agarose gel of <i>tetA</i> (G) PCR products.	99
4.13	Agarose gel of orf5-F/IS6100 (Rv-2) linkage PCR on SGI1 containing strains.	99
4.14	Agarose gel of <i>dfrA10</i> PCR products.	101
4.15	CR1 to <i>dfrA10</i> (orf513(F)/RH380) (PCR 1) and <i>dfrA10</i> to <i>sulI</i> linkage PCRs.	101
4.16	Maps of all In104 variants found in this study.	102

**Chapter 5: A molecular and epidemiological study of multiply
antibiotic resistant *Salmonella* Paratyphi BdT⁺ containing
SGI1 sourced from ornamental fish tanks and human
infections in Australia**

5.1	Structure of SGI1 region of serovar Typhimurium DT104 and primer locations for SGI1 screening of MR <i>S. Paratyphi</i> BdT ⁺ isolates.	113
5.2	Agarose gel of L2/L3 multiplex PCR on MR <i>S. Paratyphi</i> isolates.	116
5.3	Agarose gel of L1/R1 gene cassette PCR on the MR <i>S. Paratyphi</i> isolates.	117
5.4	Agarose gel of <i>RsaI</i> digestions of L1/R1 gene cassette PCR amplicons obtained for the MR <i>S. Paratyphi</i> isolates.	117
5.5	Agarose gel of S026-FW/aadA2-R2 PCR for a representative number of <i>S. Paratyphi</i> isolates.	118
5.6	Agarose gel of groEL-F/pse-R2 PCR for a representative number of <i>S. Paratyphi</i> isolates.	118
5.7	Pulsed field gel electrophoresis (PFGE) of <i>XbaI</i> digested whole cell DNA for a set of representatives of SGI1 containing <i>Salmonella enterica</i> serovar Paratyphi B dT ⁺ isolates.	119
5.8	Southern hybridisation blots of <i>PstI</i> digested whole cell DNA from <i>S. Paratyphi</i> isolates containing SGI1 hybridised with IS200 digoxigenin (DIG) labelled DNA probe.	120

List of Tables

Table No.		Page No.
<u>Chapter 1: Review of the Literature</u>		
1.1	Host adaption of <i>S. enterica</i> from a series of epidemiological assays.	2
1.2	Major antibiotic classes, their introduction and therapeutic use, and mechanisms of action.	8
<u>Chapter 2: General Methods and Materials</u>		
2.1	<i>Salmonella</i> experimental isolates (collection 1) used in this study.	30
<u>Chapter 3: Molecular analysis of Class 1 integrons in a collection of multi-drug resistant Australian <i>Salmonella</i> spp. from human, animal and environmental sources</u>		
3.1	Summary of L2/L3 <i>intI1</i> PCR results.	55
3.2	BlastN sequence search results for strains representing each of the different L1/R1 RFLP profiles and their identity to previously published cassette arrays.	58
3.3	Relationships between members of the AacC-A or AAC(3)-I protein family.	62
3.4	Antibiotic susceptibility assay for pCR-script containing <i>aacC5-aadA7</i> cassette array and pCR-Script negative control.	63
3.5	Relationships between members of the DfrB protein family.	64
<u>Chapter 4: A detailed analysis of the <i>Salmonella</i> Genomic Island SGI1 containing a multiple antibiotic resistance region and variants thereof, from a collection of different <i>Salmonella</i> spp.</u>		
4.1	SGI1 primer table.	88
4.2	PCR results for major SGI1 junctions.	93
4.3	Antibiotic resistance profiles and resistance genes of <i>Salmonella</i> spp. containing SGI1 and variants of SGI1.	100

**Chapter 5: A molecular and epidemiological study of multiply
antibiotic resistant *Salmonella* Paratyphi BdT⁺ containing
SGI1 sourced from ornamental fish tanks and human
infections in Australia**

- 5.1** A summary of genetic characteristics of *Salmonella enterica* 112
 serovar Paratyphi BdT⁺ isolates used in this study.

Abbreviations

aa	amino acid
ANGIS	Australian National Genomic Information Service
Ap	ampicillin
59 be	59 base element
bp	base pairs
CFU	colony forming units
Cm	chloramphenicol
CR	common region
5'CS	5' conserved segment
3'CS	3' conserved segment
Cp	ciprofloxacin
Cp'	intermediate resistance to ciprofloxacin
CSPD	Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1 ^{3,7}] decan}-4-yl) phenyl phosphate
DANMAP	The Danish Integrated Antimicrobial Resistance Monitoring Program
dfr	dihydrofolate reductase
DHPS	dihydropteroic acid synthetase
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
°C	degrees celsius
EDTA	ethylenediamine tetra-acetic acid
EMAI	Elizabeth Macarthur Agricultural Institute
Fl	florfenicol
<i>g</i>	<i>g</i> forces for centrifugation
<i>g</i>	gram
Gm	gentamicin
h	hour
HCl	hydrochloric acid
In	integron
Inc	Incompatibility group
IR	inverted repeats
IS	insertion sequence
JETACAR	Joint Expert Technical Advisory Committee on Antibiotic Resistance
kb	kilobases
kD	kilodalton
kg	kilogram
Km	kanamycin
kV	kilovolts
l	litre
LB	Luria Bertani
m	milli
M	molar
mm	millimetre
MDU	Microbiological Diagnostic Unit (Melbourne)
MIC	minimal inhibitory concentration
MMR's	multi-resistance regions
MR	multiply antibiotic resistant

μ	micro
min	minute
ml	millilitre
n	nano
Na	nalidixic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
NEPSS	National Enteric Pathogens Surveillance System
NNDSS	National Notifiable Diseases Surveillance System
OD	optical density
ORF	open reading frame
%	percentage
PBS	phosphate buffered saline
PCR	polymerase chain reaction
p	pico
pH	pondus Hydrogeni
PT	phage type
PFGE	pulse field gel electrophoresis
RDNC	results do not conform
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulfate
SGI1	<i>Salmonella</i> Genomic Island 1
Sm	streptomycin
Sp	spectinomycin
spp.	species
SRC	<i>Salmonella</i> reference collection
SSC	sodium citrate
Su	sulphathiazole/sulfonamides
TBE	tris-borate EDTA
TE	tris EDTA
Tc	tetracycline
Tn	transposon
Tp	trimethoprim
Tra	transposition region
U	units
UV	ultra-violet
V	volts
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organisation

Publications arising from this thesis

1. Levings, R. S., S. R. Partridge, S. P. Djordjevic, and R. M. Hall. (2007) SGI1-K, a variant of the SGI1 genomic island carrying a mercury resistance region, in *Salmonella enterica* serovar Kentucky. *Antimicrob Agents Chemother* **51**:317-23.
2. Levings, R. S., R. M. Hall, D. Lightfoot, and S. P. Djordjevic. (2006) *linG*, a new integron-associated gene cassette encoding a lincosamide nucleotidyltransferase. *Antimicrob Agents Chemother* **50**:3514-5.
3. Levings, R. S., D. Lightfoot, R. M. Hall, and S. P. Djordjevic. (2006) Aquariums as reservoirs for multidrug-resistant *Salmonella* Paratyphi B. *Emerg Infect Dis* **12**:507-10.
4. Levings, R. S., D. Lightfoot, L. D. Elbourne, S. P. Djordjevic, and R. M. Hall. (2006) New integron-associated gene cassette encoding a trimethoprim-resistant DfrB-type dihydrofolate reductase. *Antimicrob Agents Chemother* **50**:2863-5.
5. Levings, R. S., D. Lightfoot, S. R. Partridge, R. M. Hall, and S. P. Djordjevic. (2005) The genomic island SGI1, containing the multiple antibiotic resistance region of *Salmonella enterica* serovar Typhimurium DT104 or variants of it, is widely distributed in other *S. enterica* serovars. *J Bacteriol* **187**:4401-9.
6. Levings, R. S., S. R. Partridge, D. Lightfoot, R. M. Hall, and S. P. Djordjevic. (2005) New integron-associated gene cassette encoding a 3-N-aminoglycoside acetyltransferase. *Antimicrob Agents Chemother* **49**:1238-41.

Chapter 1: Review of the Literature

1.1 The Genus: *Salmonella*

Salmonellae are typical members of the family *Enterobacteriaceae*. Consisting of Gram-negative, non-spore forming bacilli (Velge et al., 2005) they are important zoonotic pathogens. *Salmonella* genome sequencing has demonstrated a median sequence identity of between 80-90% with its nearest relation *Escherichia coli* (*E. coli*) (McClelland et al., 2001). The Genus *Salmonella* is divided into two species *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is divided up into 6 subspecies; *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houetnae* (IV) and *indica* (VI) (Le Minor et al., 1982). Members of the genus *Salmonella* have three different types of antigens, the somatic O, flagellar H and capsular Vi antigen, the agglutination properties of which are used to distinguish between more than 2500 serotypes (Popoff et al., 2003). *Salmonella* serotypes can be further subdivided by using biotyping (biochemical variation between microbes of the same serotype) and phage typing (variation between organisms with the same serotype but different susceptibilities to a lytic bacteriophage) (Ward et al., 1987).

Members of *S. enterica* subspecies I (*enterica*) account for more than 99% of serovars involved in disease in warm blooded animals and are the main focus of epidemiological studies (Christensen et al., 1998; Chan et al., 2003). *Salmonella bongori* is found in the environment and is also associated with disease in cold-blooded animals but is rarely isolated from humans (Christensen et al., 1998; Chan et al., 2003).

Table 1.1 Host adaptation of *S. enterica* serotypes from a series of epidemiological assays (Rabsch et al., 2002).

Please see print copy for Table 1.1

The ability of salmonellae to adapt to different hosts varies between serotypes and this in turn affects their pathogenicity for humans and animals (Velge et al., 2005) (Table 1.1). *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi are extremely well adapted to colonisation in humans and usually cause severe

diseases such as septicaemic typhoidic syndrome (enteric fever), however they are not usually pathogenic to animals (Velge et al., 2005). *Salmonella enterica* serovar Typhi causes 16 million human illnesses and 600,000 deaths worldwide each year (Parry, 2003). Serotypes that are highly adapted to animal hosts such as *S. Gallinarum* in poultry and *S. Abortusovis* in sheep, usually produce very mild symptoms in humans (Velge et al., 2005). Strains such as *S. Enteritidis* and *S. Typhimurium* have a wider host range (humans and animals) and generally only cause uncomplicated gastroenteritis. However, they are probably the two most important serotypes transmitted from animals to humans (WHO, 2005). Limited information is available on the pathogenic mechanisms of *Salmonella* spp., however a critical virulence determinant of these organisms is the ability to invade mammalian cells (Jones, 2005).

1.2 *Salmonella* infections

Salmonella is recognised as being a major cause of food poisoning worldwide (Mead et al., 1999) and salmonellosis constitutes a major public health and economic burden in many countries. Salmonellae along with other enteric bacteria such as *E. coli* and *Campylobacter* spp. are transmitted via the faecal-oral route. Transmission of these bacteria to humans is usually due to poor sanitation of water and food supplies in underdeveloped countries, and as a result of the consumption of raw and undercooked contaminated food products (mainly meat, poultry, eggs and milk) in developed countries (see Fig 1.1) (WHO, 2005). Intensively farmed food producing animals usually acquire these pathogens by ingesting contaminated feed (Crump et al., 2002).

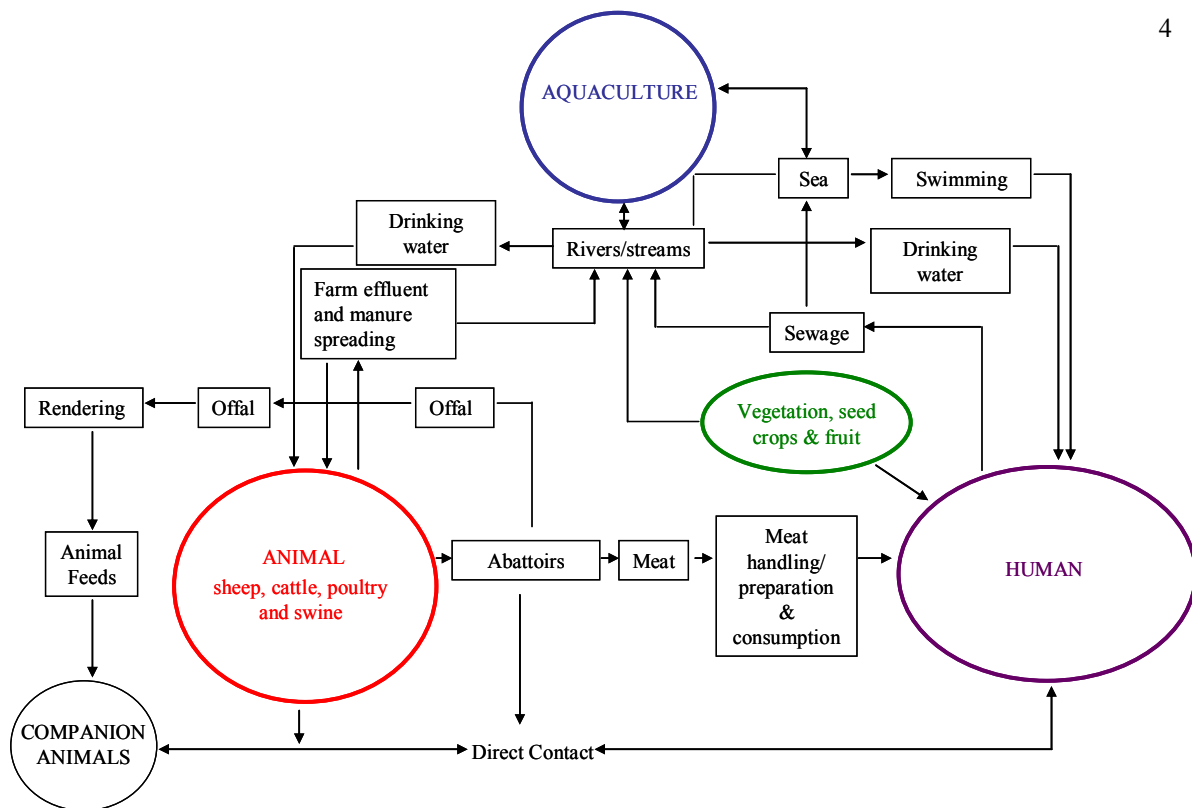


Fig 1.1 Likely routes of transmission of *Salmonella* spp. between animals and humans (Diagram adapted from Prescott, 2006; Linton, 1977).

Salmonella spp. cause infections ranging from gastroenteritis to systemic typhoid fever, which has an associated high mortality rate. The severity and outcome of a *Salmonella* infection depends on the virulence capabilities of the bacterium, infectious dose, and the immunological status of the host (Mastroeni, 2002). *Salmonella* infections are usually self-limiting but in the case of invasive infection antibiotics are administered. Human salmonellosis is usually characterised by acute onset of fever, abdominal pain, diarrhoea, nausea and vomiting and can even lead to dehydration, septicaemia and death in the young, elderly or immuno-compromised (WHO, 2005, Parry 2003).

It has been reported that there are five pathogens which are estimated to account for over 90% of worldwide food related deaths. *Salmonella* account for 31%, with *Listeria*, *Toxoplasma*, Norwalk-like viruses, *Campylobacter* and *E. coli* 0157:H7

making up the remainder (White et al., 2002). The World Health Organisation (WHO) reports that in the United States there are an estimated 1.4 million non-typhoidal *Salmonella* infections, resulting in 15,000 hospitalisations and 580 deaths costing around US\$3 billion annually (WHO, 2005). In 2003, a total of 7,011 salmonellosis cases were reported to the National Notifiable Diseases Surveillance System (NNDSS) in Australia, a rate of 35.3 cases per 100,000 people (0.0353%) (Miller et al., 2003). An Australian survey conducted on the epidemiology of 214 foodborne disease outbreaks during the 1995-2000 period indicated that 75 (35%) of the total outbreaks were due to *Salmonella* and 8 (40%) out of the 20 deaths reported were attributed to salmonellosis (Dalton et al., 2004). It is clear from these statistics that *Salmonella* pose a serious public health risk and costs involved in the treatment and management of disease associated with the bacteria are significant.

Salmonella not only affects public health expenditure it also impinges on agricultural expenditure. Where there is evidence of possible transmission from livestock (contaminated food products) to humans, the cost to the effected industry can be devastating. Several countries are now implementing surveillance and control programs to minimise the financial impact of *Salmonella* outbreaks. In Denmark, a *Salmonella* control programme, The Danish Integrated Antimicrobial Resistance Monitoring Program (DANMAP) has been in place for many years, and is estimated to save US\$25.5 million of annual public expenditure (WHO, 2005). Strategies previously used to control *Salmonella* outbreaks, generally involved the treatment of infected patients and livestock with antibiotics. However, this is thought to have compounded the problem due to the emergence and dissemination of multi-drug resistant strains of *Salmonella* as well as other bacterial pathogens.

1.3 Antibiotics and the emergence of resistance

1.3.1 Antibiotics and their usage

Antibiotics are naturally occurring, semi-synthetic and synthetic compounds with antimicrobial activity. Antibiotics are used in human and veterinary medicine (including aquaculture) to treat and prevent disease, for growth promotion in the farming of food producing animals (Wegener, 2003; Phillips et al., 2004) and also for the treatment of plant diseases in agriculture (McManus et al., 2002).

The use of antibiotics in animal husbandry is quite considerable. Tetracycline antibiotics have been used at subtherapeutic levels as growth promoters in animal feed in the United States since 1949 when they were observed to have growth promoting properties (Chopra and Roberts, 2001). The worldwide use of antibiotics for animal health in 1996 was estimated to be 27,000 tonnes and 90% of the antibiotics used in animal husbandry are randomly distributed as an additive in feed (Schwarz and Chaslus-Dancla, 2001). From 1992 to 1996, Australia imported an average of 582 kg of vancomycin per year for medical purposes and 62,642 kg of avoparcin (an antibacterial glycopeptide) per year for animal husbandry (Witte, 1998). There are many different families of antibiotics that are used in both clinical and agricultural settings.

1.3.2 Major Classes of antibiotics

Many of the antibiotics used today are structural derivatives of naturally occurring antibacterials and generally target cell wall, protein and nucleic acid synthesis. Antimicrobials are either bactericidal ie. kill the bacteria or bacteriostatic ie. inhibit cell growth.

1.3.3 Emergence of multiply antibiotic resistant bacteria

The discovery and subsequent usage of antibiotics over the last 50 years in clinical and veterinary settings, has revolutionised modern medicine and aided in the control of infectious microbial agents. However, the overuse and misuse of these antibiotics has created an enormous selection pressure for antimicrobial resistance among bacterial pathogens. In many cases resistance to the antibiotics listed in the Table 1.2 began emerging shortly after the introduction of the agent.

Resistance can be divided in two groups, intrinsic resistance (naturally inherent to the bacteria) and acquired resistance (sourced from other bacteria or environment). (Schwarz et al., 2001). Different genetic mechanisms: (i) inactivation of antimicrobial agent by a specific enzyme; (ii) presence of alternative enzymes for the enzyme that is targeted by the antibiotic; (iii) mutations in the targeted gene which reduce binding of the antimicrobial agent; (iv) post-transcriptional and/or post-translational changes in the target or absence of the target (i.e. no cell wall); (v) reduced uptake or efficient efflux of the antibiotic; (vi) overproduction of the antimicrobial agents target (Fluit et al., 2001) are involved in the development of resistance.

Table 1.2 Major Antibiotic Classes, their introduction and therapeutic use, and mechanisms of action.

Classes of antibiotics	First introduced	Historical Use	Type and mechanism of action
Sulfonamides	1932	human/ animal	bacteriostatic: limits cell growth by inhibiting the substrate <i>p</i> -aminobenzoic acid of dihydropteroic acid synthetase (DHPS).
Trimethoprim	1962	human	bacteriostatic: limits cell growth by inhibiting dihydrofolate reductase.
Aminoglycosides (eg. streptomycin, spectinomycin, kanamycin, gentamicin)	1944	human/ animal/ plant	bactericidal: inhibits translocation and protein synthesis by high affinity binding to RNA.
Chloramphenicol & Florfenicol (fluorinated derivative)*	1948	human/ animal	bacteriostatic: inhibits protein synthesis by targeting the peptidyl transferase centre of the bacterial ribosome.
Tetracyclines	1940's	human/ animal/ plant	bacteriostatic: inhibits protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site.
Beta (β)-lactams (eg. cephalosporins, monobactams and carbapenems)	1928	human/ animal	bactericidal: inhibits cell wall synthesis by covalently binding to cell wall transpeptidases and blocking transpeptidation of peptidoglycan strands.
Quinolones (eg. nalidixic acid and ciprofloxacin)	1960's	human/ animal	bactericidal: inhibits bacterial replication by targeting bacterial DNA gyrase and topoisomerase IV.

* Banned from use in human medicine.

NB. Chloramphenicol and florfenicol have bactericidal effects against some bacterial species. Sulfonamides in combination therapies are also bactericidal.

The dissemination and proliferation of antibiotic-resistant bacteria poses a serious public health threat in hospitals worldwide. Antibiotics suppress an individual's (both human and animal) normal microflora, reducing competition and often giving rise to a dominant antibiotic resistant clone. The additional selective effect of antimicrobial resistance has been estimated to increase the vulnerability to infection more than three fold among patients receiving antimicrobial therapy for unrelated reasons resulting in

an extra 29,379 non-typhoidal *Salmonella* infections in the United States each year (Barza and Travers, 2002).

The increase in antibiotic resistance has meant countries have had to develop monitoring programs in order to keep track of the emergence of resistant strains. The DANMAP program implemented in Denmark in 1995 acts as a continuous surveillance system of antimicrobial resistance among bacteria isolated from pigs, cattle and broiler chickens throughout Denmark (Aarestrup et al., 1998). A program has been set up in Australia and is known as the NSW *Salmonella* Enteritidis Free Monitoring and Accreditation Scheme (Arzey, 1999). This scheme was introduced to allow the early detection of *Salmonella* Enteritidis within the poultry industry based on reports from the United States of increasing *Salmonella* Enteritidis infections due to consumption of infected eggs. However, this scheme does not yet involve antibiotic resistance monitoring and currently there are no large surveillance programs in Australia that deal with antibiotic resistance. A major finding by the Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR), a body established in Australia in April 1998, was the need for an antibiotic resistance monitoring and surveillance program to be established within Australia (JETACAR Report 1998).

Bacterial resistance to one or more antibiotics is not uncommon. The incidence of multiple antibiotic resistance is prevalent among both Gram-negative and Gram-positive bacteria. Emerging antimicrobial resistance phenotypes have been noted among many different zoonotic pathogens including *Salmonella* spp., *E. coli*, *Campylobacter jejuni*, *Listeria monocytogenes* and *Yersinia enterocolitica* (White et

al., 2000). Studies of multiply resistant (MR) *Shigella* in Japan from 1955 to 1967 highlighted the rapid rise in MR isolates. In 1955 0.02% of clinical *Shigella* strains were resistant to one or more antibiotics, and in 1967 the number had risen to over 74% (Mitsubishi, 1971). Due to the emergence of MR bacteria many antibiotics have been made redundant with some hospitals in Europe and North America overwhelmed with infections of enterococci resistant to all available antibiotics (Moellering, 1991; Swartz, 1994).

The antibiotics that were typically administered to treat invasive salmonellosis were cotrimoxazole, aminoglycosides and chloramphenicols. The emergence of multi-drug resistance has led to fluoroquinolones and expanded-spectrum cephalosporins becoming the main antibiotics used in the treatment of life-threatening salmonellosis. Unfortunately, there have been increasing reports of *Salmonella* spp. that have decreased susceptibilities to these 'last resort' antibiotics making treatment very difficult (Fey et al., 2000).

1.3.4 Global clones of MR *Salmonella* Typhi and MR *Salmonella* Typhimurium DT104

As with other pathogens, the number of *Salmonella* serotypes isolated from humans and animals displaying a multi-drug resistance phenotype have been increasing worldwide. A recent study in Spain highlighted the increase of resistance in different *Salmonella* serotypes from 1985-1987 compared with isolates from 1995-1998. Strains resistant to ampicillin (Ap) increased from 8 (1985-1987) to 44% (1995-1998), tetracycline (Tc) resistance increased from 1.7 to 42%, chloramphenicol (Cm) resistance rose from 1.7 to 26% and resistance to nalidixic acid increased from 0.1 to

11% (Prats et al., 2000). Of particular concern are the ever increasing multiply antibiotic resistant *Salmonella* Typhi strains (resistant to four or more antibiotics), with outbreaks occurring in India, Pakistan, Bangladesh, Vietnam, the Middle East and Africa (Fig 1.2) (Parry et al., 2002). In European countries between 1999 and 2001 the number of multi-drug resistant Typhi isolates rose from 22 to 29% (Threlfall et al., 2003).

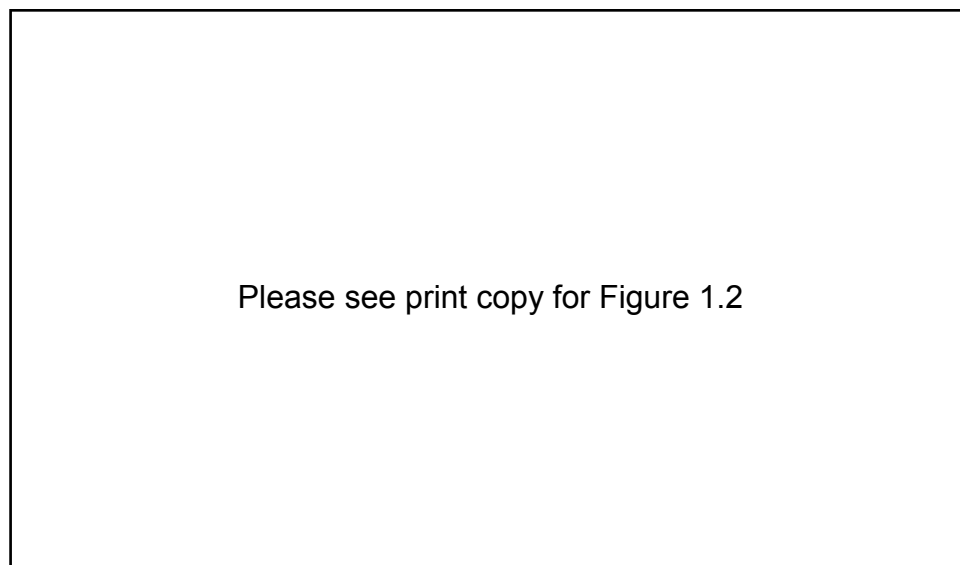


Fig 1.2 Worldwide distribution of antibiotic resistant strains of *Salmonella enterica* serotype Typhi 1990-2002. Shaded areas are areas of endemic disease (Parry et al., 2002).

The emergence of MR *Salmonella enterica* serovar Typhimurium DT104 exhibiting resistance to ampicillin, chloramphenicol, florfenicol, streptomycin, spectinomycin, sulfonamides and tetracycline is a significant public health issue. Molecular typing studies have shown that during the mid-1980's the MR *S. Typhimurium* DT104 clone appeared in the United Kingdom (UK), Europe and the United States (US) simultaneously (Davis et al., 2002). MR *S. Typhimurium* DT104 has now become the second most prevalent *Salmonella* serotype isolated from humans in the UK and its

increasingly being found in the US, South Africa, the United Arab Emirates, Trinidad, the Phillipines, the Irish Republic, Canada, Israel, Japan and European countries (Ribot et al., 2002; Threlfall, 2002; Threlfall et al., 2000). Numerous studies, utilising Pulse Field Gel Electrophoresis (PFGE) and other fingerprinting techniques have highlighted the clonal nature of this MR strain and traced its movement around the world (Lawson et al., 2004; Baggesen et al., 2000).

The MR *S. Typhimurium* DT104 clone is often incorrectly described as being “penta-resistant” due to the fact that the screening for spectinomycin and florfenicol resistance is not routine. The resistances to the seven different antibiotics in the majority of strains is due to the presence of five genes *aadA2* (streptomycin and spectinomycin resistance), *sulI* (sulfonamide resistance), *floR* (florfenicol and chloramphenicol resistance), *tetA(G)* (tetracycline resistance) and *blaP1* (resistance to β -lactam antibiotics) (Briggs and Fratamico, 1999; Boyd et al., 2001). The antibiotic resistance genes responsible for the MR *S. Typhimurium* DT104 phenotype all reside within a 13 kb region that is part of a genomic island known as *Salmonella* Genomic Island 1 (SGI1) (Briggs and Fratamico, 1999; Boyd et al., 2001) and will be discussed in detail later in Chapter 3. The acquisition and spread of antibiotic resistance determinants via mobile genetic elements is thought to be a major route for the dissemination of resistance among bacteria (Witte, 1998).

1.4 Mobile genetic elements

1.4.1 Mobile genetic elements and their role in the acquisition and dissemination of resistance genes

The genes of the bacterial genome are classified into two main groups; the essential core gene pool (consisting mainly of chromosomal genes responsible for basic cellular function) and the flexible gene pool (made up of mobile genetic elements) (Hacker and Kaper, 2000; Oelschlaeger and Hacker, 2004). Bacterial genomes are constantly evolving and exchanging genetic information. Bacteria are highly adaptable as individual cells do not need to rely on their own genetic resources and many have access to a large pool of genes that move among bacterial populations and other phyla (Fig 1.3).

The rapid dissemination of antibiotic resistance genes between bacteria of the same or different species and Genera is principally the result of horizontal gene transfer events (Schwarz and Chaslus-Dancla, 2001). The mobile genetic elements that are involved in horizontal gene transfer are phages, plasmids, transposons/IS elements, integrons/gene cassettes and genomic islands. Each of these elements consist of double stranded DNA, however differences can be seen in the size, structure, biological properties and mechanisms by which these transfer and disseminate (Schwarz and Chaslus-Danlca, 2001).

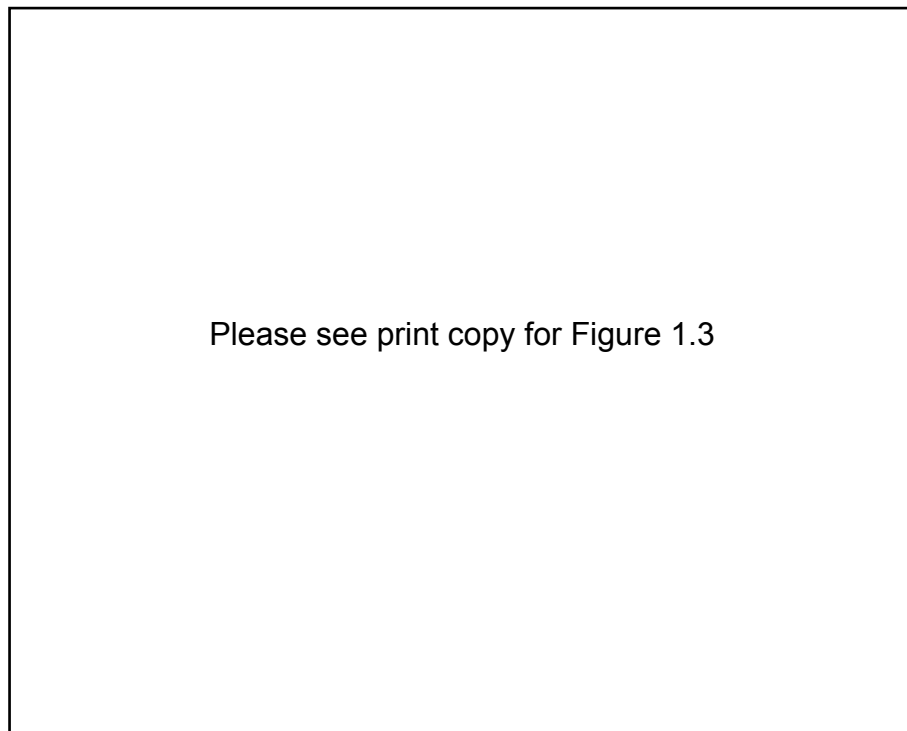


Fig 1.3. The range of gene transfer among bacteria and other taxa (taken from Davies, 1997 modified from Amabile-Cuevas & Chicurrel, 1992.)

1.4.2 Plasmids

Plasmids are one of the major driving forces in the spread of antibiotic resistance and encode a large variety of functions which are not essential for survival of the host in normal conditions. Plasmids increase host fitness in atypical or stressful environments by providing genes responsible for protection against agents such as antibiotics or heavy metals, virulence factors, metabolic properties and fertility functions (Russell, 1998).

Plasmids are capable of autonomous replication. The two main ways in which plasmids can be transferred is via conjugation and to a lesser extent transformation. The transfer of DNA by conjugation occurs after the formation of a conjugal bridge

between two bacterial cells. In order for conjugation to occur the plasmids must contain the necessary *tra* and *oriT* genes. Non-conjugative plasmids can still become mobile and transfer via conjugation if the necessary genes are present on another plasmid in the same cell (Russell, 1998). Transformation is the uptake of foreign DNA by naturally competent bacteria from the environment (Russell, 1998).

A recent study isolated a 120,592 bp incompatibility group F (IncF) plasmid pRSB107 from a sewage treatment plant sludge bacterium. Plasmid pRSB107 contained genes encoding resistance to ampicillin, penicillin G, chloramphenicol, erythromycin, kanamycin, neomycin, streptomycin, sulfonamides, tetracycline and trimethoprim, highlighting the possible survival advantages clustered resistance genes provide bacteria in hostile environments (Szczepanowski et al., 2005). Isolates of MR *Salmonella* serovar Typhi may contain plasmids harbouring antibiotic resistance genes which belong to the IncHI1 incompatibility group and range in size from 100,000 to 180,000 kD (Taylor and Brose, 1985; Hampton et al., 1988; Wain et al., 2003). Plasmids can be vehicles for the transfer of other mobile genetic elements including IS elements/transposons and integrons/gene cassettes.

1.4.3 Transposons and IS elements

Transposons, unlike plasmids, are not able to replicate independently and in order to be maintained, they must be integrated into plasmid or chromosomal DNA. The smallest type of transposable elements are insertion sequences (IS) which are usually made up of two inverted repeats and carry a transposase gene for insertion into and excision from foreign DNA (see Fig 1.4A). Larger transposable elements carry

additional genes which in many cases are responsible for antibiotic resistance. Transposons can be incorporated into a genome via site specific recombination. For example, many Gram-negative bacteria have been found to contain a transposon which belongs to a subgroup of the Tn3 family called Tn21 (Grinsted et al., 1990; Mercier et al., 1990). Tn21 is 19.7 kb in length and contains the necessary genes for transposition (4.1 kb) and a mercury resistance region (4.6 kb) (Grinsted et al., 1990; Silver and Phung, 1996; Liebert et al., 1999). Tn21 also carries a class 1 integron (In2) which also contains an aminoglycoside resistance gene *aadA1* (Liebert et al., 1999).

A.



B.

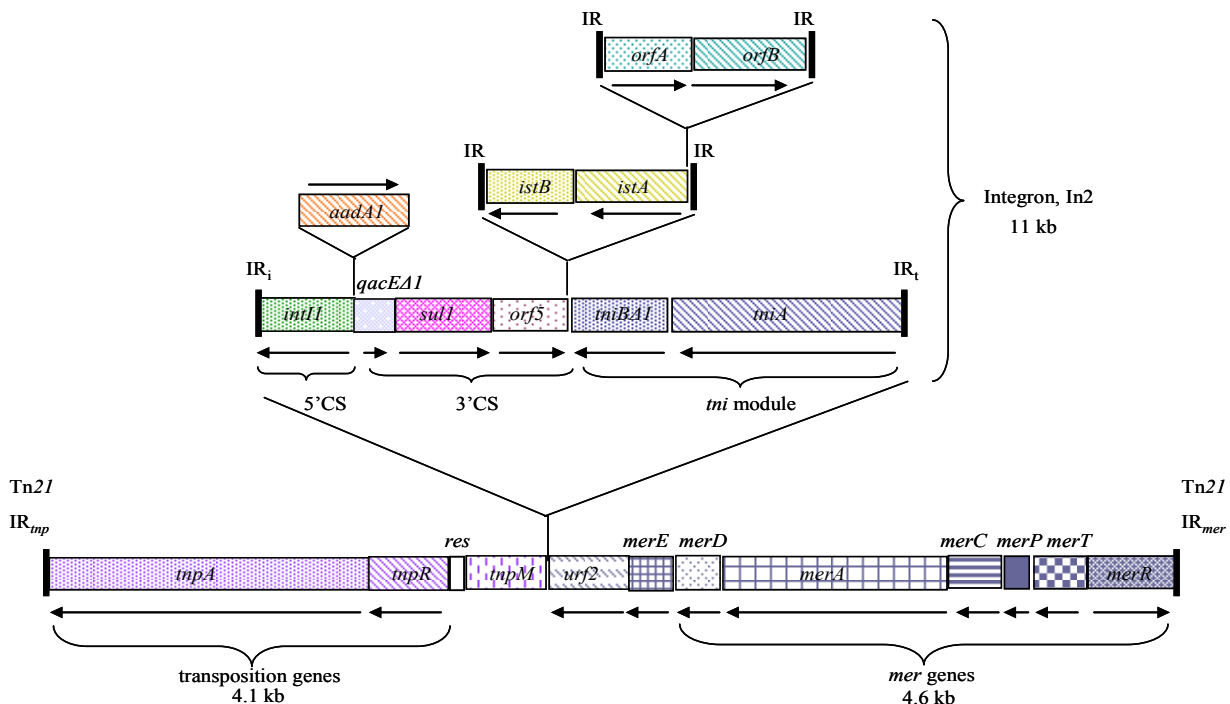


Fig 1.4 A. Insertion sequence (IS) - the simplest transposable element containing a transposase flanked by two inverted repeats (IR). B. Transposition genes, mercury resistance operon and In2 elements (5'CS, 3'CS, *aadA1* cassette and *tmi* module) that make up the 19.7 kb transposon Tn21. Diagram adapted from Liebert et al. (1999).

1.4.4 Integrons: a natural cloning and expression system

In addition to plasmids and transposons, integrons have been described as vehicles for the acquisition of new genes, predominantly antibiotic resistance genes (Hall and Collis, 1995). Integrons have been defined as genetic units that include components of a site-specific recombination system, capable of integrating, excising and rearranging genes that are contained in mobile elements called gene cassettes and are found in both chromosomal and extrachromosomal DNA (Hall and Collis, 1995; Recchia and Hall, 1995; Goldstein et al., 2001). Integrons differ from plasmids and transposons by the lack of replication systems and transposition systems respectively, and are described as transposon derivatives that are incapable of self transposition (Brown et al., 1996; Partridge et al., 2001a). In many cases integrons are plasmid or transposon borne as in the case of integrons found on plasmid R46, transposon Tn1696 (Hall and Vockler, 1987; Stokes and Hall, 1989), NR1 and Tn21 (Womble and Rownd, 1988; Liebert et al., 1999) (see Fig 1.5).

Studies using restriction mapping and heteroduplex analysis revealed that different sets of antibiotic resistance genes were often found in the same genomic environment in closely related plasmids or transposons. Sequencing of these genes revealed that identical sequences flanked the resistance genes, suggesting that they were all integrated at the same site within a common structure (Recchia and Hall, 1995). Integrons were originally identified in the transposon Tn21 and have been found in many antibiotic resistant Gram-negative bacteria including *Salmonella* Typhimurium DT104, *E. coli*, *Vibrio cholerae*, *Acinetobacter baumannii* and *Campylobacter* spp. (Sandvang et al., 1998; Chang et al., 2000; Dalsgaard et al., 2000; Gallego and Towner, 2001). At present, four classes of integrons have been identified with the

most common being the class 1 integrons (Stokes and Hall., 1989; Sundstrom and Skold, 1990).

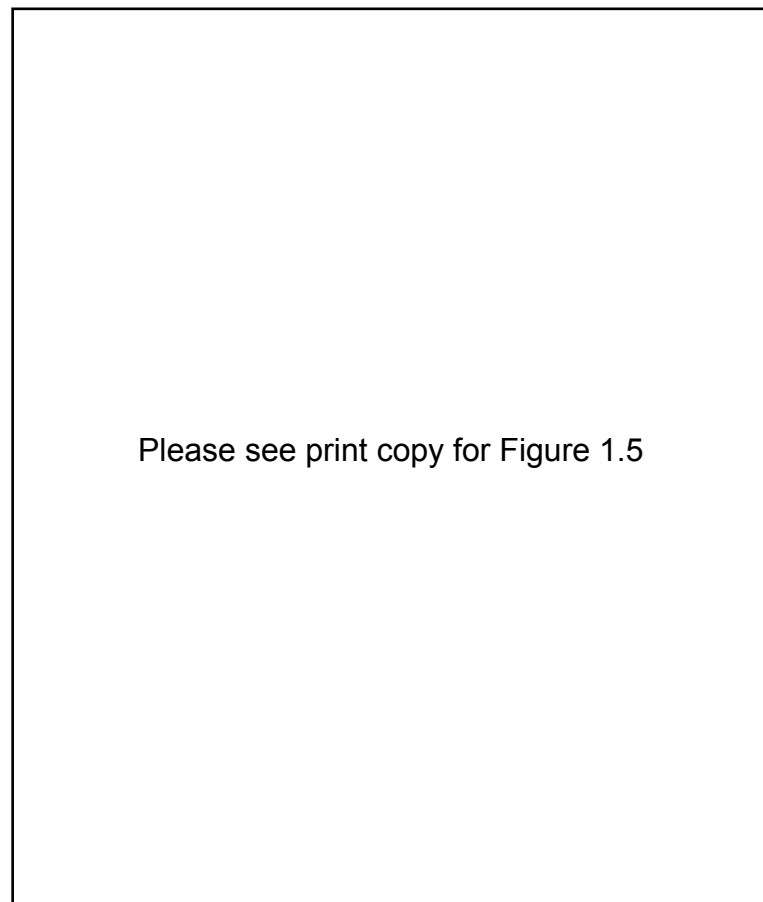


Fig 1.5 Diagram of NR1, a 94.5 kb, self transmissible, multiply antibiotic resistant plasmid carrying Tn21 (containing class 1 integron In2), the tetracycline resistance transposon Tn10 and a chloramphenicol resistance transposon Tn9 homologue, first isolated in Japan in the late 1950's (Nakaya et al., 1960; Watanabe, 1963). The *tra* genes necessary for conjugation are also shown. Diagram taken from Liebert et al. (1999).

1.4.4.1 Class 1 integrons: basic structure

Integrons from different bacterial isolates are highly variable with respect to the number, order and type of gene cassettes they possess. Integrons possess two conserved segments, which are separated by a variable region, containing the gene cassettes (Hall and Collis, 1995; Sandvang et al., 1998; Martinez-Freijo et al., 1999). The conserved regions are known as the 5' conserved segment (5'CS) and the 3' conserved segment (3'CS) (Hall and Collis, 1995; Levesque et al., 1995; Hall et al., 1996; Chang et al., 2000) (see Fig 1.6). The class 1 integron structural boundaries consist of two 25 bp inverted repeats (in relation to each other) known as IRi and IRt. Transposase binding sites found adjacent to these inverted repeats, assist in the movement of the whole class 1 integron via transposition (Kamali-Moghaddam and Sundstrom, 2001; Stokes et al., 2006).

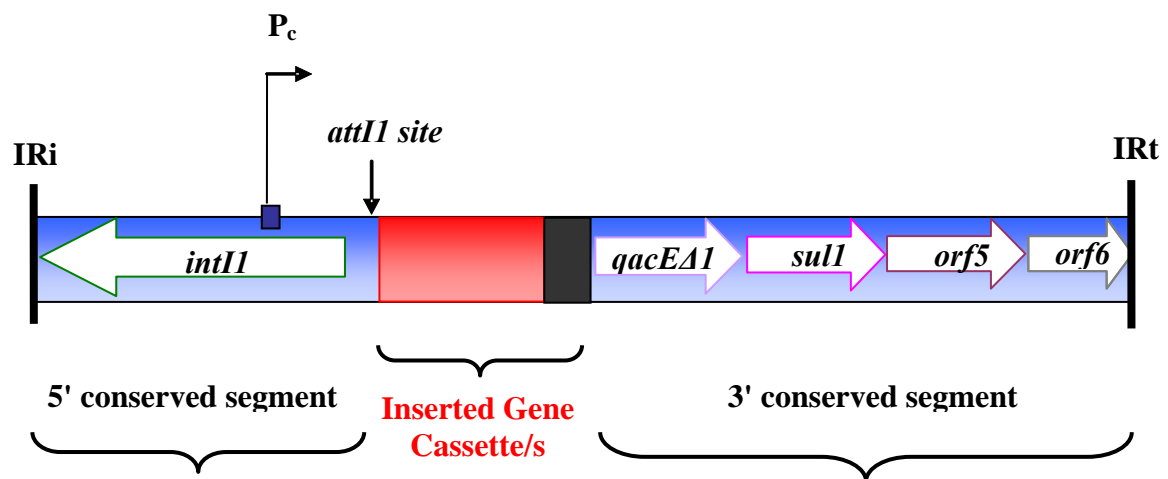


Fig 1.6 General structure of commonly described Class 1 integrons. The arrows show the direction of transcription. The black region is the cassette associated 59 base element involved in site specific recombination. The *intI1* integrase gene (green arrow) is in the 5'CS region and the genes responsible for resistance to quaternary ammonium compounds and sulphonamide are in the 3'CS region as *qacEΔ1* and *sul1*. The *orf5* and *orf6* genes are present. The promoter (P_c) and the flanking inverted repeats IRi and IRt are also represented. Diagram is not drawn to scale. Diagram modified from Levesque et al. (1995).

1.4.4.2 The 5'CS

The 5'CS contains the *intI* integrase gene, which encodes a polypeptide of 337 amino acids that has been shown to be homologous to other members of the site-specific tyrosine recombinase protein family of integrases which catalyse site specific recombination (Collis and Hall, 1992; Nield et al., 2001). Even though the integrases vary between different integron classes they still have characteristic features including the presence of the two conserved regions, the 5'CS and the 3'CS (Martinez-Freijo et al., 1999; Nield et al., 2001).

The 5'CS, which is approximately 1.36 kb in length (Collis and Hall, 1992), also contains an integration site (*attI*) and promoters for the integration and expression of all downstream cassettes (Collis and Hall, 1995; Sandvang et al., 1998; Martinez-Freijo et al., 1999; Partridge et al., 2000). The *attI* sites of the respective integron classes are different, the only common feature being a core site with a consensus sequence GTTRRRY (R-purine, Y-pyrimidine) (Stokes et al., 1997; Nield et al., 2001).

1.4.4.3 The 3'CS

The 3'CS of class 1 integrons may contain a sulphonamide resistance gene, *sulI* (Sundstrom et al., 1988; Stokes and Hall, 1989) and a truncated *qacE* gene (*qacEΔ1*) which encodes resistance to ethidium bromide and quaternary ammonium compounds (Paulsen et al., 1993). The 3'CS has also been known to include open reading frames *orf5* and *orf6* whose functions are currently unknown (Stokes and Hall., 1989; Chang et al., 2000; Partridge et al., 2001b). Class 1 integrons lacking a 3'CS have also been

reported (Rosser and Young, 1999). It is becoming more apparent that 3'CS's can be variable with deletions and IS element insertions known to occur (Hall et al., 1994; Partridge et al., 2001). The basic structural features of both the 5'CS and 3'CS of an example of a class 1 integron can be seen in Fig 1.6.

Tn402, found in plasmid R751, has been classified as both an active transposon (Kamali-Moghaddan and Sundstrom, 2000) and a class 1 integron (Radstrom et al., 1994). The 3' region of Tn402 contains a transposition module made up of four genes *tniA*, *tniB*, *tniQ* and *tniR* necessary for its transposition (Kholodii et al., 1995; Radstrom et al., 1994). Although rare, this transposon is thought to be an ancestral form of the class 1 integron increasingly isolated today (Partridge et al., 2001). The evolutionary pathway from ancestral Tn402 to class 1 integron modules has involved rearrangements and deletions including the loss of the *tni* modules or part thereof, partial deletion of *qacE*, IS element acquisitions as seen in *Pseudomonas aeruginosa* and the integration of *sulI* and other 3'CS ORFs (Stokes et al., 2006) (see Fig 1.7).

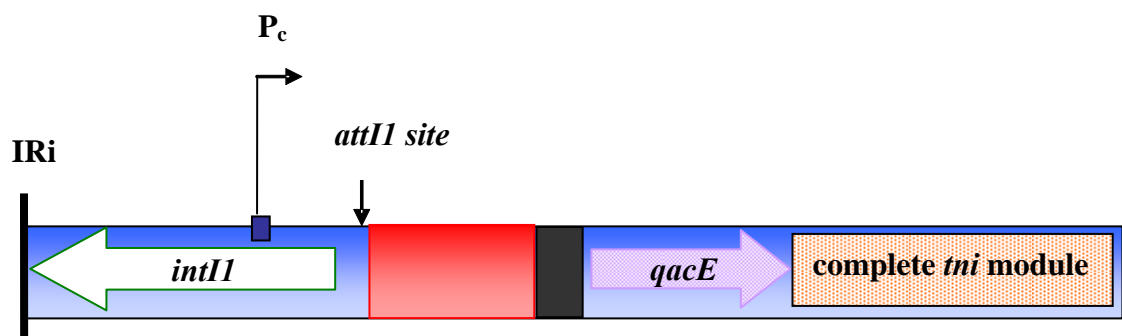


Fig 1.7 Tn402, the ancestral model for commonly found class 1 integrons. IRi, P_c promoter, *attI1* site, integrase and gene cassette are all present as in Fig 1.6. Diagram modified from Stokes et al. (2006).

1.4.4.4 Gene Cassettes

The variable regions of integrons are comprised of one or more genetic units, most commonly encoding for antibiotic resistance and otherwise known as gene cassettes (Stokes et al., 1997; Guerra et al., 2000). Gene cassettes are discrete mobile units and each comprise a gene for antimicrobial resistance or open reading frames (ORF's) which have unknown functions. A recombination site known as the 59 base element (59 be), recognised by all integrases from the 4 classes (Hall et al., 1991; Recchia and Hall, 1995; Stokes et al., 1997; Nield et al., 2001) makes up the remainder of gene cassettes. Over 115 gene cassettes have been identified in multi-drug resistant bacteria and this number is continually on the rise (Partridge and Hall, personal communication).

Gene cassettes, unlike other mobile genetic elements such as plasmids, do not include all of the functions required for their mobility and expression (Hall and Collis, 1995; Stokes et al., 1997). Most genes inserted into integrons lack their own promoter and are expressed from the common promoter region as part of a resistance operon (Levesque et al., 1995; Recchia and Hall, 1995). One exception is the *cmlA* gene (responsible for chloramphenicol resistance) found in integron In4 of Tn1696, which has both a promoter and translational attenuation signals (Bissonnette et al., 1991; Stokes and Hall, 1991).

The common promoter located in all integrons is referred to as P_c (formerly P_{ant} or P1) and is located 214 bases from the inner boundary of the 5'CS and directs transcription of the cassette genes. P_c was first identified by insertional inactivation as that responsible for the transcription of the *dfrA2* gene in the plasmid R388 (Stokes and

Hall, 1989; Collis and Hall, 1995). There are variants of the P_c promoter and their expression efficiencies differ considerably, with the strongest version displaying equal or greater strength than the *tac* promoter (Levesque et al., 1994; Recchia and Hall, 1995). The closer the cassette in an array is to the promoter, the higher the expression levels (Hall and Collis, 1995).

1.4.4.4.1 Gene Cassette integration

Gene cassettes are only formally part of an integron when they are integrated at the integron receptor site (*attI* site) (Recchia et al., 1994; Partridge et al., 2000). While gene cassettes are most commonly found as part of an integron, they can also exist free as covalently closed circular molecules (Hall and Collis, 1995; Recchia and Hall, 1995). The circular forms arise via excision of the cassette from an integron, and are recognised as important mobile contributors to the dissemination of antibiotic resistance genes (Collis and Hall, 1992; Stokes et al., 1997).

Members of the 59 base element family act as sites for site specific integration events catalysed by the integrase encoded by the integron. The most conserved features of the members of the 59 base element family are an inverse core site (RYYYAAC) (R= purine, Y=pyrimidine) and a core site (GTTRRRY). The conserved triplet GTT that is part of the core site, can be found at both ends of integrated gene cassettes with only one GTT part of the cassette (Hall et al., 1991; Recchia and Hall, 1995).

Initially, problems were encountered when trying to determine the boundaries of cassettes, however, the precise location of the crossover in recombination events was determined experimentally by involving one core site with a mutation in the GTT

sequence. The crossover was found to occur between the G and the first T. The first 6 bases of each integrated cassette come from the 59 be and have the sequence TTRRRY (Recchia and Hall, 1995; Stokes et al., 1997) (see Fig 1.8). Several distinct 59 base elements have been shown to function as specific recombination sites. *IntI* mediated recombination can take place between two 59 base elements to form arrays or between the *attI* site and a 59 base element.

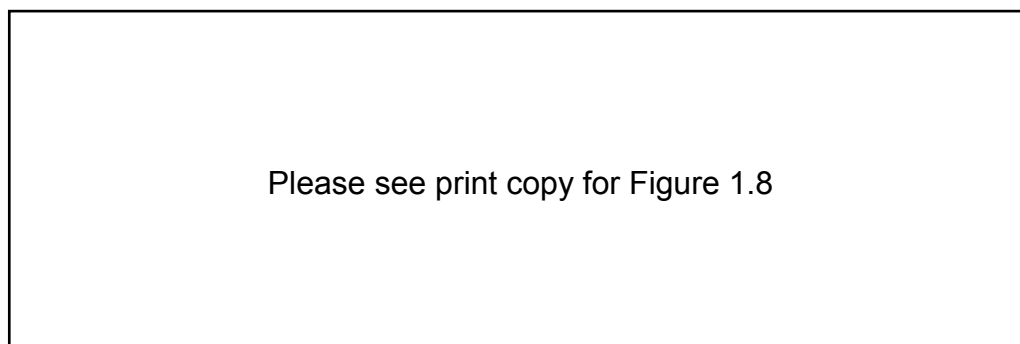


Fig 1.8 The boundaries of gene cassettes. The vertical arrows indicate the recombination crossover point and therefore the boundaries of gene cassettes in linear arrays. The core sites GTTRRRY are shown at both ends of the integrated cassette. The inverse core site of the 59 be is also shown RYYAAC. The solid line and arrow above the gene cassette indicate the extent of the 59 be that is associated with the circularised cassette. The dashed line and arrow indicate those bases making up the 59 be once the cassette has been inserted into the integron structure. Diagram taken from Recchia and Hall (1995).

1.4.4.5 Class 2, class 3 and class 4 integrons

Class 2 integrons, like class 1 integrons, act as a vehicle for the expression of antibiotic resistance genes (Gibrel and Skold, 1998; Goldstein et al., 2001). Class 2 integrons are present on transposon Tn7 and contain an integrase-like gene called *intI2* and an *attI2* site (Hall and Vockler, 1987; Tietze et al., 1987; Hall et al., 1991; Sundstrom et al., 1991). *IntI2* is similar in structure and function to the *intI1* integrase found in *sulI*-associated class 1 integrons but it is interrupted by a stop codon (Hall and Collis, 1995). This type of integrase can promote recombination between

recombination sites in gene cassettes in the same plasmid or different plasmids experimentally, only after changing the termination codon to a sense codon (Hansson et al., 2002).

Another distinguishing feature of the class 2 integrons is that they usually contain a dihydrofolate reductase gene cassette *dfrA1* that encodes resistance to the antibiotic trimethoprim, a *sat* gene providing resistance to streptothricin and *aadA1* for resistance to spectinomycin (Fling and Richards, 1985; Tietze et al., 1987; Gibreel and Skold, 1998; Dalsgaard et al., 2000; Goldstein et al., 2001). Class 2 integrons have been isolated in several strains of Gram-negative bacteria including *Salmonella typhimurium*, *Escherichia coli*, *Vibrio cholerae* and *Bacteroides* species. In a study by Goldstein et al., several multiresistant *Escherichia coli* and *Salmonella* isolates possessed multiple integron classes. Class 2 integrons were found in conjunction with class 1 integrons in strains that displayed resistance to five or more antibiotics (Goldstein et al., 2001). A hybrid class1/class2 integron found in *Acinetobacter baumannii* has also been reported. This atypical integron has the 5'CS, the *dfrA1* and *sat* gene cassettes normally associated with class 2 integrons, however adjacent to this is an *aadA1* (which differs from the one described in class 2 integrons but is identical to those previously described in class 1 integrons) and the 3'CS normally associated with class 1 integrons (Ploy et al., 2000). This study highlighted the recombination potential of class 1 and class 2 integrons in natural bacterial populations.

Class 3 integrons have been characterised in an imipenem-resistant *Serratia marcescens* strain, TN9106 in Japan (Arakawa et al., 1995). From the Japanese study, the bla_{IMP} (metallo-β-lactamase) gene was identified and characterised and was found

to encode broad-spectrum β -lactam antibiotic resistance (Arakawa et al., 1995). The *bla*_{IMP} gene is flanked by a 59-base element and an integrase gene (similar to an integrase gene found in *E. coli*) and the *aac* (6')-Ib gene, which encodes aminoglycoside resistance (Goldstein et al., 2001).

Class 4 integrons have to date only been characterised in *Vibrio cholerae*, a widely distributed bacterial group found primarily in the aqueous environment that is pathogenic to both humans and animals (Mazel et al., 1998; Goldstein et al., 2001). The *Vibrio cholera* genome has been found to contain repeated sequences in clusters that have a similar organisation to integron-gene cassette structures (Mazel et al., 1998). The repeated sequences are similar to the arrays of antibiotic resistance genes (Goldstein et al., 2001) and are separated from one another by 1-2 open reading frames. This novel class of integron also contains an *intI4* integrase, which has 45-50% identity with the three known integrases (Goldstein et al., 2001).

1.4.5 Genomic Islands

1.4.5.1 *Vibrio cholerae* SXT element and *Salmonella* Genomic Island 1 (SGI1)

Genomic islands, which vary in size between 10 and 500 kb, have been identified in many bacterial species. Genomic islands are often made up of elements derived from plasmids, transposons and bacteriophages and make a considerable contribution to bacterial genomic diversity. Increasing genome sequencing of bacterial species has highlighted the fact that microbial genomes are made up of essential cellular functional sequences i.e. house keeping genes (of fairly consistent G+C content) and

genes that have been acquired via horizontal transfer where the G+C content differs from chromosomal housekeeping genes (Hacker and Kaper, 2000).

One such chromosomal integrating element SXT found in *Vibrio cholerae* O139 is greater than 60 kb in size and contains a cluster of genes responsible for resistance to chloramphenicol, streptomycin, sulfonamides and trimethoprim (Waldor et al., 1996; Hochhut et al., 2001). This element was shown to be self transmissible, chromosomally integrated and able to be transferred to *Vibrio cholerae* O1 and *E. coli* (Waldor et al., 1996). The SXT element encodes a λ family site-specific recombinase which mediates the element's insertion and excision from the *prfC* gene in the chromosome (Hochhut and Waldor, 1999; Hochhut et al., 2001).

SGI1 of the globally disseminated MR *Salmonella* Typhimurium DT104 clone is also another genomic island playing a very important role in the dissemination of antibiotic resistance. Residing in the 43 kb genomic island are antibiotic resistance genes responsible for resistance to up to seven antibiotics (Briggs and Fratamico, 1999; Boyd et al., 2001). The MR region of SGI1 is found in a 13 kb class 1 integron called In104 (Levings et al., 2005b), containing two *attII* sites into which gene cassettes can insert (see Fig 1.9). The SGI1 element inserts into the *Salmonella* chromosome between the *thdF* and *yidY* genes (Boyd et al., 2001). SGI1 has been shown under experimental conditions to be mobilised via a site-specific manner to *E.coli* and an SGI1 negative *Salmonella* strain in the presence of a helper plasmid and an SGI1 integrase (*int*) (Doublet et al., 2005).

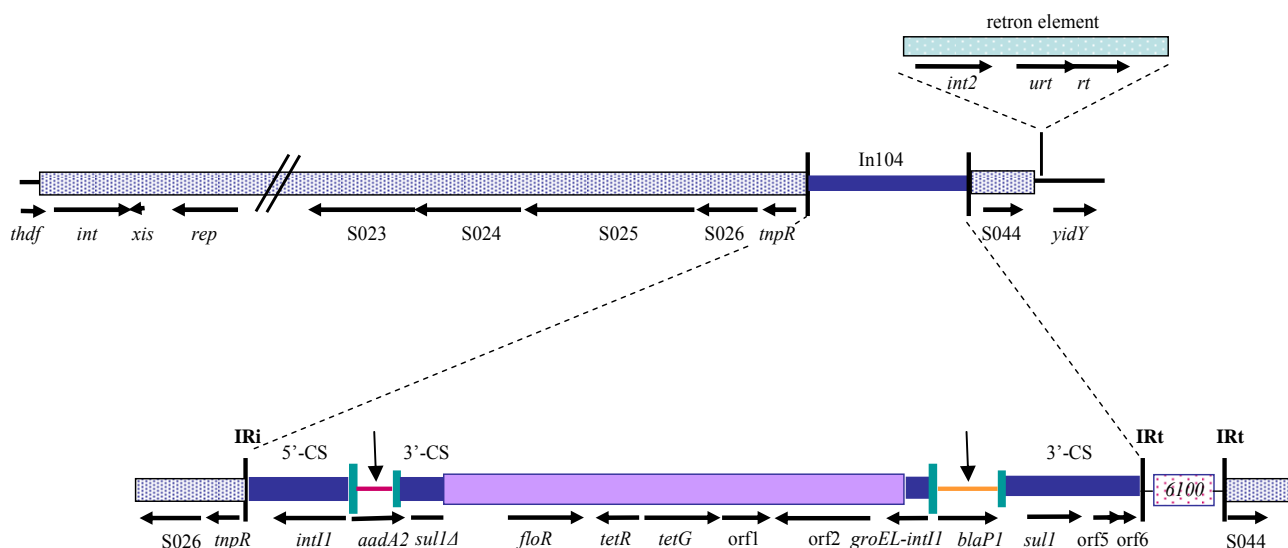


Fig 1.9 SGI1 and its major genetic components. The two gene cassettes are highlighted with vertical arrows. The chromosomal genes *thdF* and *yidY* are shown. The 13 kb MDR region (In104) has been enlarged and the integrating retron element associated with *Salmonella* Typhimurium DT104 is present. The inverted repeats IRi and IRt of In104 are also shown. Diagram modified from Levings et al. (2005b).

The increasing incidence of MR bacteria and the associated demand for an understanding of the genes and mechanisms driving this phenomenon, has highlighted the important role that mobile genetic elements and clustered resistance regions play in the development and spread of multi-drug resistance. Clustered antibiotic resistance is a contributing factor to the persistence of antibiotic resistance even after the removal of the drug from therapeutic use. Taking into account the nature of the highly variable and mobile genetic environment of bacteria, this study has set out to develop sensitive molecular tools to characterise MR regions. Genetic information gathered will ultimately play an important role in the development of selective treatment regimes in both the clinical and veterinary setting.

Chapter 2: General Methods and Materials

2.1 Bacterial strains and culture conditions

The first bacterial collection consisted of 137 drug resistant (displaying resistance to one or more antibiotic) *Salmonella enterica* collected by the Microbiological Diagnostic Unit (MDU) (Melbourne) 39 from humans, 93 from animals, 2 from effluent, 1 from imported spice and 1 from a fish tank during the period February 1999-December 2001 (Table 2.1). The source of strain SRC137 was unknown and the serovar of SRC121 was unknown. SRC89 was not viable upon arrival from MDU and was removed from the collection. The second collection described in greater detail in Chapter 5 was made up of multiply resistant *S. Paratyphi* BdT⁺ strains isolated from human infections and home aquaria. The strains in both collections were serotyped at the MDU using standard procedures to the Kauffman and White scheme (Popoff and LeMinor, 2001) and the resistance profile determined as previously described (Amavisit et al., 2001; Bettelheim et al., 2003).

Susceptibilities to ampicillin (Ap, 32 µg/ml), chloramphenicol (Cm, 10 µg/ml), gentamicin (Gm, 2.5 µg/ml), kanamycin (Km, 10 µg/ml) streptomycin (Sm, 25 µg/ml), spectinomycin (Sp, 50 µg/ml), sulphathiozole (Su, 550 µg/ml), tetracycline (Tc, 20 µg/ml), trimethoprim (Tp, 50 µg/ml), nalidixic acid (Na, 50 µg/ml) and ciprofloxacin (Cp, 2.5 µg/ml) were tested. Upon arrival from MDU isolates were cultured using MacConkey agar (Oxoid, Basingstoke, Hampshire, United Kingdom) and incubated at 37°C for 24 h. Glycerol storage stocks for each of the strains were prepared by resuspending a loopful of culture in a 10% glycerol and 1% casamino acid solution and stored in a freezer at -80°C.

Table 2.1 *Salmonella* experimental isolates (collection 1) used in this study

Isolate No.	Serovar/Phage Type	Source/Country/Year	R Phen
SRC01*	Singapore	human, F (01)	CmSmSpSuTcTp (Na)
SRC02*	Blockley	human, F Bali (99)	KmSmTc (NaCp')
SRC03*	Blockley	human, M Thai (99)	CmKmSmSpSuTcTp (NaCp')
SRC04*	Derby	human, M Malay (99)	CmGmSmSpSuTcTp
SRC05*	Cerro	human, F Thai (99)	ApCmSuTcTp (NaCp')
SRC06*	Enteritidis PT1	human, F HK (99)	SmSuTc (NaCp')
SRC07*	Hadar	human, M Indo (99)	ApSmTc
SRC08*	Hadar PT10	human, M o/s (99)	ApTc (NaCp')
SRC09*	Hadar PT2	human, F/ (99)	SmTc
SRC10*	Kiambu	human, M (99)	ApCmSmSpSuTcTp
SRC11*	Kiambu	human, M (99)	ApCmSmSpSuTcTp (NaCp')
SRC12*	Typhimurium PT135	human, F (99)	ApSu
SRC13*	Typhimurium PT135	human, F (99)	ApSmSuTcTp
SRC14*	Typhimurium PT44	human, F (99)	ApSmSuTcTp
SRC15*	Typhimurium PT9	human, F (99)	SmSu
SRC16*	Typhimurium Pt	human, M (99)	ApCmGmSpSuTcTp
SRC17*	Bovismorbificans	bovine (99)	ApCmSmSuTcTp
SRC18*	Derby	porcine (99)	ApTc
SRC19*	Emek	waste effluent (99)	CmSuTcTp (NaCp')
SRC20*	Infantis	chicken (99)	SmSpSuTc
SRC21*	Kiambu	chicken (99)	SuTcTp
SRC22*	Ohio	porcine (99)	CmGmKmSmSpSuTc
SRC23*	Seftenberg	porcine (99)	ApCmSmSpSuTcTp
SRC24*	Sofia	chicken (99)	SmSpSuTp
SRC25*	Sofia	chicken (99)	ApTc
SRC26*	Typhimurium PT1var2	bovine (99)	ApKmSmSuTcTp
SRC27*	Typhimurium PT135	equine (99)	ApCmGmKmSmSpSuTcTp
SRC28*	Typhimurium PT135	equine (99)	ApCmGmKmSmSpSuTcTp
SRC29*	Typhimurium PT141	porcine (99)	ApGmKmSmSuTcTp
SRC30*	Typhimurium PT141 v4	porcine (99)	ApGmKmSmSuTcTp
SRC31*	Typhimurium PT44	bovine (99)	ApSmSuTcTp
SRC32*	Typhimurium PT68	bovine (99)	ApKmSmSuTcTp
SRC33*	Typhimurium PT9	bovine (99)	SmSu
SRC34	Agona	human, F Africa (01)	CmSmSpSuTcTp
SRC35	Agona	human, M Malay (01)	SmSuTc (Na)
SRC36	Bovismorbificans PT13	human, F (01)	ApKmSmSuTcTp
SRC37	Derby	human, F (01)	SuTc
SRC38	Dusseldorf	human, M Malay (01)	ApCmSuTcTp (Na)
SRC39	Hadar PT10	human, M Indo (01)	SmTc (NaCp')
SRC40	Hadar PT11	human, F Bali (01)	Tc (NaCp')
SRC41	Hadar PT11	human, M (01)	CmKmSmSpSuTc (NaCp')
SRC42	Hadar PT14	human, F Bali (01)	Tc (Na)
SRC43	Hadar PT2	human, F (01)	SmTc (Na)
SRC44	Hadar PT22	human, F (01)	SmTc (Na)
SRC45	Havana	human, M (01)	SmSpSuTc
SRC46	Infantis	human, F (01)	SmSpSuTp
SRC47	Infantis	human, M (01)	ApTcTp
SRC48	Montevideo	human, M (01)	GmKmSmSpTc (NaCp')
SRC49	Paratyphi B dT ⁺	human, F (01)	ApCmSmSpSuTc
SRC50	Paratyphi B dT ⁺	human, M (01)	ApCmSmSpSuTc
SRC51	Singapore	human, M (01)	CmSmSpSuTcTp (NaCp')
SRC52	Sofia	human, M (01)	Tp
SRC53	Sofia	human, F (01)	Km
SRC54	Stanley	human, M Thai (01)	CmGmKmSmSpSuTc (Cp')

Isolate No.	Serovar/Phage Type	Source/Country/Year	R Phen
SRC55	Stanley	human, F Thai (01)	SmSuTc
SRC56	Typhimurium 104L	human, M Timor (ND)	ApCmGmKmSmSpSuTcTp
SRC57	Agona	porcine (01)	ApGmTc
SRC58	Agona	porcine (01)	Tc
SRC59	Bovismorbificans PT13	bovine (01)	ApKmSmSuTcTp
SRC60	Bovismorbificans PT13	bovine (01)	ApCmKmSmSuTcTp
SRC61	Bovismorbificans PT11	bovine (01)	ApKmSmSuTcTp
SRC62	Bovismorbificans PT32	bovine (01)	ApCmKmSmSuTcTp
SRC63	Derby	porcine (01)	SmTc
SRC64	Hadar PT11	duck (01)	SmTc
SRC65	Hadar PT14	duck (01)	SmTc
SRC66	Hadar PT2	duck (01)	SmTc
SRC67	Hadar PT22	duck (01)	SmTc
SRC68	Hadar PT33	duck (01)	SmTc
SRC69	Havana	porcine (01)	ApGmKmSmSpTc
SRC70	Infantis	feline (01)	SmSpSuTc
SRC71	Infantis	chicken (01)	SmSpSuTc
SRC72	Infantis	chicken (01)	ApSmSpSuTc
SRC73	Kentucky	imported spice, (01)	ApGmSmSpSuTc (Na)
SRC74	Ohio	porcine (01)	ApCmGmKmSmSpSuTc
SRC75	Sofia	chicken (01)	Tp
SRC76	Sofia	chicken (01)	SuTp
SRC77	Sofia	chicken (01)	SuTcTp
SRC78	Anatum	porcine (00)	Tc
SRC79	Bovismorbificans PT14	bovine (00)	ApCmKmSmSuTcTp
SRC80	Bovismorbificans PT24	bovine (00)	ApCmKmSmSuTcTp
SRC81	Bovismorbificans 24	abattoir effluent (00)	ApGmTc
SRC82	Bovismorbificans 32	bovine (00)	ApSmSuTp
SRC83	Bovismorbificans u	chicken (00)	CmSmSpSuTc
SRC84	Bredeney	porcine (00)	SuTcTp
SRC85	Derby	porcine (00)	SmTc
SRC86	Derby	porcine (00)	ApCmTc
SRC87	Derby	porcine (00)	SmTcTp
SRC88	Derby	porcine (00)	ApGmTc
SRC89	Derby	duck, (00)	Na (this isolate did not grow)
SRC90	Havana	porcine (00)	ApCmSuTcTp
SRC91	Havana	porcine (00)	GmSmSpSuTc
SRC92	Infantis	canine (00)	SmSpTcTp
SRC93	Infantis	chicken (00)	ApCmSpSuTc
SRC94	Infantis	chicken (00)	CmSmSpSuTc
SRC95	Infantis	chicken (00)	SmSpSuTc
SRC96	Infantis	chicken (00)	SmSuTc
SRC97	Kiambu	chicken (00)	SuTp
SRC98	Kiambu	chicken (00)	SuTcTp
SRC99	Kiambu	chicken (00)	ApCmSmSpSuTcTp
SRC100	Kiambu	chicken (00)	SmSuTcTp
SRC101	Paratyphi B dT ⁺	fish tank water (00)	ApCmSmSpSuTc
SRC102	Seftenberg	porcine (00)	ApGmKmSmSpSuTcTp
SRC103	Seftenberg	porcine (00)	ApGmKmSmSpSuTc
SRC104	Seftenberg	meat, bone meal (00)	Sm
SRC105	Sofia	canine (00)	Tc
SRC106	Sofia	chicken (00)	ApSmSuTc
SRC107	Sofia	chicken (00)	SmSu
SRC108	Sofia	chicken (00)	SuTcTp
SRC109	Sofia	chicken (00)	SmSuTcTp
SRC110	Sofia	chicken (00)	ApSmSuTc
SRC111	Sofia	chicken (00)	SuTcTp
SRC112	Stanley	porcine (00)	ApGmSuTcTp
SRC113	Typhimurium PT12	bovine (00)	Tc

Isolate No.	Serovar/Phage Type	Source/Country/Year	R Phen
SRC114	Typhimurium PT135	avian (00)	Sm
SRC115	Typhimurium PT135	chicken (00)	TcTp
SRC116	Typhimurium PT135	bovine (00)	ApSmSuTcTp
SRC117	Typhimurium PT141	porcine (ND)	ApGmKmSmSuTp
SRC118	Typhimurium PT141	porcine (00)	ApGmKmSmSuTcTp
SRC119	Typhimurium PT208 v1	porcine (00)	ApCmGmKmSmSpSuTc
SRC120	Typhimurium PT29	porcine (00)	ApKmSmSuTp
SRC121	N/D	porcine (00)	ApGmKmSmSuTp
SRC122	Typhimurium PT29	porcine (00)	ApKmSmSuTp
SRC123	Typhimurium PT29	porcine (00)	ApKmSmSuTcTp
SRC124	Typhimurium PT44	bovine (00)	ApSmSuTcTp
SRC125	Typhimurium PT44	bovine (00)	KmSuTcTp
SRC126	Typhimurium PT44	bovine (00)	ApSmSuTp
SRC127	Typhimurium PT44	bovine (00)	KmSuTcTp
SRC128	Typhimurium PT44	bovine (00)	ApSmSuTcTp
SRC129	Typhimurium PT44	bovine (00)	ApKmSmSuTcTp
SRC130	Typhimurium PT64	bovine (00)	SmSu
SRC131	Typhimurium PT9	bovine (00)	ApSmSuTp
SRC132	Typhimurium PT9	bovine (00)	ApSmSu
SRC133	Typhimurium PT9	bovine (00)	ApKmSmSu
SRC134	Typhimurium PT99	pheasant (00)	Tc
SRC135	Typhimurium RDNC	porcine (ND)	ApKmSmSuTcTp
SRC136	Typhimurium RDNC	porcine (00)	ApKmSmSuTp
SRC137	Typhimurium	ND/ND	Tc

* isolates partially characterised during my honours year 2001.

SRC89 failed to grow and is not included in the study.

ND- isolation dates were not available for SRC 56, 117, 135. Isolation source and date information was not available for SRC137.

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

2.2 DNA preparation

2.2.1 Crude DNA extraction procedure

Template DNA for PCR was prepared by one of two methods. Crude DNA solutions were prepared by resuspending a single colony in 1 ml of sterile purified milli-Q water, heating (100°C for 5 min) and pelleting debris by centrifugation (13,000 rpm, 10 min). Template DNA (5 µl) derived from the crude DNA extraction was used during the initial screening process for the presence of the class 1 integrase (*intI1*) and *Salmonella* specific *invA* gene multiplex PCR (see section 2.3.1).

2.2.2 Phenol/Chloroform DNA extraction procedure

The second method of DNA purification used a standard phenol/chloroform extraction method (Sambrook et al., 1989) and generated DNA of higher purity used for downstream PCR analysis of cassette containing strains. Bacteria were grown overnight on MacConkey agar (Oxoid) plates at 37°C and the cells were harvested by adding approximately 2 ml of 1 X phosphate buffered saline (PBS) (see Appendix A.1) to the plate and removing all of the bacterial growth. The liquid was transferred to a sterile tube and centrifuged at 2500 xg for 30 min and supernatant discarded. The pellet was resuspended in 4 ml of sterile 1 X PBS and centrifuge as mentioned above.

The bacterial pellet was resuspended in 10% sucrose/TE (1 ml sucrose/TE per 0.1 g wet pellet weight). To this suspension assuming a pellet weight of 0.1 g, 125 µl of freshly made lysozyme (50 mg/ml) was added along with 325 µl of 0.5 M EDTA (pH 8.0), mixed and incubated in a 37°C waterbath for 15 min. 250 µl of 10% (w/v) SDS/TE buffer and 25 µl RNaseA (10 mg/ml)(Sigma-Aldrich, St Louis, Missouri, USA) were added and the tubes mixed with gentle inversion and incubated at 37°C for 30 min until solution became clear. 12.25 µl of a 10 mg/ml solution of proteinase K (Roche Molecular Biochemicals, Mannheim, Germany) was added and tubes again mixed with gentle inversion and incubated for 1 h at 37°C. The waterbath temperature was then increased to 56°C and the tubes left to incubate overnight.

The solutions were transferred to SST Gel and Clot Activator Vacutainer Tubes (Becton Dickinson Vacutainer Systems, NJ, USA) and 2 ml of TE saturated phenol/chloroform/isoamylalcohol (25:24:1, pH 8.0) was added and mixed

thoroughly. The tubes were centrifuged at $2,800 \times g$ for 15 min. The phenol/chloroform/isoamylalcohol step was repeated twice more on the aqueous layer formed after each centrifugation. The aqueous layer was then transferred to fresh SST Vacutainer Tubes and 2 ml of TE saturated chloroform/isoamylalcohol (24:1, pH 8.0) was added and mixed thoroughly. The tubes were centrifuged at $2,800 \times g$ for 15 min. The chloroform/isoamylalcohol step was repeated twice more on the aqueous layer.

The aqueous phases for each DNA preparation were transferred to dialysis bags and dialysed against TE buffer solution (pH 8.0) with 2-3 changes of the buffer over 24-48 h. The dialysed solutions were then placed in 5 ml sterile tubes and stored in the fridge at 4°C. The DNA concentration of the solutions for each isolate were measured using the GeneQuant SpectrophotometerTM and using agarose gel electrophoresis and known concentration marker Hyperladder I (Bioline, London, UK).

2.3 PCR analysis and conditions for initial screening experiments

2.3.1 Multiplex PCR for identification of Class 1 integrons

All PCR amplification reactions were carried out in 50 µl reaction volumes made up of 1 X PCR reaction buffer (Roche) containing 160 µM dNTPS (Bioline), 20 pmole of each primer (Sigma-Aldrich), approximately 10-50 ng template DNA and 1 unit of Taq DNA polymerase (Roche). All PCR reactions were carried out on either a PC-960 or FTS-960 thermal cycler (Corbett Research, Mortlake, Australia). Primer details and conditions not listed here will be mentioned in relevant chapters. A negative control

reaction was also run for each set of PCR reactions, consisting of all of the above reagents with DNA template replaced with 5 µl of sterile purified milli-Q water.

The primers used in the multiplex PCR were L2 (5'-GACGATGCGTGGAGACC-3') (Sallen et al., 1995) and L3 (5'-CTTGCTGCTTGGATGCC-3') (Maguire et al., 2001) which amplify a 298 bp fragment of the class 1 integrase (*intI1*) (see Fig 2.1) along with *Salmonella* specific primers that amplify a 389 bp product of the *invA* gene (part of the *invA-spa* complex), Salm3 (5'-GCTGCGCGCGAACGGCGAAG-3') and Salm4 (5'-TCCCGGCAGAGTTCCCAT-3') (Ferreti et al., 2001). Thermal cycling conditions used in the multiplex PCR included an initial denaturation cycle 94°C for 5 min, followed by 30 amplification cycles and a final extension cycle of 72°C for 10 min. Each amplification cycle consisted of 30 s at 94°C (denaturation), 30 s at 54°C (annealing) and 2 min at 72°C (extension).

2.3.2 Gene cassette variable region PCR

Amplification of the gene cassette variable region was performed using standard primers in the 5'CS and 3'CS of the class 1 integron, L1 (5'-GGCATCCAAGCAGCAAGC-3') and R1 (5'-AAGCAGACTTGACCTGAT-3') (Levesque and Roy, 1993) (see Fig 2.1). Thermal cycling conditions used in the cassette PCR included a denaturation cycle 94°C for 5 min, followed by 30 amplification cycles and a final extension cycle of 72°C for 10 min. Each amplification cycle consisted of 30 s at 94°C (denaturation), 30 s at 57°C (annealing) and 1 min 30 s at 72°C (extension).

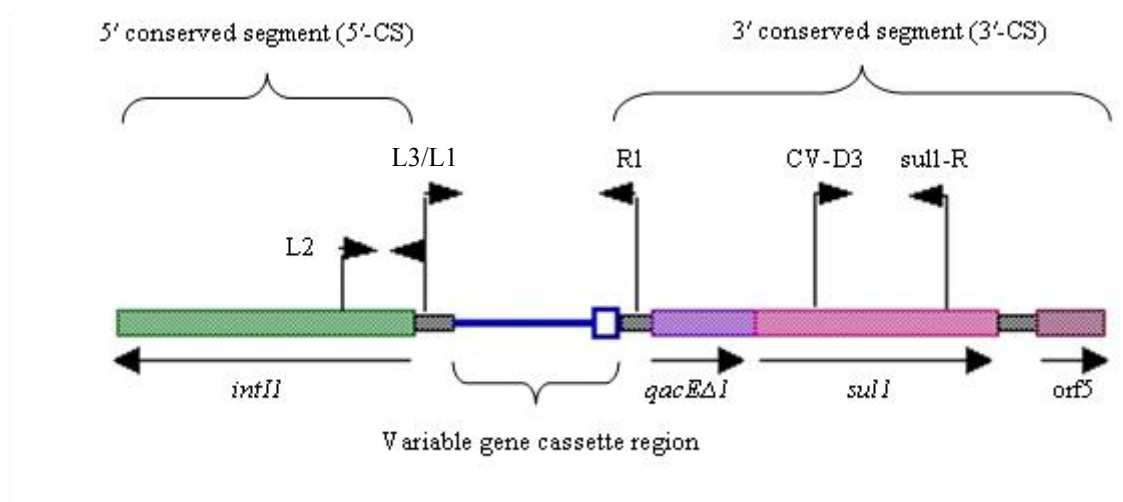


Fig 2.1 A diagrammatic representation of Class 1 integron primers and their amplification targets. Primers L2 and L3 anneal at positions 910 to 926 and 1206 to 1190, respectively, in the published sequence of integron In0 (GenBank accession number M73819). In the same sequence, L1 and R1 anneal at positions 1190 to 1206 and 1342 to 1326, respectively and the CV-D3 and *sul1*-R primers (used to create DIG-labelled probe in Chapter 3) anneal at positions 1954 to 1973 and 2406 to 2387. Arrows under primer names indicate primer direction.

2.4 Restriction enzyme analysis of L1/R1 PCR products

Restriction enzyme analysis of the variable gene cassette amplicons with a frequent 4 bp cutter, *RsaI* (Roche) and *Tsp509I* (New England Biolabs, Beverly, MA, USA) was used to distinguish between different cassette arrays. PCR products (100-200 ng) were digested in a total volume of 20 μ l with 3U of enzyme, 1 X buffer (specific to the enzyme) and were incubated overnight at 37°C or for a minimum of 4 h on a ThermolineTM dry cell heating block (Scientific Equipment Pty Ltd, Smithfield, Australia) according to manufacturer's instructions.

2.5 Agarose gel electrophoresis

10-20 µl of amplified DNA fragments and digestion products were resolved by 1-2 % w/v agarose gel using 0.5 X TBE buffer with approximately 2 µl of loading buffer (0.25% bromophenol blue, 15% ficoll). GeneRuler™ 100 bp DNA Ladder Plus (1.5 µg) (MBI Fermentas Life Sciences, Lithuania), unless otherwise stated, was used to estimate sizes of fragments. Gels were electrophoresed at approximately 90-110 V and were stained with ethidium bromide (5 µg/ml) (Bio-Rad, Hercules, CA, USA), visualized with UV illumination, and imaged using a GelDoc 1000 image analysis station (Bio-Rad).

2.6 Southern hybridisation of digested chromosomal DNA probed with digoxigenin (DIG) labelled DNA probes.

2.6.1 Nitrocellulose membrane preparation and DNA transfer

50-100 ng of digested chromosomal DNA (phenol/chloroform extracted) of experimental isolates were loaded onto a 1% w/v agarose gel and electrophoresed at 110 V for 3 h. The agarose gels were stained and visualised as above. The DNA was nicked by washing the gels with gentle agitation twice with 0.2% HCl, each wash being 5 min. The DNA was then denatured by washing the gels in denaturation solution (0.5 N NaOH, 1.5M NaCl) for 15 min (total of 3 washes) and neutralised in neutralisation solution (1M Tris [pH 8.0], 1.5M NaCl) for 7.5 min (total of 2 washes). The DNA from the gels was then transferred via a concentration gradient to a nitrocellulose membrane (Hybond N⁺) (Amersham, Buckinghamshire, UK)

overnight as described previously (Sambrook et al., 1989). The southern transfer stack was dismantled and the membrane baked in a vacuum oven at 80°C for a minimum of 2 hrs. The membranes were washed with sterile milliQ water and left to air dry.

2.6.2 DIG-labelled DNA probe preparation and hybridisation

The PCR amplicons were amplified using reaction conditions suitable for each of the primer sets described in detail in each chapter, however, standard unlabelled dNTPs were replaced with DIG-labelled dNTPs. The labelled amplicons were visualised using 1.2% w/v agarose gel and all probes used in this study were purified using the QIAquick PCR purification kit (Qiagen, California, USA) according to protocols supplied by the manufacturer. The purified DIG-labelled PCR products were eluted off the column with sterile milliQ water. After purification the PCR products were denatured by heating at 100°C for 10 min and placed on ice immediately. Hybridisation solutions were made by adding 10 µl of the denatured PCR probe to 50 ml of fresh EasyHyb pre-hybridisation solution (Roche) which was then stored at -20°C until necessary.

The membranes were transferred to clean hybridisation bottles with approximately 25 ml of fresh EasyHyb pre-hybridisation solution (Roche) and incubated at 58°C for 2h using a rotisserie hybridisation oven (Hybaid). The pre-hybridisation solutions were removed and 25 ml of hybridisation solution containing DIG-labelled probe added. The membranes were hybridised at 65°C in the hybridisation oven for 1 h and then left to incubate overnight at 42°C.

2.6.3 Development of southern membranes

The hybridisation solutions were removed and the membranes washed twice for 15 min at room temperature with 35 ml of fresh 2 X SSC (0.3M NaCl, 30mM sodium citrate, pH 7.0)/0.1% SDS. This process was repeated but the incubation temperature was increased to 68°C. The membranes were equilibrated with 50 ml of 1 X wash buffer (Roche) for 1 min at room temperature and then blocked for 1 h at room temperature with 50 ml blocking solution which was made up of 1 X blocking buffer (Roche) and 1 X maleic acid (Roche).

The anti-DIG antibody solution was made up by adding 5 µl (3.75 U) of anti-digoxigenin-AP Fab fragment solution (Roche) to 50 ml of fresh blocking solution. The membranes were incubated with this solution for 30 min at room temperature and after removal of antibody solution, washed twice for 15 min at room temperature with the 1 X wash buffer (Roche). The membranes were transferred to clean plastic bags and equilibrated for over 2 min with 5 ml of 1 X detection buffer (Roche). 50 µl of CSPD (calf spleen phosphodiesterase) chemiluminescence substrate (Roche) was added to the bags and mixed well using a sponge roller and the bags left in a dark environment for 30 min at room temperature. Excess solution was removed from the bags and X-ray film was placed over the membranes (under red light) in an x-ray cassette and left to expose for between 30 min to 1 h depending on degree of signal. The films were placed in developer solution (Eastman Kodak Company, Rochester, NY, USA) for up to 5 min with gentle agitation, washed in a stop solution of water briefly and then placed in fixer solution (Eastman Kodak Company) for up to 3 min.

The x-ray films were washed with water and left to air dry and then scanned using the GS-800 Calibrated Densitometer (BioRad).

2.7 DNA sequencing

Gene cassette PCR products representing each of the different *RsaI* profiles and any other sequenced PCR products examined were sequenced using the Big Dye Terminator Reaction Mix version 3.2 (Applied Biosystems, California, USA). Cycle sequencing reactions (20 µl final volume) contained PCR amplicon (30-90 ng), 3.2 pmol of primer and 8 µl of reaction mix. The PCR products were prepared for sequencing using the QIAquick PCR Purification Kit (Qiagen) following protocols supplied by the manufacturer. The sequencing reaction consisted of 28 cycles of denaturation (90°C, 30 s), annealing (50°C, 15 s) and extension (60°C, 240 s). The sequencing reactions were purified using ethanol precipitation and run on the ABI377TM gel sequencing system (Applied Biosystems).

DNA sequence analysis was conducted on both strands of template DNA. DNA sequence analysis was carried out using the BioEdit alignment program and BlastN features from the NCBI (National Centre for Biotechnology Information) GenBank database. Novel sequence regions were deposited into GenBank using the Sequin sequence entry program downloaded from NCBI website. A complete list of cassette sequencing primers can be found in Appendix Table A.2.

2.8 Cloning of novel gene cassettes

2.8.1 PwoI PCR amplification of L2/R1 PCR products for cloning

In order to determine function of the novel gene cassettes isolated in this study the L2 (5'-GACGATGCGTGGAGACC-3') (Sallen et al., 1995) and R1 (5'-AAGCAGACTTGACCTGAT-3') (Levesque and Roy, 1993) primers were used to amplify the gene cassette variable region as well as the *intI1* promoters in the 5'CS of the class 1 integron. PCR amplification reactions were carried out in 50 µl volumes of PCR reaction buffer (Roche) containing 160 µM dNTPS, 20 pmole of each primer, approximately 10-50 ng template and 0.5 µl (2.5U) of Pwo DNA polymerase (Roche). Thermal cycling conditions used in the PCR included a denaturation cycle 94°C for 5 min, followed by 30 amplification cycles and a final extension cycle of 72°C for 10 min. Each amplification cycle consisted of 30 s at 94°C (denaturation), 30 s – 1min at 62°C (annealing) and 1 min 30 s at 72°C (extension). PCR products were purified by either the QIAquick PCR purification kit using manufacturer's instructions and eluted with sterile milliQ water (Qiagen) or by gel extraction using an Amicon Bio-separations Ultrafree-DNA columns (Millipore Corporation, Bedford, Massachusetts).

2.8.2 Ligation of L2/R1 PCR products into pPCR-scriptTM Amp SK(+) cloning vector

The purified L2/R1 PCR product for each of the strains (SRC54, SRC70 and SRC73) was then ligated into pPCR-scriptTM Amp SK(+) cloning vector (Stratagene, La Jolla, CA, USA)(see Fig 2.2). The ligation reactions were made up to a total of volume of

10 μ l with sterile milliQ water, consisted of 1 μ l (10 ng) of vector pPCRScriptTMAmpSk(+), 1 μ l (10 X) reaction buffer, 0.5 μ l of 10 mM rATP, 1 μ l (5U) *SrfI* restriction enzyme, 1 μ l (4U) T4 DNA Ligase and 4 μ l (approximately 50 ng) of purified PCR product. A control with vector only was also included in these experiments. The tubes were incubated at room temperature for 1 h, then heated at 65°C on a PC-960G gradient thermal cycler (Corbett Research) for 10 min and stored.

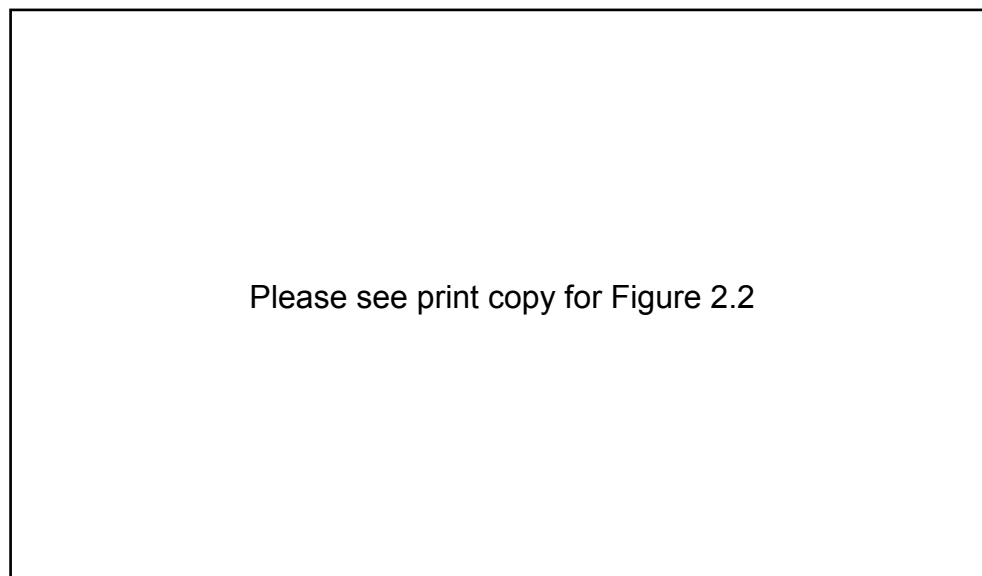


Fig 2.2 Diagram of pPCR-ScriptTM Amp SK (+) cloning vector used in this study. Diagram taken from Stratagene pPCR-ScriptTM Amp SK (+) cloning vector kit manual.

2.8.3 Transformation of pPCRScriptTMAmpSk(+) containing vector into *E. coli* 294 and *E. coli* DH5 α cells

Vector pPCRScriptTMAmpSk(+) containing the cloned insert from SRC73 was electroporated into *E. coli* 294 (rifampicin resistant: *hsdR hsdM⁺ thi endA recA*) (Talmadge et al., 1980) electro-competent cells and the vector containing insert from SRC54 and SRC70 was electroporated into *E. coli* DH5 α (*F'/endA1 hsdR17[rK-mK⁺]*)

supE44 thi-1 recA1 gyrA) (Woodcock et al., 1989) electro-competent cells using a BioRad Gene Pulsar at 2.5 kV (voltage suggested for *E. coli* by the manufacturer). The electroporation mixture consisted of 4 µl of ligation reaction mix, 50 µl of electro-competent cells and 40 µl of sterile milliQ water. The electroporation mixtures were then added to 1 ml of sterile LB media and incubated at 37°C for 1 h with shaking at 220 rpm. The cells were then plated onto appropriate selective media depending on the gene cassettes that were cloned (LB with ampicillin 100 µg/ml and streptomycin 25 µg/ml (SRC54 cloned fragment), ampicillin 100 µg/ml and trimethoprim 50 µg/ml (SRC70 cloned fragment) and ampicillin 100 µg/ml and gentamicin 8 µg/ml (SRC73 cloned fragment). Colonies were then picked and patched onto appropriate selective media for DNA extraction and PCR analysis and stored at -80°C after colonies were resuspended in a sterile solution of 10% glycerol and 1% casamino acids. Plasmid DNA for each of the clones was extracted using QIAprep Spin Miniprep columns (Qiagen) using manufacturer's protocols. A cell pellet from a 5 ml overnight culture was used. Sequencing of the plasmid templates (final concentration approximately 200-300 ng) containing cloned fragments of interest were carried out using sequencing techniques mentioned previously.

2.8.4 Antimicrobial susceptibility testing of clones and controls

In order to get approximate MIC levels for the cloned fragments two types of selective screening methods were employed. For the clones with insert PCR products generated from SRC54 and SRC70 antibiotic gradient plates were utilised. A single colony of each clone grown on appropriate selection media was resuspended in 1 ml of sterile milliQ. A sterile cotton swab was then used to spread the resuspended

culture onto a square antibiotic gradient plate, applying from the least to most concentrated end (see Fig 2.3A). The antibiotic gradient plates used were LB agar containing lincomycin 0 to 2000 $\mu\text{g/ml}$ (for clone generated from PCR amplicon from SRC54) and Mueller Hinton agar (Oxoid) containing trimethoprim 0 to 1000 $\mu\text{g/ml}$ (for clone generated from PCR amplicon from SRC70).

For the cassette array clone generated from SRC73 susceptibilities to gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin and spectinomycin were determined by using antibiotic susceptibility testing discs (Oxoid). One colony was resuspended in approximately 2.5 ml of sterile saline and then distributed evenly onto a blood sensitivity agar plate Sensitest Agar, (Oxoid). The excess liquid was removed using a sterile pipette and plates were dried (under sterile conditions) for no longer than 30 min.

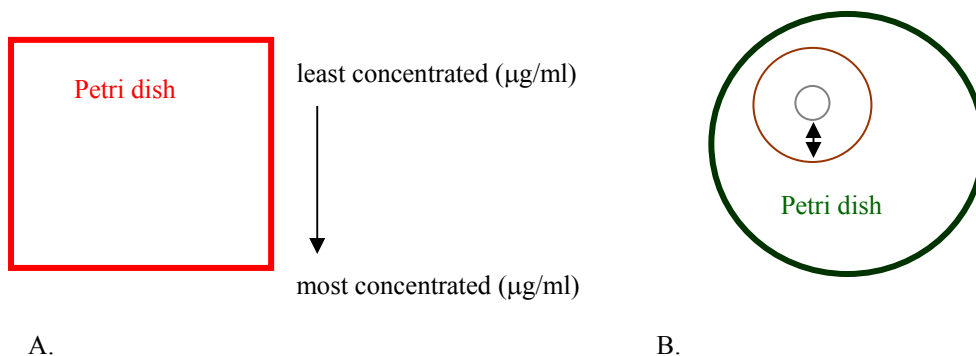


Fig 2.3 A. The gradient plate method where the direction of application is indicated by the arrow. B. Antibiotic disc susceptibility testing method. The inner grey circle represents the antibiotic disc and the outer brown circle represents the extent of confluent bacterial growth. The arrow represents the annular radius measured.

A dispenser loaded with antibiotic discs of interest were applied to the plate and securely fixed to the plate with sterile tweezers. The antibiotic discs used included amikacin 30 $\mu\text{g/ml}$ (AK30), gentamicin 10 $\mu\text{g/ml}$ (CN10), kanamycin 30 $\mu\text{g/ml}$

(K30), netilmicin 30 µg/ml (NET30), spectinomycin 25 µg/ml (SH25), streptomycin 25 µg/ml (S25) and tobramycin 10 µg/ml (TOB10) (Oxoid). The inoculum was approximately 10^5 colony forming units (CFU) per spot. The plates were incubated for 18 hrs at 37°C. The annular radius (the shortest distance from the edge of the disc to the edge of the confluent growth) was measured and results recorded (see Fig 2.3B). All susceptibility testing was performed in duplicate and controls *E. coli* containing pPCRScripTMAmpSk(+) vector only were also included.

Chapter 3: Molecular analysis of class 1 integrons in a collection of multi-drug resistant Australian *Salmonella* spp. from human, animal and environmental sources

3.1 Introduction

Salmonella is a Gram-negative bacterium and is an important zoonotic pathogen. *Salmonella* is responsible for significant food-borne illness in humans. The use of antibiotics in both human and veterinary medicine, as well as use in growth promotion over the past has created enormous pressure for the selection of antibiotic resistance within the bacterial community. Bacterial resistance to antimicrobials is emerging as a serious global health problem and of particular concern is the increasing incidence of multidrug resistance in *Salmonella* strains isolated from zoonotic foodborne infections (Baggesen et al., 2000).

Salmonella infections are a significant health concern and the most predominant serovar isolated here in Australia is *S. Typhimurium*. In 2001, 2653 (38.3%) cases of *S. Typhimurium* were identified out of a total of 6932 *Salmonella* cases. *S. Virchow* (6.9%), *S. Enteritidis* (4.2%), *S. Saintpaul* (4.1%), *S. Birkenhead* (3.6%) and *S. Bovismorbificans* (2.4%) followed, making up a large proportion of the top ten serovars isolated in Australia. Increasing antimicrobial resistance among *Salmonella* spp. is a significant problem in Australia. Of the 2354 *Salmonella* strains identified by the National Enteric Pathogens Surveillance Scheme (NEPSS) during 1999, 253 (10.7%) exhibited some level of resistance to one or more antibiotics. This figure had risen to 16.8% in 2001 (NEPSS, 2001).

The dissemination of antibiotic resistance genes among bacterial pathogens is facilitated by mobile genetic elements such as plasmids, phages and transposons. However, integrons (transposons or defective transposon derivatives) and circularised forms of their cassettes are also important vehicles in the acquisition and spread of resistance genes and have been described in a wide range of bacterial pathogens including *Salmonella* spp. (Jones et al., 1997; Partridge et al., 2001b). Integrons comprise a site specific recombination system capable of integrating and express mostly resistance genes known as gene cassettes (Recchia and Hall, 1995). The most commonly isolated is the class 1 integron.

Class 1 integrons are defined by their boundaries, which are two 25 bp inverted repeats known as IRi and IRt. Class 1 integrons have three main domains, the 5'CS and 3'CS which flank a variable gene cassette region. As mentioned in chapter 1 (see section 1.4.4.1), the 5'CS contains an integrase (*intI1*) and an integration site (*attI1*) which recombines with the 59 base element of the gene cassettes, allowing the cassette to be inserted or excised into the variable region of the integron structure and a promoter (P_c), directing transcription of inserted gene cassettes (Stokes and Hall 1989). The content of the 5'CS and its boundaries are highly conserved among all class 1 integrons (Hall et al., 1994).

Observed in class 1 integrons is the variability in the genetic content of the 3'CS (Partridge et al., 2001b). The 3'CS (see section 1.4.4.3) can contain a *qacEΔ1*, a *sulI* gene responsible for resistance to quaternary ammoniums and sulfonamides respectively and two open reading frames with unknown function orf5 and orf6. This region can also contain insertion elements such as IS6100 found in In28 (Partridge et

al., 2001b). The exact content of the original complete 3'CS is unknown however a common belief is that Tn402 (which doesn't contain any of the genes normally associated with commonly described 3'CS's) is the ancestor of the class 1 integron and certain IS mediated deletions, insertions and rearrangements have lead to the genetic diversity of the 3'CS's commonly seen today (see Fig 3.1).

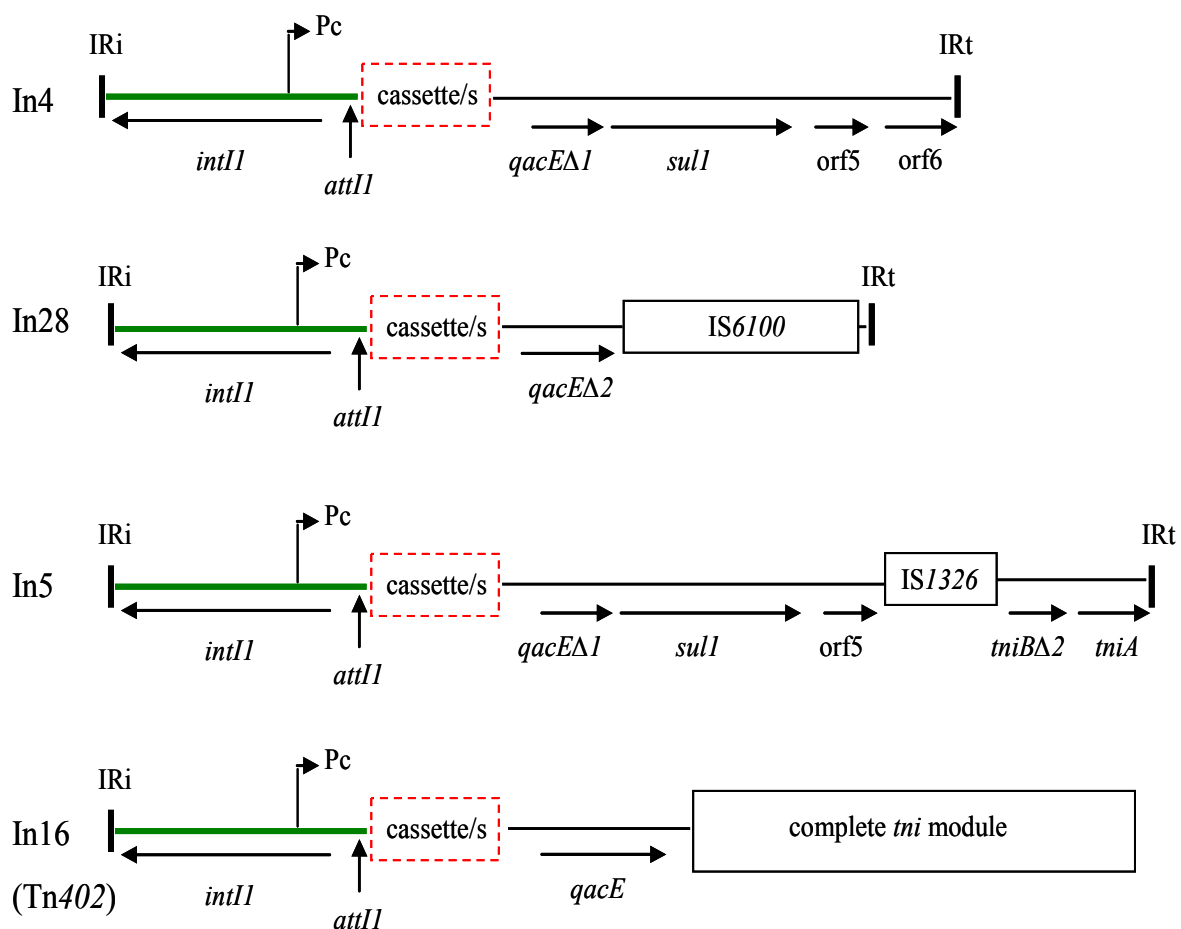


Fig 3.1 Genetic variability of the 3'CS of Class 1 integrons. The 5'CS is denoted by the thick green line for all integrons In4, In28, In5 and In16 (also known as Tn402). The 3'CS is indicated by the black horizontal lines. The IRi and IRt inverted repeats are shown as thick vertical bars. The Pc promoter and attI1 sites are shown. Diagram is not drawn to scale.

Class 1 integron screening techniques commonly involve a PCR using standard primer pairs located in the 5'CS and 3'CS, in order to amplify the gene cassette variable region. However, one needs to be aware of negative cassette PCR results due to the fact that there is variability in the genetic make-up of the 3'CS associated with class 1 integrons. In many cases the genes present downstream of the cassettes do not conform to the “typical” structure (as seen for In4 in Fig 3.1) and in many cases all or some of these genes are not present, making it difficult to develop standardised techniques for amplification of the cassette variable region.

Integrons are found in a variety of plasmids and transposons as well as bacterial chromosomes and can change their location relatively easily within the same bacterial species and between different bacterial species due to their association with these mobile genetic elements (Partridge et al., 2001b). If one or either of the repeats I_{Ri} or I_{Rt} are lost, mobility of the class 1 integron is also lost and their location remains fixed (Brown et al., 1996; Partridge et al., 2001b).

Gene cassettes within the integron (fixed or mobile) are able to be excised and incorporated into another class 1 integron via site-specific recombination catalysed by the integron (*intI1*) integrase (Collis and Hall., 1992). The role of the circularised gene cassette in the evolution of antimicrobial resistance is often underestimated. However, class 1 integron cassette stability has been demonstrated by the isolation of conserved gene cassette arrays among isolates of different origins, bacterial species and their retainment under antibiotic selection over time (Martinez-Frejo et al., 1999; Maguire et al., 2001). Genes that encode plasmid and transposon resolvases are often found adjacent to the I_{Ri} and are a favoured transposition site for class 1 integrons

(Kamali-Moghaddam and Sundstrom, 2000; Partridge et al., 2001b; Stokes et al., 2006).

It has been suggested that integron-carrying elements facilitate the acquisition of additional resistance genes, which may be the result of factors present on these elements such as integrases, recombinases and IS elements and/or the presence of class 1 integrons increases the strains survival under selective pressure (Ashraf et al., 1991; Francia et al., 1993; Hansson et al., 1997). Patterns emerging from previous studies appear to suggest that class 1 integrons may be a marker for multiple drug resistance, often as part of larger multi-drug resistance regions (Daly et al., 2005; Szczepanowski et al., 2005).

Class 1 integrons typically possess one to three gene cassettes. Studies characterising class 1 integrons have shown that inserted gene cassettes predominantly confer resistance to trimethoprim (*dfr* cassettes) and/or aminoglycosides (*aad* and *aac* cassettes) (Recchia and Hall, 1995; Chang et al., 2000; Sandvang and Aarestrup, 2000; White et al., 2001; Leverstein-van Hall et al., 2002). Other types of gene cassettes associated with class 1 integrons include chloramphenicol (*cat* and *cml* gene cassettes) and ampicillin resistance genes (β -lactamases, *bla* gene cassettes) (Randall et al., 2004).

Studies into the presence and content of class 1 integrons in Australia up to date have been limited. The overall aim of this study was to assess the role that class 1 integrons play in the dissemination of antibiotic resistance among *Salmonella* spp. and potentially other bacterial pathogens in Australia. Using molecular tools, the initial

aim was to identify class 1 integrons among a collection of 136 *Salmonella* isolates from Australian human, animal and environmental sources displaying resistance to between 1 and 9 different antibiotics using standard primers in the highly conserved 5'CS region. It also aimed to characterise the gene cassette content of the class 1 integrons (if present) among this selection of multi-drug resistant *Salmonella* spp. isolates. The strains were then screened for the presence/absence of the *sulI* resistance gene often associated with class 1 integrons to detect any variability in the 3'CS.

3.2 Material and Methods

3.2.1 PCR amplification and restriction enzyme analysis

All of the 136 strains in Table 2.1 (Chapter 2) were screened via PCR using the L2/L3 integrase and *invA* (*Salmonella* control primers) multiplex PCR and the gene cassette variable PCR using primers L1/R1 and conditions described in Chapter 2. *RsaI* digestions of L1/R1 PCR products were carried out as described in Chapter 2.

3.2.2 Southern hybridisation of *BglII* digested DNA with *sulI* DIG-labelled DNA probe and *PstI* digested *S. Infantis* DNA with IS200 DIG-labelled DNA probe

3.2.2.1 *sulI* digoxigenin DNA probe preparation

Amplification of the *sulI* gene from the 3'CS of the class 1 integron was performed using primers *sulI*F(C) (5'-CGATCAGATGCACCGTGTTT-3') (C. Venturini unpublished information) and *sulI*(R) (5'-TTTACAGGAAGGCCAACGGT-3') (Leverstein-van Hall et al., 2002) (see Fig 2.1, Chapter 2) and was expected to give a

453 bp amplicon. Thermal cycling conditions used in the PCR included a denaturation cycle 94°C for 5 min, followed by 30 amplification cycles and a final extension cycle of 72°C for 10 min. Each amplification cycle consisted of 30 s at 94°C (denaturation), 1 min at 64°C (annealing) and 1 min 30 s at 72°C (extension). The PCR amplicon using primer pair sul1F(C)/sul1R from SRC10 was amplified using primer and reaction conditions described in Chapter 2 replacing unlabelled dNTPS with DIG-labelled dNTPs (Roche).

3.2.2.2 IS200 digoxigenin DNA probe preparation

A 557 bp PCR amplicon was created using phenol/chloroform DNA isolated from SRC50 and primers IS200-F (5'-CCTAACAGGCGCATACGATC -3') and IS200-R (5'-ACATCTTGAGGTCTGGCAAC-3') (Burnens et al., 1997; Weill et al., 2005).

IS200 Southern hybridisation of *Pst*I digested chromosomal DNA is an important epidemiological tool used worldwide to track and classify different clonal lineages of *Salmonella* (Stanley et al., 1991; Bacquar et al., 1994; Weill et al., 2005). Reaction components consisted of reagent amounts previously described however standard unlabelled dNTPs were replaced with DIG-labelled dNTPs (Roche). Thermal cycling conditions used in the PCR included a denaturation cycle 94°C for 5 min, followed by 30 amplification cycles and a final extension cycle of 72°C for 10 min. Each amplification cycle consisted of 30 s at 94°C (denaturation), 1 min at 61°C (annealing) and 1 min at 72°C (extension).

3.2.2.3 BglII and PstI chromosomal DNA digestions

Approximately 50-100 ng of whole cell DNA (phenol/chloroform extracted) was digested in a total reaction volume of 50 µl containing 5 µl of 1 X restriction enzyme buffer (supplied by manufacturer), 10 U of *Bgl*III or *Pst*I (MBI Fermentas Life Sciences) restriction enzymes. The restriction enzyme digestions were carried out at 37 °C for a period of 16 h.

3.2.2.4 Nitrocellulose membrane preparation, transfer and hybridisation

*Bgl*III digested chromosomal DNA (phenol/chloroform extracted) for all of the 136 experimental isolates and *Pst*I digested DNA for *S. Infantis* isolates only, were loaded onto a 1% w/v agarose gels and electrophoresed at 110 V for 3 h. Included on all membranes probed with *sulI* was a *sulI* PCR positive isolate (SRC10 *Bgl*III digested DNA) confirmed by DNA sequence analysis (see Chapter 2 for DNA sequence analysis protocol). DNA transfer onto a nitrocellulose membrane and hybridisation of DIG-labelled DNA probe was performed as described in Chapter 2.

3.2.3 Cloning of novel gene cassettes and antimicrobial susceptibility testing.

A detailed description of cloning techniques adopted for this study and subsequent antimicrobial susceptibility testing of clones generated can be found in Chapter 2.

3.3 Results

3.3.1 L2/L3 multiplex PCR screening of *Salmonella* isolates

Of the 136 isolates screened using the L2/L3 and Salm3/Salm4 multiplex PCR, all isolates were positive for the *Salmonella* specific *invA* gene and 81 (59.6%) were positive for the class 1 integrase (*intI1*). 60 (74%) of the *intI1* positive isolates were isolated from animals (65.2% of total animal isolates), 18 (22.2%) were human isolates (46.2% of total human isolates) and 1(1.2%) isolate from each of the following environmental sources, effluent, imported spice and fish tank water made up the remainder. An agarose gel electrophoresis of select strains screened using the L2/L3 multiplex PCR is given in Fig 3.2.

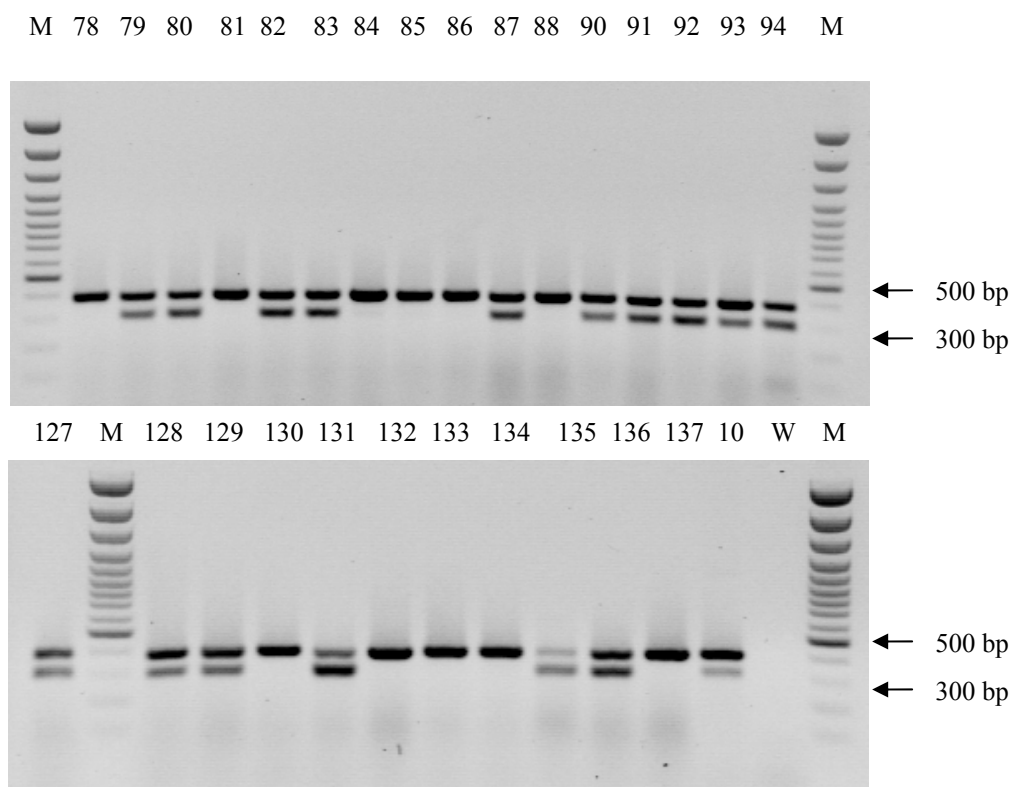


Fig 3.2 Agarose gel electrophoresis results for the L2/L3 and Salm3/Salm4 multiplex PCR for a select number of strains from the total *Salmonella* collection and negative water control (W). Molecular weight markers are labelled (M). The numbers above the lanes represent the SRC number of the strains loaded. The higher PCR amplicon represents the 389 bp *invA* *Salmonella* control product and the lower amplicon, where present represents the 298 bp L2/L3 integrase product.

Table 3.1 Summary of L2/L3 *intI1* PCR results. Isolate SRC numbers of both positive and negative strains indicated as well as total % positive/negative PCR results .

	Isolate SRC Numbers	% of total isolates screened
Integrase (L2/L3) +ve isolates	1, 4, 5, 10, 11, 14, 16, 17, 19, 21, 22, 23, 24, 26, 27, 28, 29, 30, 31, 32, 34, 38, 41, 45, 46, 49, 50, 51, 53, 54, 56, 60, 61, 62, 69, 70, 71, 72, 73, 74, 77, 79, 80, 82, 83, 87, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 108, 111, 112, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 131, 135, 136	81/136 (59.6%)
Integrase (L2/L3) -ve isolates	2, 3, 6, 7, 8, 9, 12, 13, 15, 18, 20, 25, 33, 35, 36, 37, 39, 40, 42, 43, 44, 47, 48, 52, 55, 57, 58, 59, 63, 64, 65, 66, 67, 68, 75, 76, 78, 81, 84, 85, 86, 88, 104, 105, 106, 107, 109, 110, 113, 114, 130, 132, 133, 134, 137	55/136 (40.4%)

3.3.2 Southern Hybridisation of *Bgl*II digested chromosomal DNA with the DIG-labelled *sulI* DNA probe

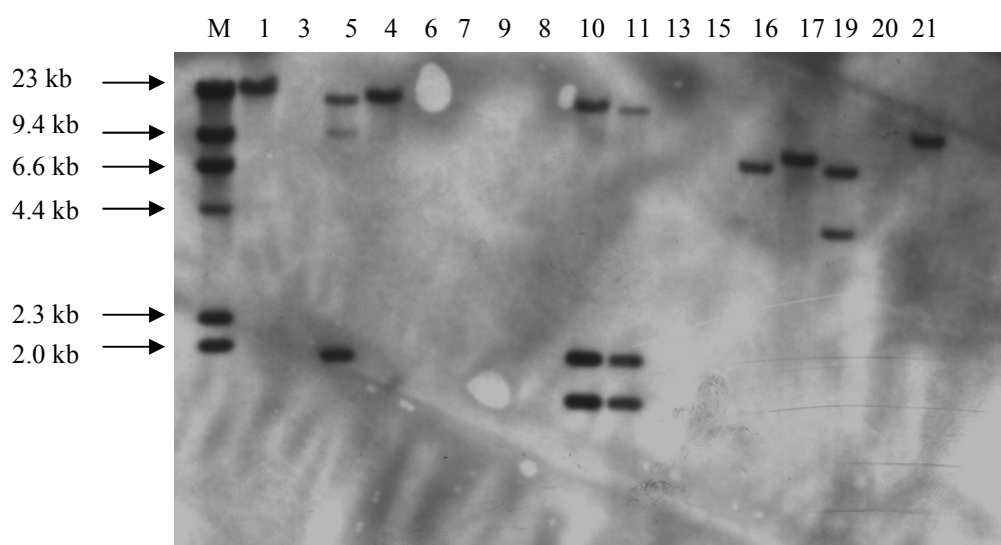


Fig 3.3 Southern blot of *Bgl*II digested DNA for representative *Salmonella* strains using a DIG-labelled *sulI* PCR probe. The DIG-labelled marker II (bacteriophage lambda DNA digested with *Hind*III) is in the lane marked (M). SRC numbers are represented above the appropriate lanes. The control SRC10 (for which the *sulI* amplicon was sequenced) is included in the lane marked 10.

The scan of a representative of one of the developed membranes is given in Fig 3.3.

The positive control SRC10 *Bgl*II digested chromosomal DNA can also be seen. The sequenced PCR product for SRC10 displayed 100% identity to the *sulI*

dihydropteroate synthase gene responsible for sulfonamide resistance from pR388 (GenBank accession no. X12869) (Sundstrom et al., 1988). In some cases as seen for SRC19, there appears to be more than one copy of *sulI*.

3.3.3 L1/R1 gene cassette PCR

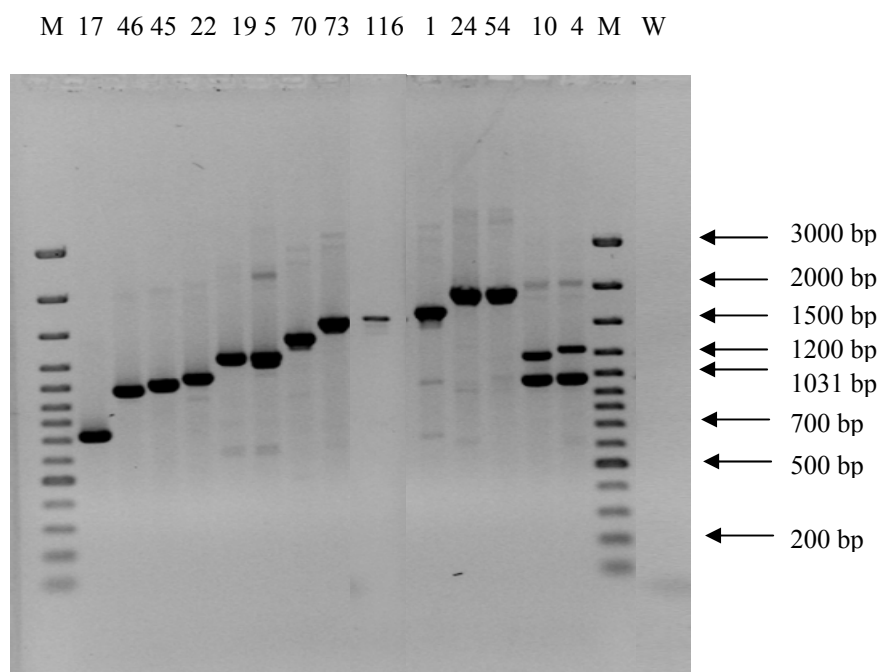


Fig 3.4 An agarose gel of all the representative L1/R1 PCR amplicons. A negative dH₂O control is labelled (W) and the molecular weight markers are also labelled (M). The molecular weight sizes are indicated in bp.

Of the 136 experimental isolates 70 (51.4%) gave a PCR amplicon for the L1/R1 gene cassette primers and all of these were positive for the *intI1* PCR. A total of 11 isolates (SRC14, 31, 41, 51, 53, 82, 103, 124, 126, 128 and 131) were positive for the *intI1* PCR but negative for the L1/R1 gene cassette variable region. There were 10 different sized PCR products for the L1/R1 primer set ranging in size from approximately 750 bp to 2 kb. 7 isolates (serovars Kiambu, Paratyphi B dT⁺ and Derby) produced 2 amplicons of approximately 1 and 1.2 kb (see Fig 3.4). A detailed summary of the

gene cassette PCR results for all experimental isolates is given in Table A.3.1 in the appendix.

3.3.4 *RsaI* restriction enzyme digestion analysis of gene cassette amplicons

There are 14 different *RsaI* restriction profiles (designated A-N) for the L1/R1 PCR product (Fig 3.5). Digestion with *Tsp509I* (data not shown) supported the *RsaI* restriction digestion data.

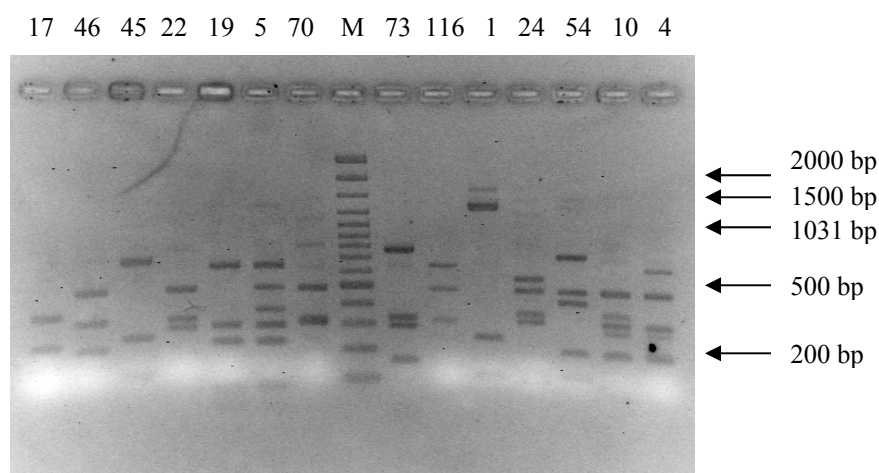


Fig 3.5 An agarose gel of the 14 different L1/R1 amplicons digested with *RsaI*. The molecular weight marker sizes are indicated and are labelled (M). The SRC numbers for the strains loaded are indicated above the lanes.

3.3.5 DNA sequence analysis of gene cassette amplicons with different *RsaI* profiles

The DNA sequencing results and subsequent BlastN analysis for representatives for each of the different L1/R1 amplicons revealed the presence of 3 novel genes in the cassette arrays for SRC54, SRC70 and SRC73, with cassette identity matches against known entries in the database <98% (see Table 3.2) (also see Tables in A.3

Appendix). The novel cassettes for these strains were cloned and sequenced in section 3.3.6. 7 other *S. Infantis* strains (SRC71, 72, 92, 93, 94, 95 and 96) appeared to display an *RsaI* digestion profile for the L1/R1 gene cassette amplicon identical to SRC70. The PCR amplicons for SRC54, SRC73 and SRC70 were cloned and characterised and are discussed below.

Table 3.2 BlastN sequence search results for strains representing each of the different L1/R1 RFLP profiles and their identity to previously published cassette arrays.

Sequenced Isolate	Cassette arrays	Accessions containing cassette/% identity	Source Bacteria
SRC17	<i>dfrA5</i>	X12868/(100%) AJ419169/(100%) AY968807/(100%)	<i>Enterobacteriaceae</i> (pLM020) <i>Escherichia coli</i> <i>Klebsiella oxytoca</i>
SRC46	<i>aadA2</i>	X68227/(100%) AF261825/(100%) CR376602/(100%)	<i>Escherichia coli</i> <i>Salmonella typhimurium</i> DT104 <i>Aeromonas caviae</i>
SRC45	<i>aadA4</i>	Z5082/(99.64%) positions 1615-1616 (CG ↑ AT) 1678 (G ↑ T)	<i>Escherichia coli</i>
SRC22	partial <i>orfF-aadA2</i>	AF284063 (partial deletion, see section 3.3.6.4)	<i>Serratia marcescens</i>
SRC19	<i>dfrA1-orfC</i>	AF455254 (100%) AB161449 (100%) AB186122 (100%)	<i>Vibrio cholerae</i> <i>Escherichia coli</i> <i>Salmonella</i> spp.
SRC70*	<i>dfrB5-aadA1</i>	AY943084 (92% <i>dfrB5</i>) AF313471 (100% <i>aadA1</i>)	<i>Pseudomonas aeruginosa</i> <i>Pseudomonas aeruginosa</i>
SRC73*	<i>aacCA3-aadA7</i>	AJ511268 (55.6% <i>aacC-A3</i>) AF234167(100% <i>aadA7</i>)	<i>Pseudomonas aeruginosa</i> <i>Escherichia coli</i> (0157:H7)
SRC116	<i>dfrA1-aadA1</i>	DQ166553, AJ884723 (100%) DQ388124, AJ879461 (100%) AY994155 (100%)	<i>Escherichia coli</i> <i>Salmonella</i> spp. <i>Klebsiella pneumoniae</i>
SRC24	<i>dfrA12-orfF-aadA2</i>	AF284063 (100%) AF550415 (100%) AB191048 (100%)	<i>Serratia marcescens</i> <i>Citrobacter freundii</i> <i>Staphylococcus aureus</i>
SRC54*	<i>aadA2-linG</i>	L06822 (100% <i>aadA2</i> and 93.4% <i>linF</i>)	<i>Escherichia coli</i>

NB. Amplicons marked with an (*) with a cassette identity match <98% were cloned for further analysis.

3.3.6 Analysis of new cassette arrays

3.3.6.1 Analysis of cassette array amplicon from SRC54

3.3.6.1.1 Protein alignment of cassette array and 59 base element from SRC54 with cassettes with similar identities

The first cassette in the array was identical to the *aadA2* cassette in GenBank accession no. L06822. The second cassette was 937 bp long and 93.4 % identical to the *linF* gene cassette. The ATG start codon was found at positions 77-9 in relation to the beginning of the cassette and encoded a 273 aa protein that is 93.1 % identical to LinF (17 aa differences). An alignment of these proteins with LinB is shown in Fig 3.6. The sequence of the *aadA2-linG* cassette array was identical to a region found in GenBank accession no. AY522431, except the ORF that it was identical to had not been annotated and identified as a lincosamide resistance gene (this has since been corrected). Using the current nomenclature system in place for *lin* genes, the new cassette was named *linG* (Levings et al., 2006c). The new accession no. for the *aadA2-linG* array deposited in GenBank is DQ836009.

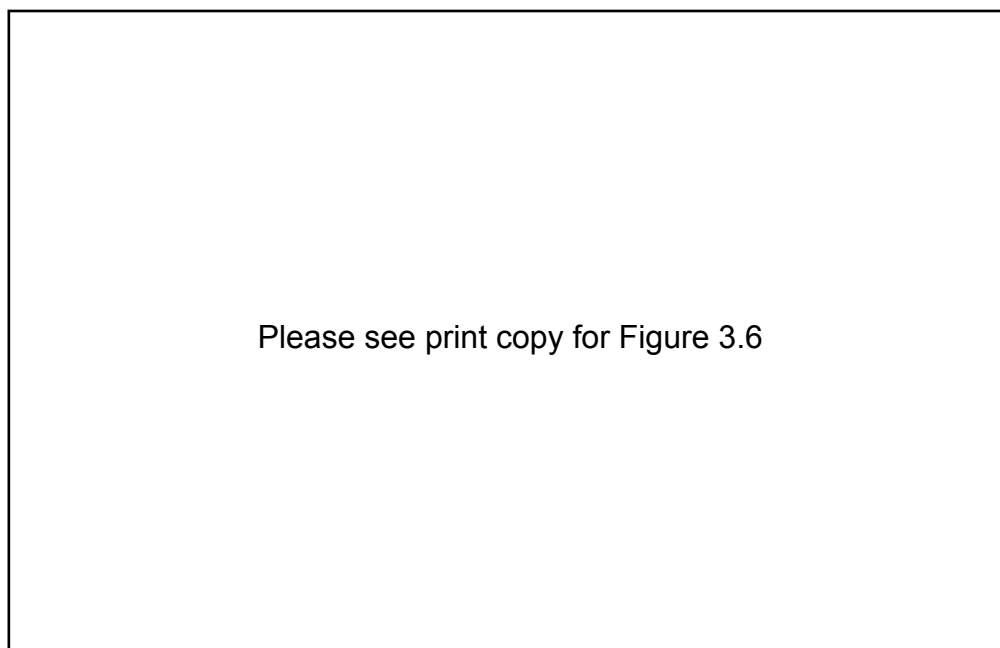


Fig 3.6 Alignment of the Lin proteins. Amino acids that are completely conserved across all sequences are shown in white letters on a black background with upper case letters below the sequence. The protein sequences of LinB and LinF were obtained from GenBank and have accession numbers AJ238249 and AJ561197 respectively. The LinG protein sequence is from this study. Alignment taken from Levings et al. (2006c).

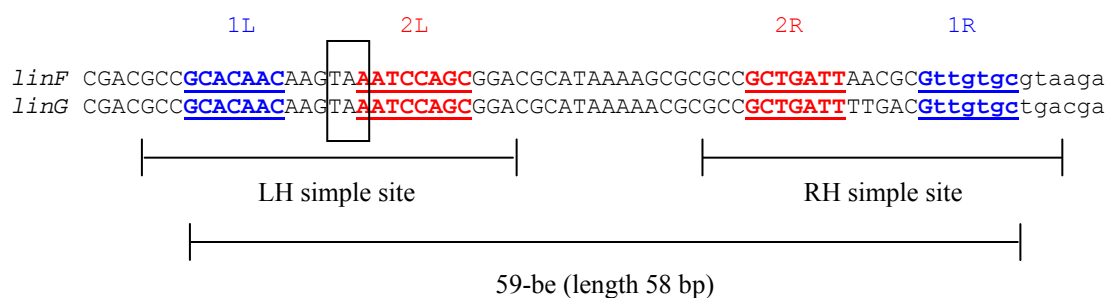


Fig 3.7 Alignment of *linF* and *linG* 59-be. The core sites are coloured and in bold font and are labelled 1L, 1R (blue), 2L and 2R (red) (Stokes et al., 1997). The boundaries of the LH (left hand) and RH (right hand) simple sites and the 59-be are indicated by bars. The bases in lowercase are those derived from the beginning of the cassette. The LinG and LinF stop codons are boxed. The sequence for each of the 59 base elements came from sources given in the alignment figure above.

The 59-be (*attC* sites) of the most closely related *linG* and *linF* cassettes (accession no. AJ561197) are both 58 bp long with only a few base pair changes (Fig 3.7). Both retain the complementary sites 1L/1R and 2L/2R, important features of 59 be (Stokes et al., 1997).

3.3.6.1.2 Gradient plate antibiotic susceptibility results for the *E. coli* DH5 α strain containing cloned *aadA2-linG* cassette array.

E. coli strain DH5 α containing pPCR-Script with the *aadA2-linG* cassette array was at least 10-fold more resistant to lincomycin (MIC \geq 2000 μ g/ml) than the control DH5 α strain containing only pPCR-Script (MIC 180 μ g/ml). The original strain SRC54 was resistant to 1100 μ g/ml of lincomycin.

3.3.6.2 Analysis of cassette array amplicon from SRC73

3.3.6.2.1 Protein alignment of cassette array and 59 base element from SRC73 with cassettes with similar identities

DNA sequencing of the 1.6 kb amplicon revealed the presence of two gene cassettes. The first cassette, 564 bp in length, contained an open reading frame whose start

codon (GTG) was found at positions 22 to 24 relative to the cassette and encoded a protein of 158 amino acids related to the known AacC-A proteins. It had the closest identity at 55.6% to the *aacC-A3* cassette. 65 amino acids were completely conserved upon alignment of these proteins and can be seen in Fig 3.8. A table of similarities and identities between members of the AacC-A family of proteins can be found in Table 3.3. The second cassette in the array was found to be identical to the *aadA7* cassette found in *E. coli* and Shiga toxin *E. coli* 0157:H7 (GenBank accession nos. AF224733 and AF234167) (Zhao et al., 2001). The accession number for the new *aacCA5-aadA7* cassette array deposited in GenBank is AY463797.

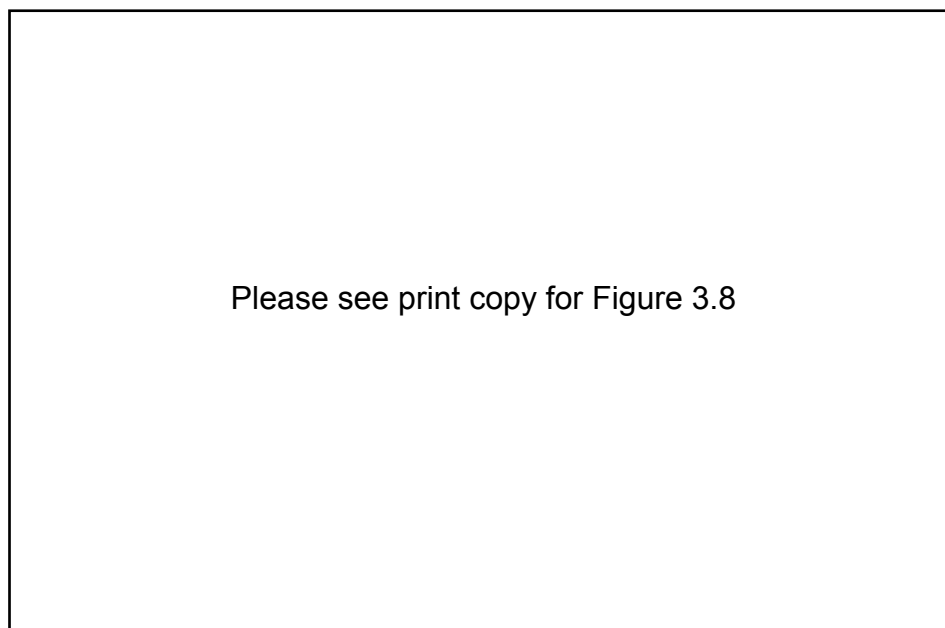


Fig 3.8 Alignment of AacC-A proteins in the AAC(3)-I family. Amino acids that are completely conserved are shown in white letters on a black background and are in uppercase letters below the sequence alignment. The sequences of AacC-A1 (AAC(3)-Ia), AacC-A2 (AAC(3)-Ib), AacC-A3 (AAC(3)-Ic) and AacC-A4 were all obtained from Genbank and have accession nos. U12338, L06157, AJ511268 and AF318077 respectively. The AacC-A5 (AAC(3)-Ie) protein is from this study. Alignment taken from Levings et al. (2005a).

Table 3.3 Relationships between members of the AacC-A or AAC(3)-I protein family. The numbers represent the % amino acid identities (top right) and similarities (bottom left) between the different AacC-A proteins.

Protein		AacC-A1	AacC-A2	AacC-A3	AacC-A4	AacC-A5
AacC-A1	AAC(3)-Ia	-	71.6	59.4	95.5	51.0
AacC-A2	AAC(3)-Ib	87.1	-	60.1	72.1	49.0
AacC-A3	AAC(3)-Ic	74.2	77.1	-	60.4	55.6
AacC-A4	AAC(3)-Id	98.7	86.4	74.0	-	51.6
AacC-A5	AAC(3)-Ie	64.1	61.4	71.9	64.1	-

The 59-be of the *aacC-A5* cassette is 78 bp in length (see Fig 3.9) and contains two simple sites and a central region typical of 59-be (Stokes et al., 1997). The *aacC-A5* 59-be does not appear to be closely related to any members of the *aacC-A* gene cassette family, nor is it related to any other known cassette in the database.

Please see print copy for Figure 3.9

Fig 3.9 Alignment of the 59-be of the six known *aacC-A* gene cassettes. The core sites are coloured and in bold font and are labelled 1L, 1R (blue), 2L and 2R (red) (Stokes et al., 1997). The boundaries of the LH (left hand) and RH (right hand) simple sites and the 59-be are indicated by bars. The bases in lowercase are those derived from the beginning of the cassette. The sequence for each of the 59 base elements came from sources given in the alignment figure above. Complete sequence for *aacC-A2* 59-be is not available. Diagram taken from Levings et al. (2005a).

3.3.6.2.2 Antibiotic susceptibility disc results for *E. coli* 294 containing the cloned *aacCA5-aadA7* cassette array

The following zone distances were recorded for each of the strains (Table 3.4). The cloned fragment conferred resistance to gentamicin with a 5 mm zone size versus an 11mm zone size for the *E. coli* 294 pCR-Script (negative control). It also conferred resistance to spectinomycin/streptomycin with 0mm/7mm zone sizes versus the negative controls 9mm/11mm zone sizes respectively.

Table 3.4 Antibiotic susceptibility assay for pCR-script containing *aacC5-aadA7* cassette array and pCR-Script negative control

	Am (30 µg/ml)	Gm (10 µg/ml)	Km (30 µg/ml)	Ne (30 µg/ml)	Sp (25 µg/ml)	Sm (25 µg/ml)	Tb (10 µg/ml)
pCR-Script + <i>aacCA5</i> <i>-aadA7</i>	13mm	5.5mm	12mm	13mm	0mm	7mm	10mm
pCR-Script (-ve control)	12mm	11mm	12mm	14mm	9mm	11mm	11mm
SRC73	7 mm	3 mm	7 mm	9 mm	0mm	0mm	6 mm

Am: amikacin, Ne: netilmicin, Tb: tobramycin

3.3.6.3 Analysis of cassette array amplicon from SRC70

3.3.6.3.1 Protein alignment of cassette array and 59 base element from SRC70 with cassettes with similar identities

DNA sequence analysis of the gene cassette amplicon obtained from SRC70 revealed the presence of two gene cassettes. The first cassette (410 bp long) contained an open reading frame with an ATG start codon at positions 71-3 relative to the start of the cassette that was related to the known DfrB proteins (approx 77-92% identical). Upon alignment (see Fig 3.10) the protein was found to differ by 7 and 10 amino acids to its nearest relatives the DfrB5 and DfrB1 proteins and using the next available number

was named DfrB6 (the gene *dfrB6*). A table of identities and similarities for the DfrB6 family of proteins is given (Table 3.5). The greatest protein divergence is found close to the N-terminus. The second cassette in the integron array was identical to the *aadA1* in GenBank accession AF313471 (Partridge et al., 2002).

Please see print copy for Figure 3.10

Fig 3.10 Alignment of DfrB proteins in the DfrB family. Amino acids that are completely conserved are shown in white letters on a black background and are in uppercase letters below the sequence alignment. The sequences of DfrB1 (dhfrIIa, dfr2a), DfrB2 (dhfrIIb, dfr2b), DfrB3 (dhfrIIc, dfr2c), DfrB4 (dfr2d) and DfrB5 were all obtained from GenBank and have accession nos. AY139601, J01773, X72585, AJ429132 and AY943084 respectively. Alignment taken from Levings et al. (2006b).

Table 3.5 Relationships between members of the DfrB protein family. The numbers represent the % amino acid identities (top right) and similarities (bottom left) between the different DfrB proteins using the BLOSUM62 similarity matrix.

Protein	DfrB1	DfrB2	DfrB3	DfrB4	DfrB5	DfrB6
DfrB1	-	78.2	78.2	76.9	88.4	87.2
DfrB2	88.5	-	85.9	74.3	79.5	82.1
DfrB3	88.5	89.7	-	79.5	82.1	83.3
DfrB4	83.3	84.6	88.5	-	76.9	76.9
DfrB5	92.3	87.2	89.7	89.7	-	92.0
DfrB6	91.0	88.5	89.7	85.9	93.6	-

The *dfrB6* 59-be is identical to that in the *dfrB1* cassette and very closely related to those of other *dfrB* cassettes, which form a group that are the shortest known at 57 bp

and again contained the necessary simple sites and central regions characteristic of 59-be (Recchia and Hall., 1997) (Fig 3.11).

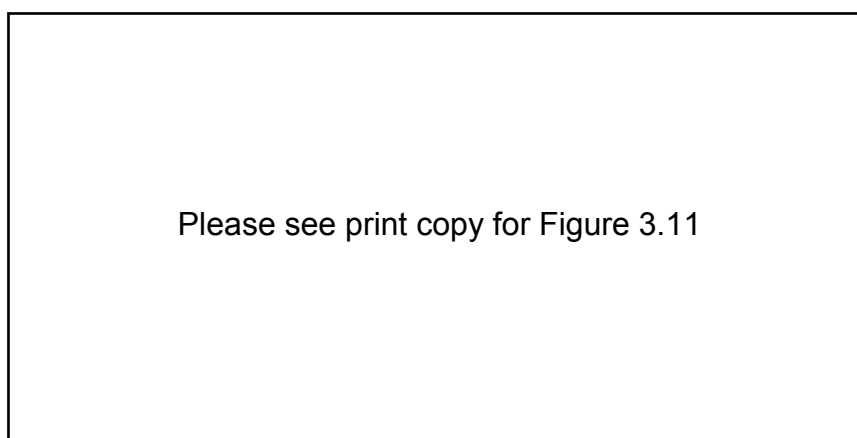


Fig 3.11 Alignment of the 59-be of the six known *dfrB* gene cassettes. The core sites are coloured and in bold font and are labelled 1L, 1R (blue), 2L and 2R (red) (Stokes et al., 1997). The boundaries of the LH (left hand) and RH (right hand) simple sites and the 59-be are indicated by bars. The bases in lowercase are those derived from the beginning of the cassette. The sequence for each of the 59 base elements came from sources given in the alignment (Fig 3.10) above.

3.3.6.3.2 Gradient plate results for *E. coli* strain DH5 α containing *dfrB6-aadA1* cassette array

E. coli strain DH5 α containing pPCR-Script with the *dfrB6-aadA1* cassette array was considerably more resistant to trimethoprim (MIC 550 μ g/ml) than the control DH5 α strain containing only pPCR-Script (MIC <1 μ g/ml). The new accession no. for the *dfrB6-aadA1* array deposited in GenBank is DQ274503.

3.3.6.4 SRC22 Δ orfF-*aadA2* cassette array

DNA sequencing and subsequent analysis of the 1 kb L1/R1 PCR amplicon obtained for SRC22 revealed an array with identity to the *dfrA12*-orfF-*aadA2* array in GenBank accession AF284063. However, 77 bp of the standard 5'CS (including the *attI1* site), the entire *dfrA12* cassette and 155 bp of the orfF cassette have been deleted

in SRC22. A schematic diagram of the deletion event and the proposed homologous recombination cross-over region leading to the deletion can be found in Fig 3.12 and 3.13 respectively.

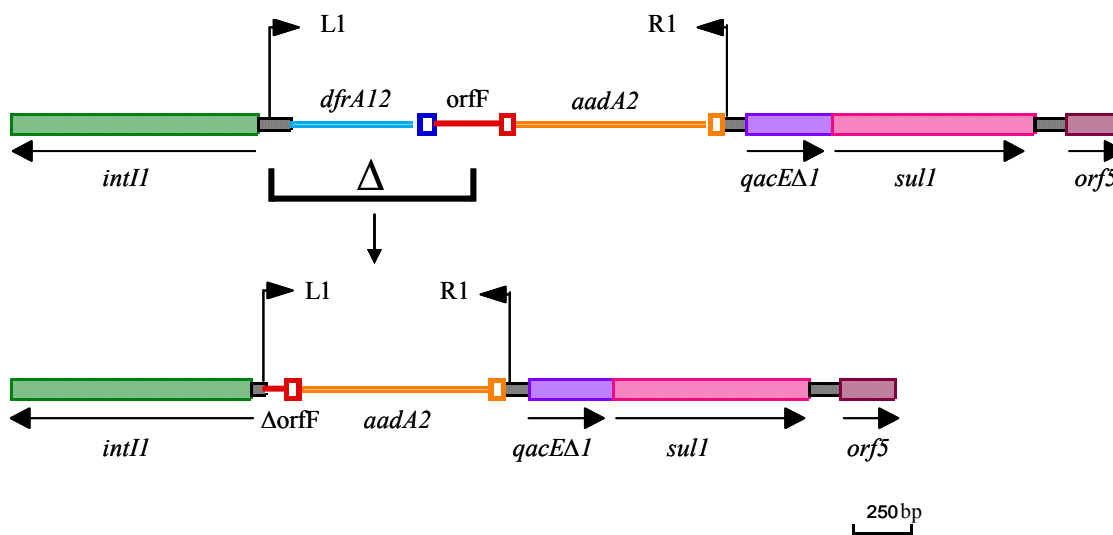


Fig 3.12 A diagrammatic representation of the putative deletion event in the *dfrA12-orfF-aadA2* cassette array, generating the Δ orfF-*aadA2* array as seen for the variable region of the class 1 integron for SRC22. Flags depict priming sites.

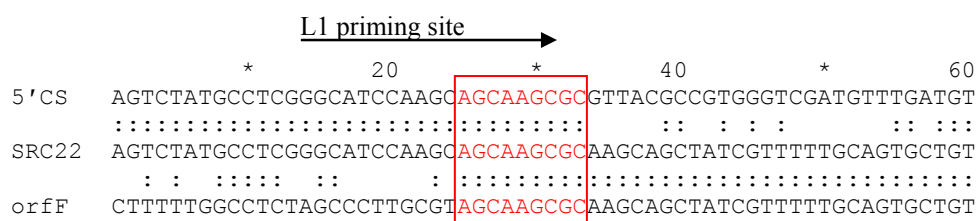


Fig 3.13 Crossover region leading to the formation of 5'CS-orfF boundary in SRC22. 5'-CS (positions 112-171) and orfF (positions 937-996) sequences from GenBank (Accession no. AF284063) were aligned with the relevant sequence from SRC22. Nine bases (boxed in red) common to 5'CS and orfF depicts the site of a putative homologous recombination event that led to the loss of 77 base pairs from standard 5'-CS, *dfrA12* and 155 bp of the orfF gene. The arrow defines the L1 priming site.

3.3.7 Potential SGI1 containing strains

Isolates SRC4, 10, 11, 49, 50, 99 and 101 that produced two PCR products (namely a 1 and 1.2 kb product) typical of the amplicons seen in the presence of SGI1 containing strains (Briggs and Fratamico 1999; Boyd et al., 2001) were not sequenced. A detailed characterisation of these isolates and the gene cassettes they contain can be found in Chapter 4. The amplicon for SRC5 and SRC38 was also not determined due to difficulty in sequencing what appears to be multiple gene cassette (L1/R1) PCR products of the same size. The restriction enzyme profiles for these strains also strengthen this belief and an in depth analysis of the gene cassettes present in these isolates can also be found in Chapter 4.

3.3.8 IS200 profiles and the genetic relationship between the *dfrB6-aadA1* containing *S. Infantis* strains

*Pst*I digested chromosomal DNA, probed with an IS200 DIG-labelled DNA probe produced identical Southern hybridisation profiles for all of the *dfrB6-aadA1* containing isolates. Each of these isolates share the same 3 bands (ie. 3 IS200 elements) and all appear to be clonal (see Fig 3.14). SRC46, also an *S. Infantis*, but lacking the *dfrB6-aadA1* array contained an extra two copies of IS200 and although related appears not to be identical to the *dfrB6-aadA1* containing strains as expected. A summary of all the gene cassettes and *intI1* PCR and *sulI* Southern hybridisation data can be found in Tables A.3.1 and A.3.2-A.3.5 in Appendix A.3.

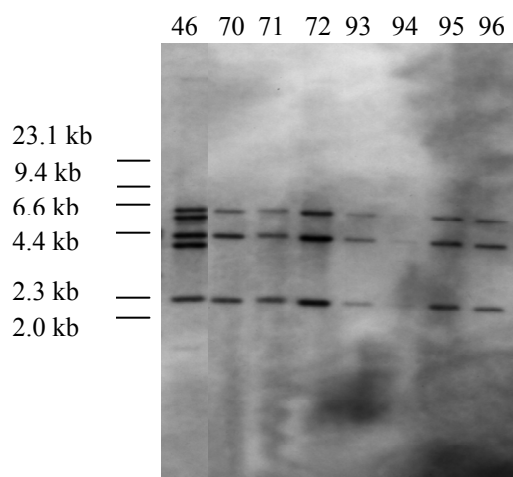


Fig 3.14 *Pst*I digested DNA of a representative of all the *S. Infantis* strains after hybridisation with the dig-labelled IS200 probe. Banding signals are indicative of the number of IS200 elements present in the strain. SRC92 and SRC94 were shown to have identical profiles to the *dfrB6-aadA1* containing *S. Infantis* strains above in subsequent blots (data not shown). SRC46 was used as a *S. Infantis dfrB6-aadA1* negative control. Molecular weight marker positions corresponding to bands from DIG-labelled marker II (Roche) are indicated.

3.4 Discussion

In this study the gene cassettes identified were almost exclusively gene cassettes conferring resistance to trimethoprim and aminoglycosides and the majority of these arrays have been described in detail in the literature. Global class 1 integron studies support our findings and also report a predominance of trimethoprim and aminoglycoside gene cassettes among class 1 integrons (Recchia and Hall, 1995; Chang et al., 2000; Sandvang and Aarestrup, 2000; White et al., 2001; Leverstein-van Hall et al., 2002).

The *dfrA5* gene cassette was among the most prevalent gene cassette identified among animal isolates in our collection. Overseas, the *dfrA5* cassette has been isolated from *Salmonella enterica* (Vo et al., 2006; Peirano et al., 2006), *Vibrio cholerae*

(Thungapathra et al., 2002) and *E. coli* (Gassama et al., 2004) although not as often as other *dfrA* cassettes. It appears that this cassette has been in circulation for quite some time within the Australian food producing animal population with a total of 31 isolates (22.8%), all from animal sources (porcine (12); bovine (11); chicken (8)) containing this cassette. The *dfrA5* gene cassette has been found previously in Australia in *E. coli* strains isolated from bovine faecal samples (Barlow et al., 2004) and in an *E. coli* human urinary isolate (White et al., 2001). The *dfrA5* cassette is also readily isolated from enterobacterial pathogens in South East Asia. *Escherichia coli* recovered from patients with urinary tract infections in Korea were reported to contain *dfrA1*, *dfrA5*, *dfrA7*, or *dfrA12* (Yu et al., 2004). To our knowledge, there are no previous reports of *dfrA5* among non-typhoidal *S. enterica* isolates isolated in the southern hemisphere.

51 of the 81 (approximately 63%) *intI1* positive strains contained *sul1*. A majority of these also contained a *dfrA* trimethoprim resistance gene cassette. The high occurrence of trimethoprim resistant gene cassettes located in class 1 integrons, which contain the *sul1* resistance gene in the 3'CS is most likely related to the trimethoprim and sulfonamide drug regimes used since 1968 to treat common urinary tract pathogens such as *E. coli* and other Gram-negative bacteria (Huovinen et al., 1995). The widespread use of this combination drug therapy co-trimexazole in the 1970's through to the 1980's, exerted a strong co-selection pressure for these two classes of antibiotic resistance genes and possibly strong selection pressure for the class 1 integron structure itself. The increase in antimicrobial resistance and hypersensitivity reactions due to sulfonamides forced health authorities in the UK to only use this combinational drug therapy in cases where absolutely necessary and even though this

led to a decrease in the use of sulfonamides considerably, resistance still persisted (Enne et al., 2001). Sulfonamides and trimethoprim, are still individually administered in human medicine (Enne et al., 2001; Guardbassi and Courvalin, 2006).

The continued use of trimethoprim and sulfonamides in agriculture and aquaculture is also contributing to the selection pressure placed on bacteria harbouring these resistance genes, with close to 80 tonnes of trimethoprim/sulfonamides being sold in the UK in 2004 for therapeutic/prophylactic use in agriculture (<http://www.vmd.gov.uk/Publications/Antibiotic/AntiPubs.htm>). In Australia the purchase of trimethoprim/sulfonamides for therapeutic/prophylactic use in food producing animals was 15 tonnes (2001/2002). Although lower than UK sales it is still significant with the majority administered randomly among herds as an additive in water supplies (http://www.apvma.gov.au/registration/antibiotics_1999_2002).

Aminoglycoside resistance genes encode enzymes which modify aminoglycosides by acetylation, adenylation and phosphorylation (Shaw et al., 1993; Recchia and Hall, 1995). The group of genes responsible for aminoglycoside resistance via acetylation include the *aacA* and *aacC* group and those responsible for resistance via adenylation include *aadA* and *aadB* (Recchia and Hall, 1995).

The *aadA1* and *aadA2* gene cassettes (responsible for resistance to streptomycin and spectinomycin) have been located on two of the earliest known resistance plasmids NR1 (Liebert et al., 1999) and pSa (Bito and Susani, 1994) and are still readily isolated today (Gestal et al., 2005). It is a common belief that the removal of an antibiotic from use should resolve the problem of resistance associated with that

particular antibiotic. However, antibiotic resistance studies and the identification of multi-drug resistance regions in bacteria suggest that removal of an antibiotic doesn't necessarily mean the disappearance of the corresponding resistance gene.

Streptomycin an aminoglycoside used quite readily in the 1950's to 1960's (Chiew and Hall, 1998) and spectinomycin (used for the treatment of gonorrhea) are rarely used in the current hospital environment (Guardbassi and Courvalin, 2006). Sales for use in agriculture are also quite low. A total of 4 tonnes was purchased in 2001-2002 for use in Australian food-producing animals (http://www.apvma.gov.au/registration/antibiotics_1999_2002), yet these antibiotic resistance genes are constantly being isolated.

The continual isolation of aminoglycoside resistance genes, such as the *aadA* genes is probably attributed to the fact that these cassettes are linked to other resistance genes (in class 1 integron arrays), such as sulfonamide and trimethoprim resistance genes, whose target antibiotics are still in use. Interestingly, the genetic location of the *aadA1* and *aadA2* is highly variable but generally they are associated with only a few cassettes in arrays. The arrays being *oxa-1-aadA1*, *dfrA1-aadA1* and *dfrA12-orfF-aadA2*. All of the arrays are globally distributed (Gestal et al., 2005). In this study examples of integrons carrying both trimethoprim and aminoglycoside resistance gene cassette arrays *dfrA12-orfF-aadA2*, *dfrA1-aadA1* and *dfrA17-aadA5* were isolated. The *aadA1* and *aadA2* cassettes can be found as individual cassettes as seen with the isolation of the *aadA2* gene cassette on its own in this study.

Identified in this study was the existence of 3 new class 1 integron associated gene cassettes, designated *aacCA5*, *dfrB6* and the third cassette *linG* (Levings et al., 2005a; Levings et al., 2006b; Levings et al., 2006c). The *aacC* genes produce a subset of enzymes that confer resistance to this group of aminoglycoside antibiotics by modification of the 3-amino group and are referred to as acetyltransferases. The known 3-*N*-amino-glycoside acetyltransferases (AAC(3) enzymes) are classified into several groups based on differences in phenotype and their ability to modify different aminoglycosides. However, at the protein level they fall into two distinct categories, category A and category B. The four members of category A (*aac(3)-I* family) are *aacCA1* (also known as *aac(3)-Ia*) (Wohlleben et al., 1989), *aacCA2* (*aac(3)-Ib*) (Schwocho et al., 1995), *aacCA3* (*aac(3)-Ic*) (Riccio et al., 2003) and *aacCA4* which is a variant of *aacCA1* (97.6% identical) (Gibb et al., 2002; Poirel et al., 2002). The protein products of the *aacCA* genes are small (usually 154 to 156 amino acids) and confer resistance to gentamicin, sisomicin and fortimicin but not to tobramycin, amikacin and kanamycin. The group A genes are all found in gene cassettes. The group B *aacC* genes are not found in gene cassettes, do not appear to be closely related to the group A *aacC* genes (less than 25% identity) and encode larger proteins (between 261 and 300 amino acids) (Levings et al., 2005a).

Since the completion of this work the *aacCA5-aadA7* cassette array has been found in *Vibrio fluvalis* (GenBank Accession no. AB114632), however the *aacCA5* cassette was incorrectly designated *aac(3)-Id* (Ahmed et al., 2004). It has also been described in *Vibrio cholerae* (AY605683) and *Salmonella enterica* serovar Newport (AY458224) (Doublet et al., 2004). All cassettes were identical apart from a few base pair differences. The 59 bp of the *accCA5* cassette is not closely related to any of the

other 59 be's from other *aacC* cassettes. Although not examined in this study, resistance to sisomicin has been demonstrated for the new *aacCA5* cassette (Ahmed et al., 2004).

Another novel cassette identified in this study was a lincosamide antibiotic resistance gene *linG*. Lincosamide antibiotics include lincomycin (a naturally derived compound produced by several actinomycetes) and its semi-synthetic chlorinated derivative, clindamycin (Bozdogan et al., 1999). These antibiotics inhibit protein synthesis by blocking the peptidyl-transferase activity of the 50S subunit of the bacterial ribosome and are active against most Gram-positive cocci and anaerobes (Dhawan and Thadepalli, 1982), however are not so effective against Gram-negative bacilli. Resistance to these compounds is generally due to an alteration of the ribosome (N⁶ dimethylation of an adenine residue in the 23S rRNA) and usually confers broad spectrum cross resistance to macrolides, lincosamides and streptogramin B antibiotics (MLSB phenotype). Bacterial modifications such as phosphorylation (Lerclercq, 2002) of the lincosamide compound have also been described as mechanisms of resistance.

To date there has only been one integron borne lincosamide resistance gene cassette *linF* described in the literature (Hier et al., 2004). This gene cassette was isolated from an *Escherichia coli* blood isolate in Norway, in an array also containing an *aadA2* gene cassette. The *linF* gene cassette encoded a putative 273 amino acid lincosamide nucleotidyl-transferase resistance protein, conferring reasonably low levels of resistance to both lincomycin and clindamycin. The LinF amino acid sequence shared approximately 35% identity to the *Enterococcus faecium* and *Enterococcus faecalis*

nucleotidyl-transferases encoded by *linB* (GenBank accession no. AF110130) (Bozdogan et al., 1999) and *linB'* (GenBank accession no. AF408195) and no significant identity with the staphylococcal lincosamide nucleotidyl-transferases encoded by *linA* and *linA'* (Brisson-Noel and Courvalin, 1986; Brisson-Noel et al., 1988).

In Australia, lincomycin is a commonly used antibiotic for the treatment of dysentery and mycoplasmal pneumoniae in swine and the isolation of a functional *lin* antibiotic resistance gene in *Salmonella* is concerning particularly for the Australian porcine industry. In this study it was found that the *E. coli* DH5 α containing the pPCR-Script plasmid without the cloned array did display a level of intrinsic resistance to lincomycin (180 μ g/ml). Intrinsic resistance to lincomycin is not uncommon for many Gram-negative bacteria, hence lincosamides are mainly used for the treatment of infections attributed to Gram-positive bacteria. The clone containing the *aadA2-linG* cassette array, however, was considerably more resistant (10-fold) to the antibiotic leading to the conclusion that the *linG* cassette in this array was contributing to increased lincomycin resistance. Although the *linG* cassette may not directly contribute to the problem of Gram-negative bacterial resistance, the fact that this cassette is in circulation in the bacterial population and is mobile and has the ability to transfer to bacteria normally susceptible to these antibiotics (ie certain Gram positive bacteria) is concerning.

The third new cassette isolated in this study is the *dfrB6* responsible for trimethoprim resistance. There are two different subgroups designated DfrA and DfrB (Howell, 2005), which are not related to each other (Grape et al., 2003a) and are encoded by

dfrA and *dfrB* genes (Recchia and Hall, 1995). The DfrA is the most prevalent subgroup. It is speculated that the *dfrA* mobile trimethoprim resistant genes found in integrons today originated as chromosomal genes taken up by recombination events into plasmids which were then transferred to commensal and pathogenic organisms (Sundstrom et al., 1995).

The second DfrB group encodes proteins of 78 amino acids that form a tetramer which binds both the substrate (dihydrofolate), and co-factor, NADP in a characteristic position allowing reduction of dihydrofolate to occur (Howell, 2005). The origin of this group of proteins is unknown but they have been isolated from bacteria that affect humans, fish (L' Abee-Lund and Sorum, 2001) and other agricultural animals (Barlow, 2004; Kadlec et al., 2005; Sunde, 2005). Before this study there had only been five *dfrB* genes identified (Swift et al., 1981; Radstrom et al., 1994; Grape et al., 2003a; Tennstedt et al., 2003; Lolans et al., 2005) and hence the reason this new cassette was designated *dfrB6*.

Interestingly, initial antibiotic susceptibility testing (and repeated susceptibility testing experiments) performed on the *S. Infantis* strain containing the *dfrB6* gene cassette only identified one strain, SRC92, as being resistant to trimethoprim. The strain from which the PCR product was cloned, SRC70 did not display significant resistance to trimethoprim. The strain containing the cloned *dfrB6-aadA1* array however, displayed resistance to up to 550 µg/ml of trimethoprim compared to the plasmid control strain <1 µg/ml, leading to the conclusion that *dfrB6* is responsible in the clone for conferring resistance to trimethoprim. The promoter in the 5'CS of the integron was

sequenced and was found to be the strong version of the P_c promoter (data not shown) (Collis and Hall, 1995).

Of the 5 previously identified *dfrB* cassettes, all appear to be resistant at considerably lower levels of trimethoprim compared with the *dfrA* cassettes (Alonso and Gready, 2006). The fact that the majority of these *S. Infantis* strains appeared to be susceptible to trimethoprim in the antibiotic susceptibility tests, even though the *dfrB6* cassette is present, tends to support the findings in the literature that this subgroup of *dfr* cassettes confer lowered resistance to trimethoprim. Furthermore, the *dfrB6* cassette is under the control of the strong P_c promoter and is also the first cassette in the array (the first cassette is often expressed at higher levels than cassettes further downstream in the array) (Recchia and Hall, 1995). The reason behind the lower resistance levels of the *dfrB* cassettes is yet to be determined and fully understood.

As mentioned previously, IS200 Southern hybridisation of *Pst*I digested chromosomal DNA is an important epidemiological tool used worldwide to track and classify clonal lineages of *Salmonella* (Stanley et al., 1991; Bacquar et al., 1994; Weill et al., 2005). These insertion elements are usually located on the chromosome and were originally thought to be specific to *Salmonella*, although in a small number of cases IS200 elements have been found in *E. coli* and *Shigella* spp. (Bisercic and Ochman, 1993) and located on plasmid DNA (Stanley et al., 1991). Despite these findings, IS200 is still considered to be a fairly stable element and this simple technique generally provides discriminative fingerprints between and within *Salmonella* serovars (Millemann et al., 1995). Identifying clonal lineages using this molecular tool is very useful especially in characterising *Salmonella* responsible for large infection

outbreaks not only domestically, but internationally with suggestions of creating an international IS200 profiling database (Stanley et al., 1993). A fingerprinting study in Finland, using IS200 to determine clonal relationships among *S. Infantis* isolates, has indicated that this serovar can contain anywhere up to 7 copies of IS200 with the majority of these containing three or more (Pelkonen et al., 1994). In this study all of the *dfrB6-aadA1* containing strains examined contained 3 copies of this IS element.

The IS200 profiling conducted in this study among the *S. Infantis* strains highlighted the clonal nature of the *dfrB6-aadA1* containing strains and discriminated between the *Infantis* control strain SRC46, not containing the unusual array. Although related (contained 3 similar bands and two extra bands not seen in the *dfrB6-aadA1* containing strains) the difference in the phylogenetic relationship between the strains became apparent. With the majority of these strains being sourced from chickens and the clonal nature of these strains it is speculated that the *S. Infantis* containing *dfrB6-aadA1* strains isolated from the companion animals (a dog and a cat) may have come from the consumption of chicken meat (Levings et al., 2006b).

The Δ *orfF-aadA2* array, another unique array isolated from 7 *Salmonella* isolates in this collection appears to have been created due to a homologous recombination event that has lead to the deletion of 77 bp of the standard 5'CS (including the *attII* site), the entire *dfrA12* cassette and 155 bp of *orfF*. Six of these isolates were recovered from pigs, which suggests that it is probably circulating quite freely in this environment among *Salmonella* spp. containing integrons. The resistance phenotypes for nearly all of the isolates supports the deletion event hypothesis with all (except for one, SRC102), appearing to be sensitive to trimethoprim at levels tested.

Increasing numbers of antibiotic resistances are being transferred by gene cassettes and when studying resistance it is important to look for and understand what cassettes are present in integrons. Rare cassettes such as the *dfrB6-aadA1* array and unique recombination events as seen with the Δ orfF-*aadA2* array, could serve as biological markers for potentially clonal strains. Identifying new cassettes is vital in trying to track and monitor resistance in the bacterial community even if the cassettes do not pose an immediate threat. The combination of rapid and effective molecular techniques help the scientific community keep up with the constantly evolving bacterial environment. Due to the overwhelming occurrence and increasing number of gene cassettes isolated, there is an urgent need for an international bioinformatics database containing all known gene cassettes and any other relevant information (strains, sources etc) that clinicians and scientists researching antimicrobial resistance could easily access.

Highlighted in this study is the variability in the genetic make-up previously associated with 3'CS of class 1 integrons (Partridge et al., 2001b; Daly et al., 2005). As mentioned earlier, due to rearrangements and IS mediated deletions, the 3'CS of class 1 integrons are often variable with one of the longest 3'CS being from In5 (Brown et al., 1996; Partridge et al., 2001b). It becomes evident when looking at the collective data for the presence of *intI1*, gene cassettes and *sulI* that 21 of the 70 (30%) *intI1*, gene cassette containing isolates within this collection do not appear to contain the *sulI* gene (see Table A.3.4).

The loss of *sulI* from the 3'CS has also been reported previously in RSF1010 derived plasmids (Daly et al., 2005). The class 1 integron structure in these constructs has

been linked to other resistance genes including *strA/strB* (responsible for resistance to streptomycin but not spectinomycin), *bla_{TEM}* (conferring resistance to β -lactam antibiotics), tetracycline resistance determinants and *sul2* (a second gene responsible for resistance to sulfonamides). Sulfonamide resistance is still observed for many of the isolates that were negative for the *sul1* gene. The resistance genes responsible, may be residing elsewhere in the genome supporting the possible presence of one of the RSF1010 related constructs. Further detailed molecular analysis (particularly on the *S. Typhimurium* strains in the collection) is being carried out at present within the laboratory to characterise the MR regions within these strains and preliminary data suggests that many of these strains contain derivatives of the RSF1010 derived structures and IS elements such as IS26 causing deletions in the 3'CS (J.Liu, personal communication).

Although not as common, a third sulfonamide resistance gene *sul3* harboured on a 54 kb conjugative plasmid originally isolated from *E. coli* in a pig population in Switzerland (Perreten and Boerlin, 2003), later found on a larger plasmid recovered from human isolates (Grape et al., 2003b) and more recently discovered in *Salmonella* isolated from livestock and food in Germany (Guerra et al., 2004), could also be responsible for the sulfonamide resistance phenotype we are seeing in our collection in *Salmonella* isolates not containing *sul1* gene. Sul3 shares approximately 40% amino acid identity with previously described resistant dihydropteroate synthase enzymes Sul1 and Sul2 (Perreten and Boerlin, 2003; Grape et al., 2003b).

11 isolates contained *int11* but an amplification product for the gene cassette variable region was not obtained. 9 of these isolates did not contain the *sul1* gene. It is possible

that these isolates contain a RSF1010 related construct (as mentioned above), whereby IS mediated events (IS26 in particular) have lead to various deletions in the 3'CS, removing the R1 priming site. The remaining 2 isolates (SRC51 and SRC103) in this subgroup, were positive for *sulI*. It is possible that these isolates contain much larger arrays that were unamplifiable with standard *Taq* polymerase. It could also be PCR failure due to sequence divergence in the R1 priming site. Further work is continuing with this subgroup of isolates.

Within the collection a total of 55 *intI1* negative isolates were examined. Interestingly a number of these displayed a multiply resistant phenotype. In some instances the resistances can be attributed to other genes like *strA/strB*, *sul2* or *sul3* (J. Liu personal communication), genes not associated directly but often linked to class 1 integrons as part of large multi-resistance regions (Daly et al., 2005; Szczepanowski et al., 2005). Spectinomycin resistance to date has only been associated with gene cassettes (*aad* and *aac*). SRC3, SRC20 and SRC48 all appear to be phenotypically resistant to spectinomycin but *intI1*/gene cassette negative and most likely have lost a plasmid carrying an integron from their mobile genome. It is known that bacteria regularly lose genetic information in the form of plasmids when the genes are not vital for the bacteria's survival. However, it is possible that there is another gene and/or mechanism responsible for the spectinomycin resistance phenotype of these strains that has yet to be identified.

It can clearly be seen that as resistance to the number of antibiotics increases, the percentage of integron positive isolates also increases. Caution needs to be taken when drawing conclusions from statistical correlations between multiple antibiotic

resistance phenotypes and the presence of class 1 integrons using this sample set, due to the non-random nature of the selection process of these strains and the inadequate size of the sample set. The importance of adequate sample collections in order to be able to draw significant statistical relationships can be seen in a study in the UK which screened over 700 *E. coli* isolates from patients in order to examine the relationship between sulphonamide resistance and a national prescribing restriction on co-trimoxazole (Enne et al., 2001). Due to the large number of strains and the careful selection of isolates they were able to examine the statistical relationship between these two variables. In our study we are able to note the patterns emerging however, analysis on a larger number of randomly selected samples would need to be undertaken in order to examine a direct statistical link between multiple antibiotic resistance phenotypes and the presence of class 1 integrons.

These initial screening and characterisation experiments have set the platform and raised some very important questions in regards to antibiotic resistance in Australian *Salmonella* spp. and potentially other bacterial isolates recovered from humans and animals. The screening techniques adopted in this study (*intI1*, gene cassette PCR and restriction enzyme analysis) appear to be a highly effective and rapid way of grouping cassettes and identifying any new cassette arrays not appearing in the GenBank database. The *intI1* PCR in conjunction with the *sulI* hybridisation is very useful also in identifying possible integron/cassette containing strains that may have deletions in the 3'CS (not detected with the L1/R1 cassette PCR), as seen in cases of RSF1010 related structures (Daly et al., 2005). Further detailed molecular work is being carried out in the laboratory involving screening for other common resistance genes not necessarily found in integrons such as tetracycline resistance determinants, *strA/strB*

(streptomycin resistance), *bla*TEM (β -lactamases resistance) and *tetA*(A) (tetracycline resistance) to determine whether these resistance determinants including integron related resistance are linked to form large, complex multi-drug resistance regions.

Chapter 4: A detailed analysis of the *Salmonella* Genomic Island SGI1, containing a multiple antibiotic resistance region and variants thereof, from a collection of different *Salmonella* spp.

4.1 Introduction

The emergence of multiply antibiotic resistant (MR) *Salmonella enterica* serovar Typhimurium DT104 exhibiting resistance to ampicillin, chloramphenicol, florfenicol, streptomycin, spectinomycin, sulfonamides and tetracycline is a significant public health issue. Strains of *Salmonella* Typhimurium DT104 susceptible to antibiotics have been isolated from cases of infection since the early 1960's. However, the multi-drug resistant (MR) *S. Typhimurium* DT104 strain was first identified in the early 1980's from gulls and exotic birds from Indonesia and Hong Kong (Threlfall, 2000; White et al., 2002). Apart from a small outbreak in humans in Scotland during the mid-1980's there was no reported outbreaks in humans until 1989, although it was well known to have become distributed among cattle herds in the United Kingdom by then (Threlfall, 2002).

In the United Kingdom, MR *S. Typhimurium* DT104 is now prevalent among poultry, pigs and sheep and is the second most prevalent *Salmonella* serotype isolated from humans and is increasingly isolated from various foods including meat/dairy products and salad ingredients (Threlfall et al., 2000; Threlfall, 2002). The dominant MR *S. Typhimurium* DT104 clone has also been isolated from numerous infected humans and animals in European countries, United States, Israel, Canada, Turkey, Trinidad, the Phillipines, the United Arab Emirates, the Irish Republic and Japan (Threlfall, 2000; Ribot et al., 2002). In Australia, however MR *S. Typhimurium* DT104 has not taken hold and has only been

detected in travellers returning from overseas and imported foods (D. Lightfoot, unpublished observations). Apart from displaying the multi-resistance phenotype another particular concern is the fact that *S. Typhimurium* DT104 appears to be highly virulent, especially in cattle (Evans and Davies, 1996). It has been found that humans infected with *S. Typhimurium* DT104 are up to three times more likely to be hospitalised (Wall et al., 1994; Varma et al., 2005).

MR *S. Typhimurium* DT104 has been shown to be resistant to 7 different antibiotics, is often incorrectly referred to as “penta-resistant”. There is in fact only five genes responsible for the multi-drug resistance in most MR *S. Typhimurium* DT104 strains, those being *aadA2*, *sulI*, *floR*, *tetA(G)* and *blaP1* and confer resistance to streptomycin/spectinomycin, sulfonamides, chloramphenicol/florfenicol, tetracyclines and β -lactam antibiotics, respectively (Briggs and Fratamico, 1999; Boyd et al., 2001) (see Fig 1.9). However, the lack of routine screening for resistance to the spectrum of antibiotics that genes in MR *S. Typhimurium* DT104 are known to confer resistance to, such as spectinomycin and florfenicol has meant that many of these strains have been incorrectly described.

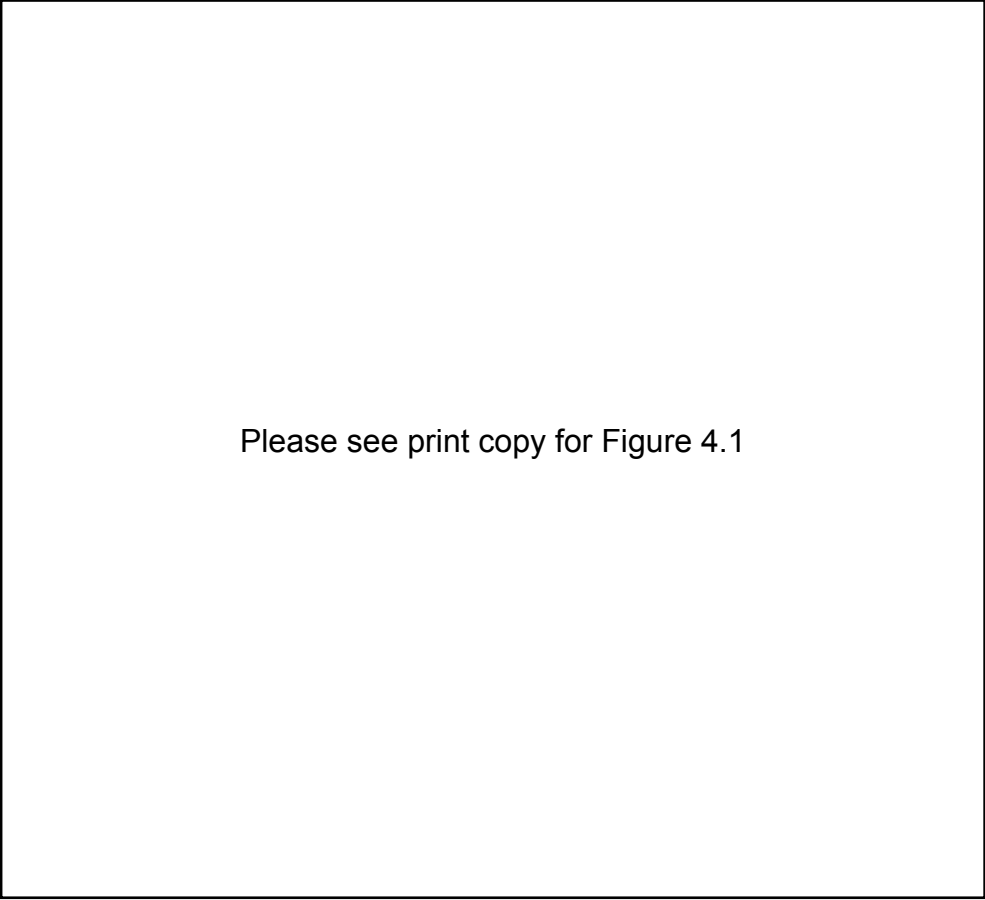
The resistance genes responsible for the multiple antibiotic resistant phenotype of the MR *S. Typhimurium* DT104 clone all reside within a 13 kb class 1 integron, recently named In104 (Levings et al., 2005b). DNA sequence analysis has shown that the structure of In104 (Briggs and Fratamico, 1999; Boyd et al., 2001), although related to In4 (Partridge et al., 2001a; Partridge et al., 2001b) is quite complex due to duplications of part of the *intI1* gene from the 5'CS and part of the 3'CS (*qacEAI* and partial *sulI* genes). These duplications have meant that In104 contains two *attI1* sites into which gene cassettes can be inserted or excised (Collis et al., 1993; Hall and Collis, 1995; Recchia and Hall, 1995; Hall and Collis,

1998). The *aadA2* cassette is found inserted in the left hand side *attI1* site of In104 (often referred to as InC) and the *blaP1* gene cassette is inserted in the right *attI1* site (often referred to as InD) (Ridley and Threlfall, 1998; Sandvang et al., 1998). These two cassettes produce a characteristic pair of gene cassette PCR products (1.0 and 1.2 kb) when using standard primers in the 5'CS and 3'CS, which was observed for some of the strains examined in Chapter 3. In between the two integron-derived regions lie the *floR* and *tetRA(G)* genes (Briggs and Fratamico, 1999) along with two open reading frames orf1 and orf2 whose functions at present are unknown, although it is speculated that orf2 is an unusual insertion sequence CR3 (Partridge and Hall., 2003).

The In104 integron lies within a genomic island known as SGI1 (GenBank accession no. AF261825) and appears to have moved to this location via transposition. This is based on the fact that there is a 5 bp direct duplication flanking the 25 bp inverted repeats (IRs) of the integron and it is located in a *res* site adjacent to a resolvase gene (*tnpR*) in the SGI1 backbone, which is where many class 1 integrons are commonly found (Kamali-Moghaddam and Sundstrom, 2000; Partridge et al., 2001b).

The complete sequence of SGI1 revealed that it is 43 kb in length, is chromosomally located residing between the *thdF* (responsible for thiopene and furan oxidation) and *yidY* (putative drug translocase) and contains 44 open reading frames (of known and unknown function) (Boyd et al., 2001). SGI1 has most likely been integrated via site-specific recombination due to the presence of an integrase (*int*) gene responsible for encoding a site-specific recombinase on the left hand end of SGI1 adjacent to the *thdF* gene (see Fig 4.1) (Boyd et al., 2001). In *S. Typhimurium* DT104 strains there is a second integrating element (retron element) which is often referred to as a retron phage located between SGI1 and the

gidY genes (Boyd et al., 2001) although is not usually present in other *S. enterica* serovars. To date SGI1 has been identified in serovars Typhimurium (DT104, DT1, DT12, DT120 and U302) (Boyd et al., 2002; Carattoli et al., 2002; Lawson et al., 2002) Agona (Boyd et al., 2002; Doublet et al., 2004a), Paratyphi B dT⁺ (Meunier et al., 2002), Albany (Doublet et al., 2003), Melegradis (Ebner et al., 2004) and Newport (Doublet et al., 2004b) suggesting that SGI1 has the potential to transfer horizontally between serovars.



Please see print copy for Figure 4.1

Fig 4.1 A theoretic model of SGI1 excision from and integration into the chromosome of *S. enterica* and *E. coli*. Integration occurs at the 3' end of *thdF* (*attB* site). DR-L and DR-R are formed by the recombination of the chromosomal *attB* site and *attP* site in SGI1. A mismatched base in the *attB* and *attP* sites is highlighted in larger font. Diagram taken from Doublet et al. (2005).

Numerous variations in the DNA structure of the integron in SGI1 have been reported. There have been 8 different variants of SGI1 described in the literature SGI1-A, SGI1-B, SGI1-C, SGI1-D, SGI1-E, SGI1-F, SGI1-G and SGI1-H (Boyd et al., 2002; Carattoli et al., 2002; Doublet et al., 2003). In some cases the entire region containing the *floR*, *tetA(G)*, *orf1* and *orf2* genes are missing (Boyd et al., 2002). Another variation includes the addition of an *orf513-dfrA10* array (Boyd et al., 2002). The *orf513* (CR1) originally described in In7 (Stokes et al., 1993) is a putative transposase gene and the *dfrA10* is a trimethoprim resistance gene (Partridge and Hall, 2003).

It has been reported recently that SGI1 can be conjugally transferred and integrated into a recipient chromosome via a site-specific manner from an *S. enterica* donor strain to both non-SGI1 *S. enterica* and *Escherichia coli* strains in the presence of a helper plasmid and an SGI1 encoded integrase (*int*) (Doublet et al., 2005). Horizontal transfer is thought to occur via *intI* mediated excision of SGI1 in the form of a circular molecule (see Fig 4.1). The conjugal transfer of SGI1 occurred at a frequency of 10^{-5} - 10^{-6} transconjugants per donor (Doublet et al., 2005).

Using detailed PCR mapping techniques, DNA sequencing and Southern hybridisation, the purpose of this study was to try and ascertain whether the characteristic 1 and 1.2 kb multiple PCR products obtained for some strains in Chapter 3 were in fact due to the presence of SGI1 and to determine whether the collection contained any other SGI1 derivatives previously described in the literature. It was also of interest to examine whether SGI1 was distributed among serovars that previously had not been mentioned in association with SGI1.

4.2 Materials and Methods

4.2.1 SGI1 specific screening PCRs

All of the *intI1* positive experimental strains from SRC1 to SRC76 screened by PCR for class 1 integrons (L2/L3 multiplex and L1/R1 PCRs and subsequent *RsaI* digestions) were included in this study. In order to minimise unnecessary PCR screening of non SGI1 containing isolates, primers linking SGI1 to the chromosome listed previously in the literature were employed. These PCR primers were U7-L12/LJ-R1 (SGI1 LJ), 104-RJ/104-D (SGI RJ) (primers linking SGI1 with chromosome) and 104-RJ and C9-L2 (retron element PCR) (Boyd et al., 2001) (see Fig 4.2 A). Primers that linked the SGI1 backbone to the In104 on both the left S026-FW/int-RV (In104LJ) and right hand DBT1/MDR-B- (In104RJ) sides were also used in the initial screening process (see Fig 4.2 B). All primers and their respective positions, used to map SGI1 and its variants for this particular study are listed in Table 4.1. PCR mapping of the CR1 (orf513)-*dfrA10* region was performed by using primer pairs pse-L/RH380, orf513-F/orf5-R2, orf513-F/RH380 and sul1F/RH380 (see Fig 4.3).

PCR conditions were carried out with reagent concentrations and volumes as previously described in Chapter 2. Thermal cycling conditions consisted of an initial denaturation cycle of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing temperatures ranging from 57 to 65 °C for 30 to 60s and extension at 72°C for 60 to 120s. Annealing temperatures for all primers can be seen in Table 4.1. A *Salmonella* MR *S. Typhimurium* DT104 isolate containing SGI1 taken from a human source and traced back to imported food (halva from Turkey) (DT02) (Fisher et al., 2001) was used as a control.

Table 4.1 SGI1 primer table (Adapted from Levings et al., 2005b)

Primer	Sequence (5'-3')	Location	Nucleotide Position	Accession #	Reference	Annealing temperatures
U7-L12	ACA CCT TGA GCA GGG CAA AG	<i>thdF</i>	1-20	AF261825.2	Boyd et al., 2000	60°
LJ-R1	AGT TCT AAA GGT TCG TAG TCG	<i>Int</i>	500-480	AF261825.2	Boyd et al., 2000	60°
SGI19-F3	TTC TTC AAT GCG CGC TTT GG	S023	19009-19028	AF261825.2	this study	60°
S026-FW	TCG GGT AAT CTC AGC AGA GC	S026	25021-25040	AF261825.2	Carattoli et al., 2002	60°
S024-RV	GGT ACG GTA TCG CCT AAG TG	S024	21930-21911	AF261825.2	Carattoli et al., 2002	60°
int-RV	GGG CAT GGT GGC TGA AGG ACC	<i>intI1</i>	27266-27246,	AF261825.2	Carattoli et al., 2002	60°
L1	GGC ATC CAA GCA GCA AGC	5'-CS	27892-27909, 37164-37181	AF261825.2	Levesque and Roy, 1993	57°
R1	AAG CAG ACT TGA CCT GAT	3'-CS	28900-28883, 38360-38343	AF261825.2	Levesque and Roy, 1993	57°
aadA2-L	TGT TGG TTA CTG TGG CCG	<i>aadA2</i>	28146-28163	AF261825.2	Ng et al., 1999	60°
aadA2-R2	TGC TTA GCT TCA AGT AAG ACG	<i>aadA2</i>	28683-28663	AF261825.2	Boyd et al., 2002	60°
QS-1	ATG AAA GGC TGG CTT TTT CTT G	<i>qacEΔ1</i>	28953-28974, 38413-38424	AF261825.2	Boyd et al., 2001	60°
sul1-F	GTG ACG GTG TTC GGC ATT CT	<i>sul1</i>	29297-29316, 35757-35776	AF261825.2	Leverstein-van Hall et al., 2002	60°
QS-2	TGA GTG CAT AAC CAC CAG CC	<i>sSul1</i>	29674-29655, 31934-31915	AF261825.2	Boyd et al., 2001	60°
StCM-L	CAC GTT GAG CCT CTA TAT GG	<i>flor</i>	30640-30659	AF261825.2	Boyd et al., 2002	60°
StCM-R	ATG CAG AAG TAG AAC GCG AC	<i>flor</i>	31527-31508	AF261825.2	Boyd et al., 2002	60°
tetG-L	CAG CTT TCG GAT TCT TAC GG	<i>tetA(G)</i>	32819-32838	AF261825.2	Ng et al., 1999	60°
tetG-R	GAT TGG TGA GGC TCG TTA GC	<i>tetA(G)</i>	33662-33643	AF261825.2	Ng et al., 1999	60°
orf2-F2	ATG CAG TGA GAA GCC GC	orf2	35291-35307	AF261825.2	this study	64°
orf2-R2	TAC TCG AGC ACG GCT TC	orf2	36024-36008	AF261825.2	this study	64°
groEL-F	ATG CCG CCC ATA CCG CCA GC	<i>groEL</i>	36490-36509	AF261825.2	this study	65°
pse-L	AAT GGC AAT CAG CGC TTC CC	<i>blaP1</i>	37495-37514	AF261825.2	Ng et al., 1999	60°
pse-R2	ACA ATC GCA TCA TTT CGC TC	<i>blaP1</i>	38147-38128	AF261825.2	Boyd et al., 2002	60°
sul1-R	TTT ACA GGA AGG CCA ACG GT	<i>sul1</i>	39424-39405	AF261825.2	Leverstein-van Hall et al., 2002	64°
orf5-F	AGG TTG TGC GGC TGA TGC	orf5	39776-39793	AF261825.2	this study	60°
orf5-R2	CGA GTT CTA GGC GTT CTG C	orf5	40213-40195	AF261825.2	this study	60°
orf6-R	ACT ATC TTC GGC CTT CAC ACG	orf6	40508-40488	AF261825.2	this study	60°
DB-T1	TGC CAC GCT CAA TAC CGA C	IS6100	41120-41138	AF261825.2	Boyd et al., 2002	60°
IS6100-F	AAG GGA TTC GAA GTC ATG C	IS6100	41322-41340	AF261825.2	this study	60°
IS6100 -Rv2	AAT GGT GGT TGA GCA TGC C	IS6100	41475-41457	AF261825.2	this study	62°
MDR-B ^b	GAA TCC GAC AGC CAA CGT TCC	S044	41905-41884	AF261825.2	Boyd et al., 2002	60°
104-RJ	TGA CGA GCT GAA GCG AAT TG	S044	42373-42392	AF261825.2	Boyd et al., 2002	60°
C9-L2	AGC AAG TGT GCG TAA TTT GG	retron element	42868-42887	AF261825.2	Boyd et al., 2000	60°
104-D	ACC AGG GCA AAA CTA CAC AG	<i>gidY</i>	47130-47111	AF261825.2	Boyd et al., 2002	60°
dfrA1-F	CGA AGA ATG GAG TTA TCG G	<i>dfrA1</i>	621-639	X17477.1	this study	60°
dfrA1-R	TTA GAG GCG AAG TCT TGG	<i>dfrA1</i>	1029-1012	X17477.1	this study	60°
orfC-F	CAT TAC GAA GCG AAT GCA CC	orfC	1172-1191	X17477.1	this study	60°
orfC-R	TCT CGA ATC AAG CAG GAA CC	orfC	1534-1515	X17477.1	this study	60°
orf513-F	ATG TCG CTG GCA AGG AAC G	orf513	3521-3539	L06418.4	this study	62°
RH379	ACC AGA GCA TTC GGT AAT CAA G	<i>dfrA10</i>	5530-5551	L06418.4	this study	60°
RH380	GCT TCA GAT AAT AAA CCA ACA CCA CC	<i>dfrA10</i>	5828-5803	L06418.4	this study	60°

^a MDR-7 targets the sequence preceding *floR*^b the sequence from AF261825.2 is GAATCCGACAGCCAACGCTTCC

A.

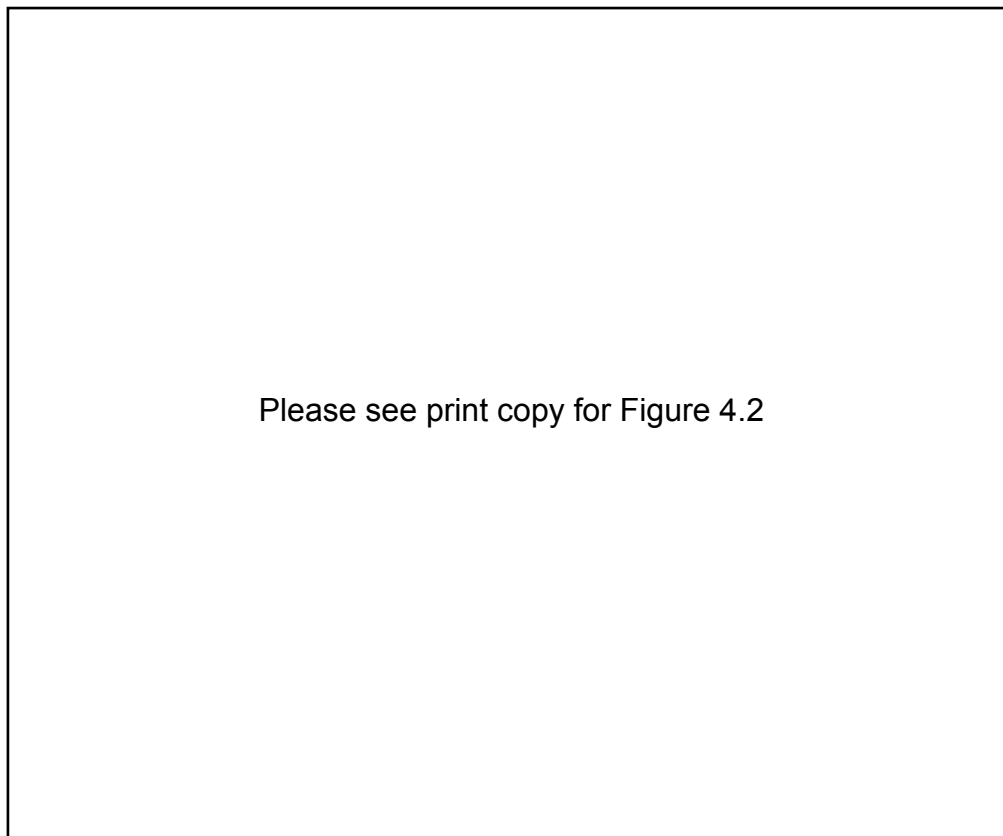


Fig 4.2 A/B Diagrammatic representation of all major linkage PCR fragments amplified to characterise the genetic content of In104 in SGI1 containing isolates. *BsaI* restriction enzyme recognition sites of interest are marked with (B). The light blue dotted region represents the SGI backbone, the 5'CS and 3'CS are represented by the dark blue boxes. The resistance region (containing *floR* and *tetA(G)*) separating the two *attI* sites is represented by the purple boxed region. Diagram is drawn to scale. Diagram taken from Levings et al. (2005b).

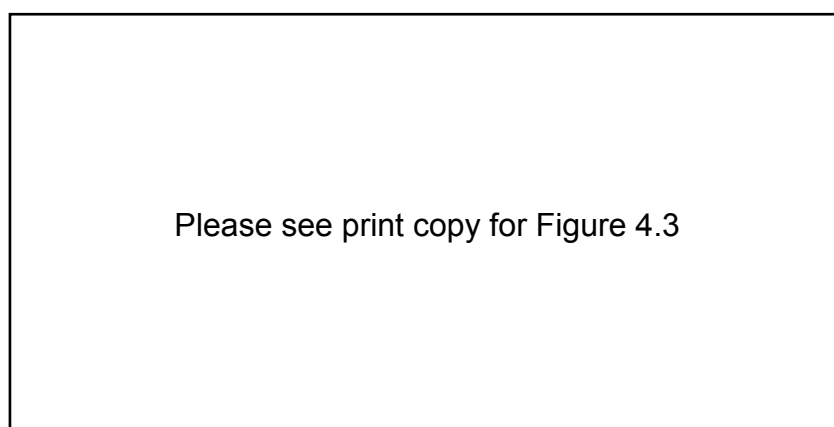


Fig 4.3 Diagram of primer amplification products for the CR1(orf513)-*dfrA10* insertion found in variants of SGI1. Diagram taken from Levings et al. (2005b).

4.2.2 Antimicrobial resistance testing for florfenicol

Florfenicol resistance was tested by streaking isolates that contained SGI1 onto LB agar containing 15 µg/ml florfenicol (Sigma). All other antibiotic resistance testing was carried out as mentioned in Chapter 2 (Materials & Methods).

4.2.3 Southern hybridisation of *Bsa*I digested chromosomal DNA probed with *tetA*(G) and CR3 (orf2) DIG-labelled DNA probes

tetA(G)-orf2 linkage was established using Southern hybridisation. 50-100 ng of *Bsa*I digested whole cell DNA was transferred to a nitrocellulose membrane (see Chapter 2, Materials and Methods) and hybridised to DIG-labelled *tetA*(G) and CR3 (orf2) PCR probes generated by using tetG-L/tetG-R and orf2-F2/orf2-R2 primer pairs (see Table 4.1 for primer annealing conditions). The boundaries of the 4.1 kb *Bsa*I fragment can be seen above in Fig 4.2 B. Hybridisation and development of the membranes were conducted as previously described in Chapter 2.

4.2.4 DNA Sequencing

A number of linkage PCRs were characterised and sequenced when PCR products were generated that were not of the expected size based on the original SGI1 database entry AF261825. Both strands of DNA sequence were obtained via primer walking techniques under conditions mentioned in Chapter 2.

4.3 Results

4.3.1 SGI1 major junction PCRs

Initial PCR screening of *intI1* positive isolates SRC1-SRC76 using the SGI1 LJ PCR and SGI1 RJ PCR suggested that there were possibly 10 SGI1 containing isolates (see Fig 4.4 and Table 4.2), all producing the expected product for each PCR. SGI1 regions for 9 of these isolates were characterised in detail. The remaining isolate SRC73 (*S. Kentucky*) found to be positive for the SGILJ and SGI1RJ, was at the time still being investigated and due to the complexity of the regions surrounding this particular In104, will not be discussed any further in relation to this study. The cassette information for this strain was highlighted in detail in Chapter 3.

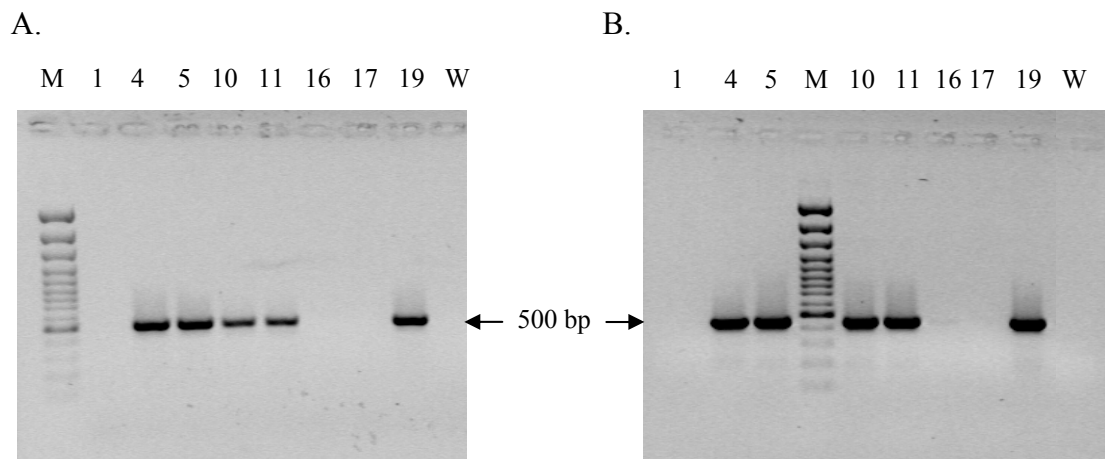


Fig 4.4 A. SGI1 left junction PCR (U7-L12/LJ-R1).B. SGI1 right junction PCR (104-RJ/104-D) on a selection of *intI1* positive strains. A molecular marker (M) and negative water control (W) are also featured in both gels. SRC4, SRC5, SRC10, SRC11 and SRC19 are positive in these gels for both products. SRC1, SRC16, SRC17 and water control are negative. DT02 was found to be positive for the LJ and negative for the RJ PCR in a subsequent gel (data not shown). See Table 4.2 for other positive strains.

The *S. Typhimurium* DT104 control strain (DT02) was the only isolate that was positive for the retron element PCR. It was also negative for the normal SGIRJ PCR

due to a substantial (~4 kb) increase in amplicon size when the retron element is present. The expected In104LJ and In104RJ, seen for Typhimurium DT104 (see Fig 4.2), were not obtained for *S. Emek* (SRC19). A series of primers designed along the backbone heading out to the left of S026 were used in conjunction with the int-RV primer. A 1.4 kb amplicon was obtained using the SGI19 (F3) primer in S023 and the int-RV primer for SRC19. IS6100-F and S024-RV were then used to link the right hand side of In104 to the backbone for SRC19 for which a 2.5 kb amplicon was obtained. The sequences revealed the presence of a 5 bp duplication CCATG adjacent to both the IRi and IRt repeats of In104, consistent with the hypothesised mechanism of insertion of In104 via transposition (see Fig 4.5). The DNA sequence for the sequence obtained for the junction between S023 and IRi of In104Emek can be found in GenBank accession AY963803. A diagrammatic representation of the new insertion site of In104Emek can be seen in Fig 4.6.

Table 4.2 PCR results for major SGI1 junctions. Taken from Levings et al.(2005b).

Please see print copy for table 4.2

Of the 9 experimental strains positive for any of the SGI1 and In104 junction PCRs, 6 produced the characteristic 1.0 and 1.2 kb amplicons for the L1/R1 gene cassette PCR. The *S. Infantis* strain (SRC46) produced a single 1.0 kb amplicon and the *S. Cerro*, *S. Dusseldorf* and *S. Emek* strains produced a 1.2 kb amplicon (see Fig 4.7A).

Please see print copy for Figure 4.7

Fig 4.7 A. L1/R1 and B. *RsaI* digestion products for isolates that were positive for the left (SGI1 LJ) and right arm (SGI1 RJ) PCRs for SGI1. Molecular weight markers are indicated and the *S. Typhimurium* DT104 control (DT02) included. Diagram taken from Levings et al. (2005b).

Restriction enzyme analysis of the PCR amplicons generated using the *RsaI* restriction enzyme (see Fig 4.7 B) showed identical profiles for the isolates producing the characteristic 1.0 and 1.2 kb PCR products, indicative of the presence of the *aadA2* and *blaP1* gene cassettes. SRC46, produced a profile that was suggestive of only the *aadA2* gene cassette, sharing some of the bands that were present in the *aadA2* and *blaP1* containing strains. This was ultimately confirmed by DNA sequencing. A single L1/R1 amplicon for SRC19 (Emek) was sequenced and found to

be identical to the *dfrA1-orfC* array found in *Vibrio cholerae* (GenBank accession no. AF455254). In order to be able to determine the structure of the right hand side *attI1* site in SRC19, the 1.2 kb groEL-F/QS-2 PCR product was sequenced and found to contain a deletion that removes both the gene cassette and 472 bp of adjacent sequences including the complete *attI1* site and all of *qacEΔ1*. This deletion is identical to one found in Tn610 from a *Mycobacterium* (GenBank accession no. X53635) (Martin et al., 1990). No amplicon was obtained for SRC46 using groEL-F/QS-2 primers (see Fig 4.8B).

The *S. Derby* isolate (SRC4) produced a profile with banding patterns representative of both the *dfrA1-orfC* and the *aadA2* cassette. The PCR products for the *S. Cerro* and *S. Dusseldorf* were unable to be sequenced due to the presence of two identical sized PCR products, however restriction enzyme analysis and DNA sequence information already obtained for the other isolates suggested that these both contained the *dfrA1-orfC* and *blaP1* gene cassettes.

4.3.3 Location of the gene cassettes within In104

In order to designate the cassettes either to the right or left hand *attI1* sites within In104, linkage PCRs using a primer in S026 (S026-FW) and one in groEL (groEL-F) with a primer internal to the appropriate gene cassette was employed (see Fig 4.8 A & B). All of the serovars apart from Cerro (SRC5), Dusseldorf (SRC38) and Emek (SRC19) contained the *aadA2* cassette in the left hand *attI1* site. The Cerro, Dusseldorf and Emek strains were found to contain the *dfrA1-orfC* array in this position (for primer positions see Fig 4.2). Paratyphi BdT⁺ (SRC49 and 50), Kiambu

(SRC10 & 11), Cerro (SRC5) and Dusseldorf (SRC38) strains all contained the *blaP1* cassette in the right hand *attII* site and the Derby (SRC4) isolate contained the *dfrA1*-*orfC* array in this position. As mentioned above the Emek strain did not contain a gene cassette in the right hand position and the Infantis only contained a single *attII* site as deduced from the *RsaI* digestion of the L1/R1 and no PCR amplicon was obtained for the groEL-F/pse-R2 or groEL-F/QS-2 PCR.

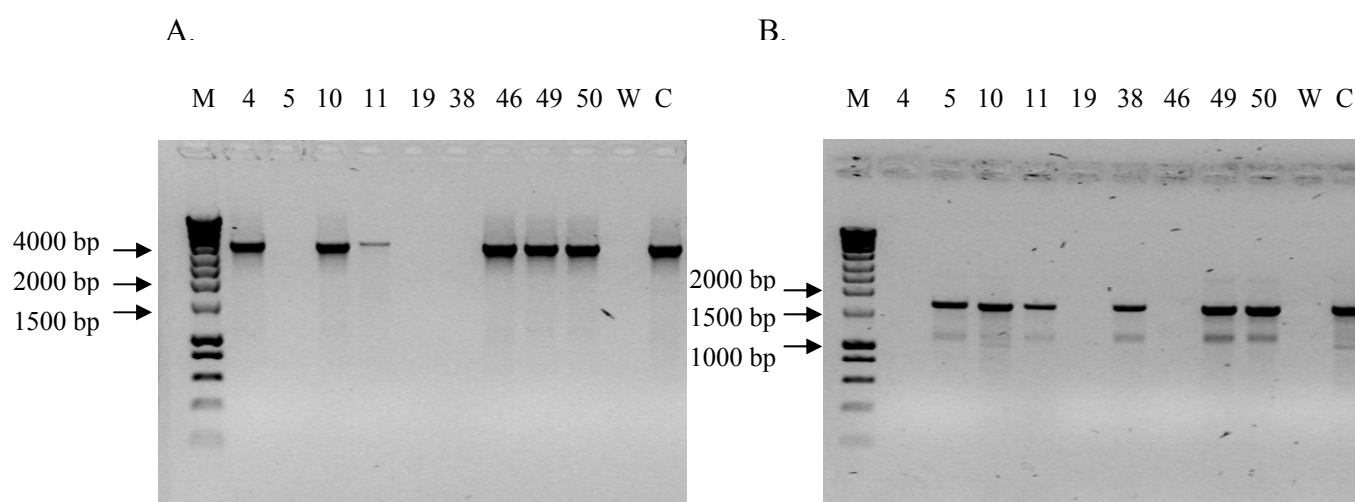


Fig 4.8 A. S026-FW/aadA2-R2 and B. groEL(F)/pse-R2 linkage PCRs on SGI1 containing strains. SRC11 gave a stronger amplicon product in subsequent experiments. The Hyperladder I DNA marker (Bioline) is labelled (M), Typhimurium DT104 control is labelled (C) and the negative water control is labelled (W).

4.3.4 Southern hybridisations of *BsaI* digested chromosomal DNA probed with *tetA*(G) and CR3 DIG labelled PCR products

Southern hybridisation results confirmed the linkage between *tetA*(G)-CR3 (*orf2*). As expected from the published DNA sequence in GenBank a 4.1 kb *BsaI* fragment hybridised with both the *tetA*(G) and CR3 (*orf2*) DIG-labelled probes. A diagrammatic representation of the probe positions within the 4.1 kb *BsaI* fragment can be seen in Fig 4.9. Hybridisation results can be seen in Fig 4.10 and 4.11. Isolates that were positive for the 4.1 kb *BsaI* fragment using both probes were SRC4, 5, 10, 11, 19, 38, 49, 50 and DT02 control. Strains SRC46 and SRC73 were negative.

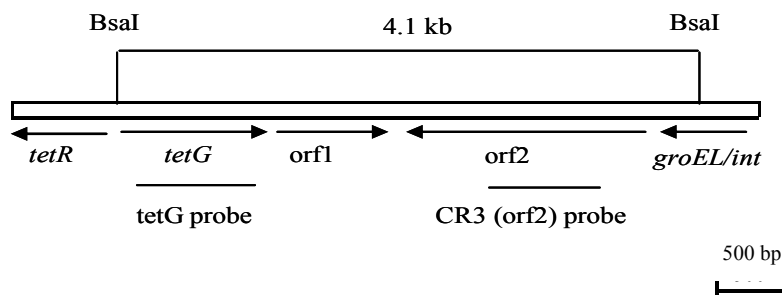


Fig 4.9 Diagrammatic representation of the 4.1 kb *BsaI* fragment that hybridised with the *tetA*(G) (*tetG*-L/*tetG*-R) and CR3 (*orf2*)(*orf2*-F2/*orf2*-R2) DIG labelled DNA probes. Diagram is drawn to scale (from accession no. AF261825).

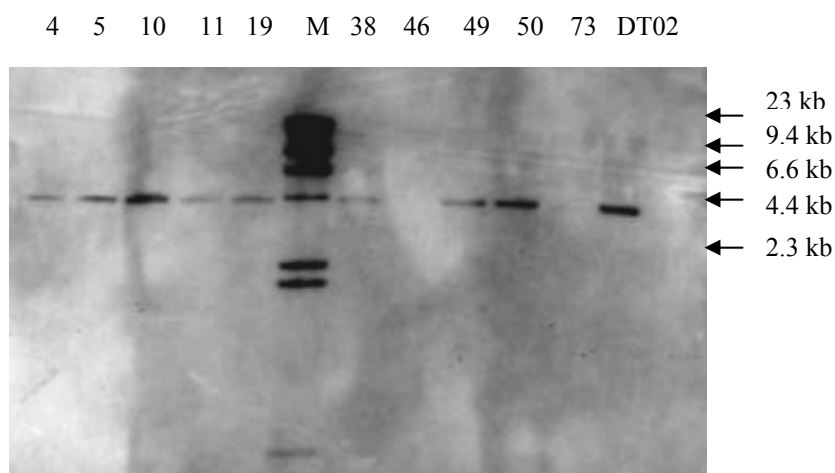


Fig 4.10 Southern hybridisation blot of CR3 (*orf2*) DIG-labelled probe to *BsaI* digested DNA for all experimental SGI1 containing strains and *S. Typhimurium* DT104 control (DT02). The DIG-labelled marker II is in lane marked (M).

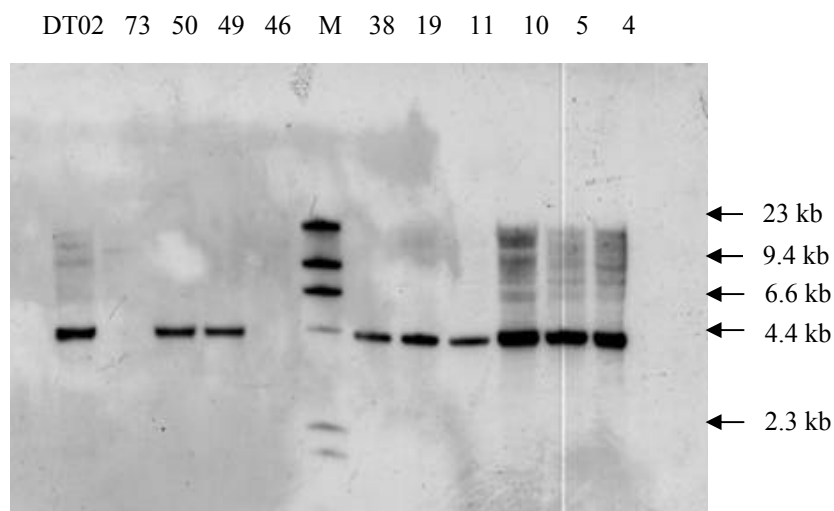


Fig 4.11 Southern hybridisation of *tetA*(G) DIG-labelled probe to *BsaI* digested chromosomal DNA for all experimental SGI1 containing strains and *S. Typhimurium* DT104 positive control DT02. The DIG-labelled marker II is in lane marked (M).

4.3.5 Summary of resistance genes and other gene linkages identified in SGI1 containing *Salmonella* spp.

Resistance genes (not cassette associated) typically found in SGI1 were identified using PCR primers internal to the genes and are listed in Table 4.1. Only a representative of these PCRs will be shown (see Fig 4.12). Linkage PCRs were also adopted by using appropriate primer pairs in adjacent open reading frames and are all listed in Table 4.1.

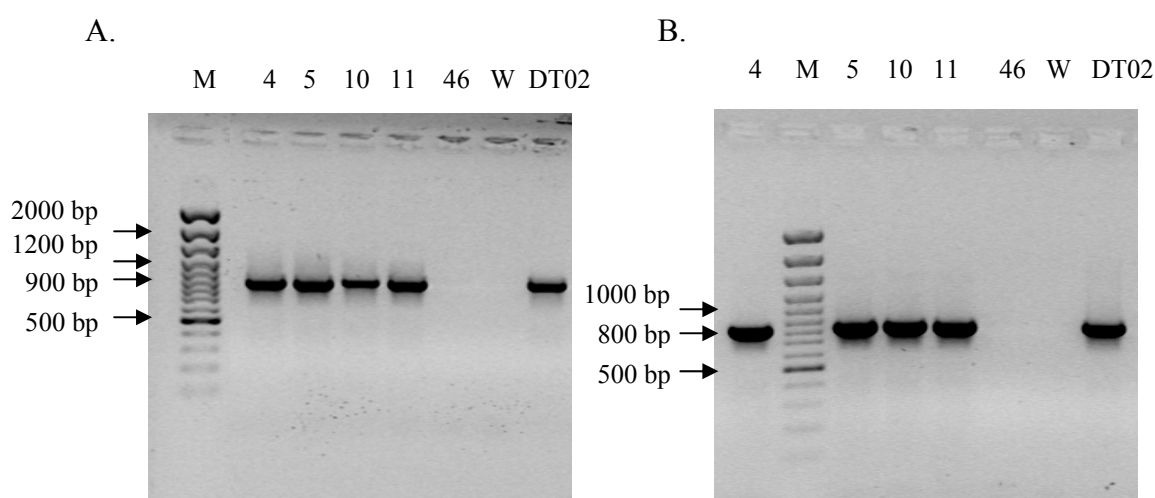


Fig 4.12 A. *floR* and B. *tetA(G)* PCR using StCM-L/StCM-R and tetG-L/tetG-R primers respectively on a representative number of SGI1 containing strains. A negative water control is also present. SRC4, 5, 10 and 11 and SGI1 control DT02 are positive for both PCRs in these gels (see Table 4.3 for other results).

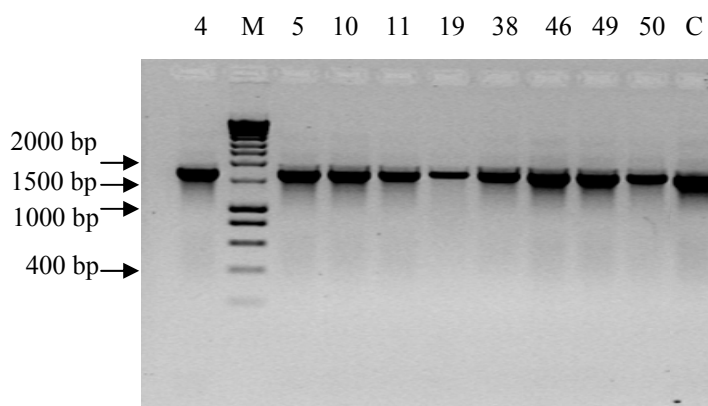


Fig 4.13 *orf5-F/IS6100* (Rv-2) linkage PCR on SGI1 containing strains. The Hyperladder I molecular marker is labelled (M) and the DT104 control strain labelled (C). Sizes are indicated. All SGI1 containing strains (SRC4, 5, 10, 11, 19, 38, 46, 49, 50 and DT02) are positive for this internal gene linkage PCR.

In order to identify whether the right hand side of In104 was the same as in *S. Typhimurium* DT104, a linkage PCR between orf5 and IS6100 using the primer pairs orf5(F) and IS6100(Rv-2) listed in Table 4.1 was conducted. Amplicons of the predicted size (approximately 1.7 kb) were obtained for all SGI1 containing strains and can be seen in Fig 4.13. A summary of all resistance genes identified for all strains can be seen in Table 4.3.

Table 4.3 Antibiotic resistance profiles and resistance genes of *Salmonella* spp. containing SGI1 and variants of SGI1. Table taken from Levings et al. (2005b).

Please see print copy for Table 4.3

4.3.6 Further linkage analysis of trimethoprim resistant SGI1 strains not containing the *dfrA1*-orfC cassette array.

PCR products of the expected sizes, based on sequence information from GenBank accession no. AF261825 were obtained for the major open reading frames in the MR region. Trimethoprim resistant strains not containing the *dfrA1*-orfC cassette array, namely, the Kiambu and Infantis isolates (SRC10, 11 and 46), were screened for the presence of the CR1(orf513)-*dfrA10* region associated with SGI1-A from an *Agona* strain (Boyd et al., 2002). The predicted PCR products obtained for the internal

dfrA10 PCR along with linkage PCRs to CR1 (orf513) and to *sulI* can be seen in Fig 4.14 and 4.15. Based on PCRs internal to major open reading frames, linkage PCRs, DNA sequencing and southern hybridisations collectively, the structures for each of the SGI1 containing strains can be seen in Fig 4.16.

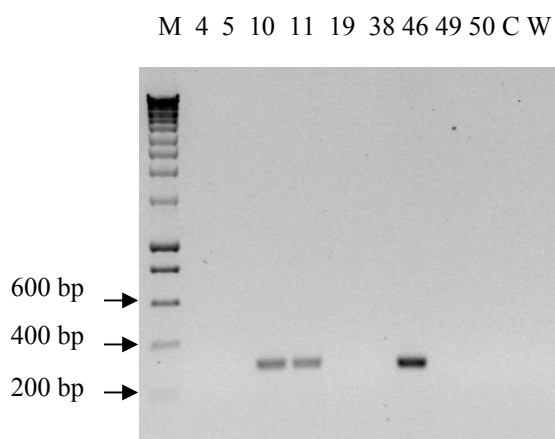


Fig 4.14 *dfrA10* PCR using primers RH379 and RH380 on SGI1 containing strains. The Hyperladder I DNA ladder is loaded in the lane labelled (M). The control strain (DT02) is loaded in the lane labelled (C). A negative water control is also included. SRC10, 11 and 46 were the only positive strains for the *dfrA10* PCR.

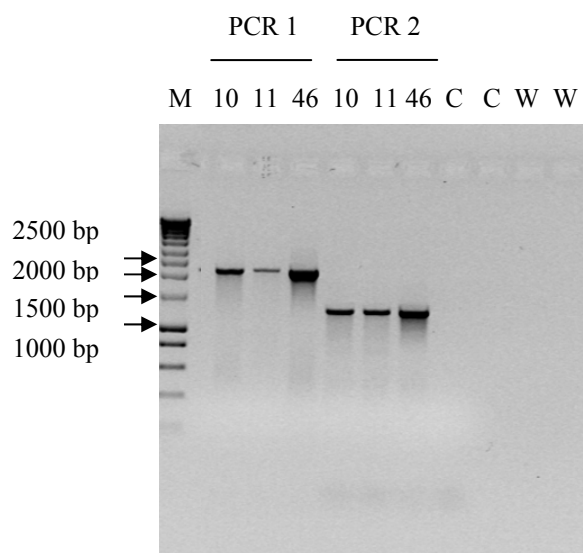
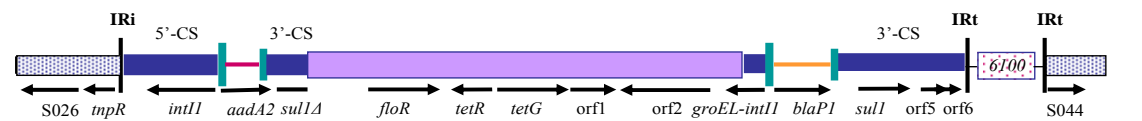
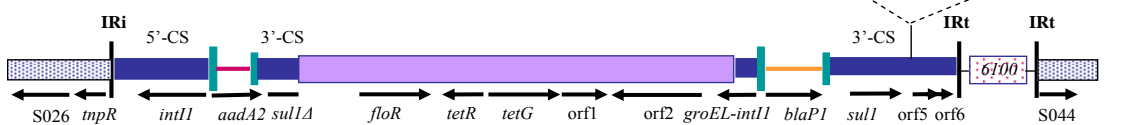


Fig 4.15 PCR linking CR1 to *dfrA10* (orf513(F)/RH380) (PCR 1) and *dfrA10* to *sulI* (RH379/QS-2) (PCR 2) in trimethoprim resistant strains not containing *dfrAI-orfC* array. The Hyperladder I DNA ladder is loaded in the lane labelled (M). The control strain (DT02) is loaded for the two PCRs and are in the lanes labelled (C). The negative water controls are also included. As expected from previous results SRC10, 11 and 46 were the only SGI1 strains positive for these *dfrA10* linkage PCRs.

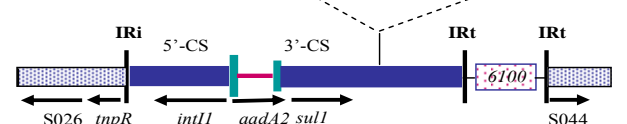
Paratyphi B dT⁺ (SRC 49 & 50)
SGI1



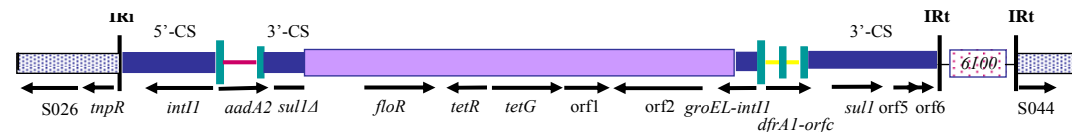
Kiambu (SRC10 & 11)
SGI1-A



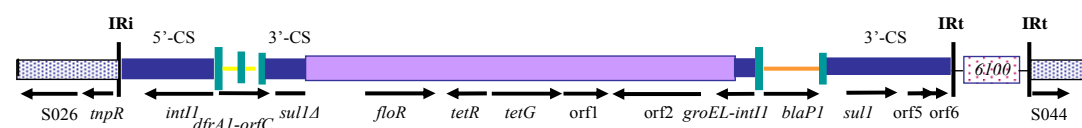
Infantis (SRC46)
SGI1-D



Derby (SRC4)
SGI1-I



Dusseldorf/Cerro (SRC5 & 38)
SGI1-F



Emek (SRC19)
SGI2 (formerly SGI1-J)

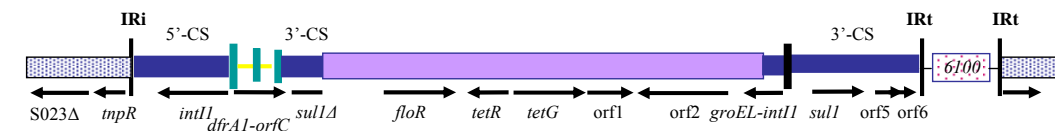


Fig 4.16 Maps of all In104 variants found in this study. Narrow vertical bars represent IRs (IRi and IRt). The 5'CS and 3'CS are indicated by filled blue boxes. The resistance region (containing *floR* and *tetA(G)*) separating the two *attI1* sites is represented by the purple boxed region. SRC numbers are indicated.

4.4 Discussion

This study highlights the wide distribution of SGI1 among *Salmonella* serovars and adds further strength to the belief that SGI1 has the ability to move from strain to strain. This study highlighted the importance of screening for the left and right chromosomal integration junctions of SGI1. Previous methodologies attempting to identify MR regions using standard gene cassette PCRs, fail to detect SGI1 variants in the absence of the expected 1.0 and 1.2 kb PCR amplicons. In the case of SRC5 and SRC19 for example if the left and right junction PCRs (SGI1LJ and SGIRJ) had not been performed, the SGI1 variants present in these strains would have gone undetected. It has become clear, that relying solely on the gene cassette information and the presence of the standard 1.0 and 1.2 kb amplicons, are not very effective tools when screening for SGI1.

Based on the *RsaI* restriction enzyme profiles it appears that the 1.0 and 1.2 kb amplicons obtained in Chapter 3 for isolates SRC99 (*S. Kiambu*) and SRC101(*S. Paratyphi* BdT⁺) are in fact the *aadA2* and *blaP1* gene cassettes associated with SGI1-A. Since the completion of the study on the 76 isolates discussed in this chapter, the rest of the original 136 isolates within the larger collection described in Chapter 3 have been screened using the SGI1-LJ and SGI1-RJ PCR. These two isolates were the only ones that produced the expected size amplicons for the PCR and it has been concluded that these strains also contain SGI1. No other SGI1 containing strains have been identified in the larger collection.

To date there have been a number of different variants described in the literature “SGI1-A to SGI1-H” and this study has revealed the existence of two new variants namely SGI1-I and SGI1-J. Since the completion of this study, a further three new variants have been identified and characterised, SGI1-K (Levings et al., 2007), SGI1-L (Cloeckaert et al., 2006) and SGI1-M (Vo et al., 2007). SGI1-K was recovered from the isolate mentioned earlier in this chapter, *S. Kentucky* (SRC73). It is a variant that contains the *aacCA5-aadA7* cassette array only and is missing the middle portion of In104 (namely the *floR* and *tetRA(G)* resistance determinants and the *orf1* and *orf2* open reading frames). It has also been found to reside next to a *Tn501/Tn21* hybrid mercury resistance operon (Levings et al., 2007). The mercury resistance operon present in this strain resides to the right of a truncated *IS6100* and has lead to the disruption of the In104/SGI1 boundary, which explains the negative result obtained for the In104RJ PCR. The SGI1-L variant was recovered from a *S. Newport* isolate and contained a *dfrA15* cassette incorporated at the left hand *attI1* site and the standard *blaP1* cassette on the right (Cloeckaert et al., 2006). The *S. Typhimurium* DT104 containing SGI1-M was recovered from an equine source in The Netherlands and the *aadA2* gene cassette has been replaced by an *aadB* cassette (Vo et al., 2007).

SGI1 has more recently been identified in *Proteus mirabilis* (Ahmed et al., 2007; Boyd et al., 2008), a Gram-negative pathogen commonly associated with hospital acquired infections. The *dfrA15* cassette has replaced the *aadA2* at the left hand *attI1* site as seen for SGI1-L in the *S. Newport* strain mentioned above (Cloeckaert et al., 2006; Ahmed et al., 2007). As expected the island has inserted into an alternate site in the *Proteus mirabilis* chromosome. A gene that is 70% identical in nucleotide sequence to the *Salmonella thdF* gene has been identified and proposed as a potential

SGI1 integration site (Doublet et al., 2007). Bioinformatic analysis by Doublet et al, identified potential SGI1 insertion sites in the chromosomes of other bacterial pathogens such as *Shigella* spp., *Vibrio* spp., *Pseudomonas* spp., *Brucella* spp., *Legionella pneumophila* and *Klebsiella pneumoniae* (Doublet et al., 2007). Interestingly, in the absence of antibiotic pressure, the SGI1 in *P. mirabilis* is unstable and readily lost from the strain, which does not seem to be the case for SGI1 stability in *S. Typhimurium* DT104 (Ahmed et al., 2007).

In *Salmonella* it appears that the chromosomal location of SGI1 potentially enhances the stability of the antibiotic resistance phenotypes of SGI1 containing strains even in the absence of antibiotic selective pressure (Ahmed et al., 2007). Incorporation of genes into the chromosome usually means that loss of these genes from the host cell occurs at a much lower rate than would be expected for genes residing on mobile genetic elements such as plasmids that are readily mobile.

S. Emek (SRC19) strain was negative for the standard SGI1 backbone and integron junction PCRs (In104LJ and In104RJ PCRs). In order to link the In104 to SGI1 backbone a new primer SGI119 (F3) was designed and used in conjunction with a primer in the *intI1* integrase (int-RV), the product of which was sequenced. From this sequence a second PCR was designed to link IS6100 to S024 on the right hand side of the integron. This amplicon, also sequenced, revealed the duplicated 5 bp (CCATG) adjacent to IRt. It appears that In104 has at some stage inserted into the SGI backbone at a different position, S023 instead of S026. The presence of the 5bp duplications adjacent to both IRi and IRt further support the hypothesis that the integron In104 is inserted via transposition (Levings et al., 2005b). The insertion of the integron in a

different location suggests that the SGI in Emek evolved independently from all other SGI described in the literature. Based on the new insertion site we have designated a new name SGI2 to the SGI chromosomal island described here for SRC19 (formerly SGI1-J) (Levings et al., 2005b).

S023 has been grouped as a possible conjugal transfer ORF and probably of plasmid origin (Mulvey et al., 2006). The presence of conjugal transfer genes in the SGI1 backbone tends to suggest that SGI1 is of plasmid origin (Mulvey et al., 2006). The G+C content of the genes present in the SGI1 structure is completely different to that of the *Salmonella* chromosome suggesting that these genes have been horizontally acquired (Boyd et al., 2001).

The global distribution of *Salmonella* isolates possessing SGI1 and variants of it have led to speculation that the island has played an important role in increased fitness and/or virulence of isolates that contain it (Boyd et al., 2001; Mulvey et al., 2004; Levings et al., 2005b; Golding et al., 2007). Studies have already begun in trying to determine whether the open reading frames in the SGI1 backbone are involved in virulence and functional studies involving knock-out mutants are underway (Golding et al., 2007). SGI1 in association with rumen protozoa is thought to be involved in the increased virulence capabilities of the bacterium (Rasmussen et al., 2005; Carlson et al., 2007). The fact that possible virulence determinants have linked up with antibiotic resistance determinants in the genomic island is particularly concerning.

Although MR *Salmonella* Typhimurium DT104 is not endemic in Australia, it is clear from this study that SGI1 containing strains are appearing in samples collected from

Australian residents and should be monitored closely with rigorous surveillance strategies. The association of resistance genes with possible virulence determinants within the island and its movement between bacteria in a circular form means SGI1 and variants of it could become a significant public health problem as well as being a threat to agricultural and veterinary settings. *Salmonella* Kambu a serovar readily isolated from the gut flora of poultry also appears to have acquired the island and looms as a possible threat to the poultry industry, which is renowned for its heavy use of antimicrobials for growth promotion. The global observation of a possible association of SGI1 containing MR *Salmonella* Paratyphi BdT⁺ (ApFICmSmSpSuTc) with human infections sourced from home aquaria, which are now being increasingly isolated here in Australia (D. Lightfoot, unpublished observations) is also of growing concern.

Chapter 5: A molecular and epidemiological study of multiply antibiotic resistant *Salmonella* Paratyphi BdT⁺ containing SGI1 sourced from ornamental fish tanks and human infections in Australia

5.1 Introduction

The global trade in ornamental fish is significant, with an estimated 8-10 million ornamental fish being imported annually into Australia (Love et al., 2004). To date the potential risks and hazards associated with this sizeable influx have not been fully examined. With such a large number of ornamental fish being imported and sourced from over 100 different countries (Whittington and Chong, 2007), one could assume there would be an increased risk of disease from newly introduced pathogens not only for native fish species but potentially for human hosts, that come in contact with the imported fish and their water environment.

Antibiotics are used in aquaculture to treat fish diseases caused by bacteria such as *Aeromonas* spp. and *Vibrio* spp (Akinbowale et al., 2006). Different countries have different regulations when it comes to the use of antibiotics in aquaculture. In developed countries, including members of the EU, USA, Canada and Norway the regulatory control on antibiotics and their administration is considered strong (Burka et al., 1997; WHO 2002; Lillehaug et al., 2003). However, up to 90% of aquaculture production occurs in developing countries where the use of antibiotics is widespread and unregulated (Bondad-Reantaso et al., 2005). It can not be disputed that the discovery and introduction of antibiotics revolutionised disease management regimes. However, the potential for these agents to be overused has created significant

evolutionary selection of bacteria containing genetic material responsible for resistance to these agents.

One bacteria of interest to human health, displaying resistance to antimicrobials is *Salmonella enterica* Paratyphi BdT⁺, formerly known as *Salmonella enterica* serovar Java (Chart, 2003). *Salmonella* Paratyphi BdT⁺ although not pathogenic to fish can reside as part of the normal gut flora in fish, whose make-up is largely dependant on the surrounding environment. Since the late 1990's multiply antibiotic resistant isolates of *S. Paratyphi* BdT⁺ have been increasingly isolated from humans around the world and are a major causative agent of gastroenteritis (Mulvey et al., 2004; Weill et al., 2005; Musto et al., 2006).

Two strains of multiply resistant *S. Paratyphi* BdT⁺ with distinctively different resistance phenotypes have been isolated from sources around the world. The first being a strain that exhibits resistance to streptomycin (Sm), spectinomycin (Sp), trimethoprim (Tp) and sulfonamides (Su) and is known to contain a chromosomally located Tn7 associated class 2 integron containing the *dfrA1-sat1-aadA1* gene cassette array (van Pelt et al., 2003). This clone is predominantly isolated from poultry and poultry products in Germany and The Netherlands (Miko et al., 2002; van Pelt et al., 2003).

The other multiply resistant *S. Paratyphi* BdT⁺ strains increasingly isolated from Canada (Mulvey et al., 2004), the United Kingdom (Threlfall et al., 2005), France (Weill et al., 2005) and Australia (Levings et al., 2005b) have been found to be resistant to Ap, Cm, Sm, Sp, Su and Tc. The genes responsible for this resistance phenotype in these strains has been attributed to the presence of the *blaP1*, *floR*,

aadA2, *sul1* and *tetA*(G) genes located in the complex class 1 integron In104 (Levings et al., 2005b), within the *Salmonella* genomic island 1 (SGI1) originally identified in *Salmonella* Typhimurium DT104 (Boyd et al., 2001). This particular strain of *S. Paratyphi* BdT⁺ is commonly associated with human gastroenteritis.

The first reported case of *S. Paratyphi* BdT⁺ with the ApCmSmSpSuTc resistance phenotype was isolated from a tropical fish in Singapore in 1997 (Meunier et al., 2002), highlighting the potential for tropical fish and aquariums as a possible source of infection. It has been suggested in the literature that *S. Paratyphi* BdT⁺ may be linked to aquacultural practices, however antibiotic resistance profiles for these particular strains were not reported and the molecular data if any to confirm this was not made available (Gaulin et al., 2002; Senanayake et al., 2004).

Sporadic cases of *S. Paratyphi* BdT⁺ with the resistance phenotype ApCmSmSpSuTc have been noted in various states in Australia since 1997 and surveys have highlighted a potential association with the ownership of domestic aquariums (D. Lightfoot, unpublished observations), with 13 cases in 2003-2004 investigated by State and Commonwealth Health Departments, all found to be associated with home aquariums containing tropical fish (Musto et al., 2006). The aim of this study was to molecularly characterise a number of *S. Paratyphi* BdT⁺ isolates with the resistance profile ApCmSmSpSuTc from both human and aquacultural sources from cases in the Australian Capital Territory (ACT) and Victoria in 2000 and 2003, to establish if there is any direct genetic link between the multiply resistant *S. Paratyphi* BdT⁺ strains isolated from human infections and those isolated from domestic aquariums (directly associated to cases of human disease). In turn, examining the possibility that

domestic aquariums act as a reservoir for multiply antibiotic resistant *S. Paratyphi* BdT⁺.

5.2 Methods and Materials

5.2.1 Bacterial collection

Water and/or gravel were collected from domestic aquariums of patients infected with *S. Paratyphi* BdT⁺ with the resistance phenotype ApCmSmSpSuTc in 2000 and 2003 in Victoria and ACT. *S. Paratyphi* strains from both humans and fish tanks were isolated using methods described previously (Musto et al., 2006) and serotyping and antibiotic resistance profiling was conducted by the MDU in Melbourne. 4 matched sets of isolates (each set comprised of human isolates and isolates obtained from aquarium environments), 2 from 2000 and 2 from 2003 were examined (see Table 5.1). Strain SRC233A was not made available for molecular analysis and will no longer be discussed. A *S. Paratyphi* BdT⁺ isolate with the resistance phenotype ApCmSmSpSuTc (SRC50) previously characterised in Chapter 4 was used as a control (Levings et al., 2005b). DNA was extracted from bacteria via the phenol/chloroform method described in Chapter 2.

5.2.2 SGI1 PCR amplification and In104 cassette analysis

The L2/L3 multiplex PCR amplifying the *intI1* integrase and *Salmonella* specific *invA* gene was performed on all MR *S. Paratyphi* isolates listed in Table 5.1. The gene cassettes associated with In104 were amplified using standard primers in the 5' and 3'

conserved segments of class 1 integrons (L1/R1) (see Fig 5.1). Fragments generated by these primers were digested using *RsaI*. All PCRs and restriction enzyme analysis were carried out under conditions described previously in Chapters 2 and 4 (Levings et al., 2005b). To confirm that the *blaPI* cassette was inserted in the right hand *attII* site in the integron and to compare whether these isolates contained the same SGI1 structure as *S. Paratyphi* BdT⁺ isolates previously described in Chapter 4, the groEL-F/pse-R2 PCR was employed (see Fig 5.1).

Table 5.1 A summary of information on *Salmonella enterica* serovar Paratyphi BdT⁺ isolates used in this study.

Isolate No ^d	Source	Phage type ^c	State	Date of isolation	Age y/sex
Set 1					
SRC229	Human	Aus2	ACT	2000	<1/F
SRC230	Human	Aus2	ACT	2000	1/M
SRC231	Fish tank	Aus2	ACT	2000	-
Set 2					
SRC232 ^a	Human	Aus2	Vic	2000	11/F
SRC233 ^a	Human	Aus2	Vic	2000	11/F
SRC233A ^b	Fish tank	Aus2	Vic	2000	-
Set 3					
SRC145	Human	Aus3	Vic	2003	74/F
SRC142	Fish tank	Aus3	Vic	2003	-
SRC143	Fish tank	Aus3	Vic	2003	-
Set 4					
SRC149	Human	Aus3	Vic	2003	12/M
SRC147	Fish tank	Aus3	Vic	2003	-
SRC148	Fish tank	Aus3	Vic	2003	-
Control					
SRC50	Human	RDNC	Vic	2001	14/M

^a isolates from the same individual.

^b this strain was not available for molecular analysis.

^c RDNC Aus2 and Aus3 are 1 var and 3b var phage typing variants, respectively and are identifiable and reproducible phage typing patterns awaiting formal assignment by the World Health Organisation.

^d All the isolates listed in the table have the resistance profile ApCmSmSpSuTc.

NB. The sets represent groups of isolate/s from an infected individual matched with isolate/s from their aquarium.

To ascertain whether the gene cassettes identified in these isolates was in fact due to the presence of the SGI1 associated In104 integron (Boyd et al., 2001; Meunier et al.,

2002; Mulvey et al., 2004; Levings et al., 2005b; Threlfall et al., 2005), PCR primers for the left (U7-L12/LJ-R1) and right (104-RJ/104-D) junctions of the SGI1 structure with the chromosome and a screen for the left and right junctions (S026FW/aadA2-R2 and DBT1/MDR-B respectively) for SGI1 and In104 were undertaken (see Fig 5.1).

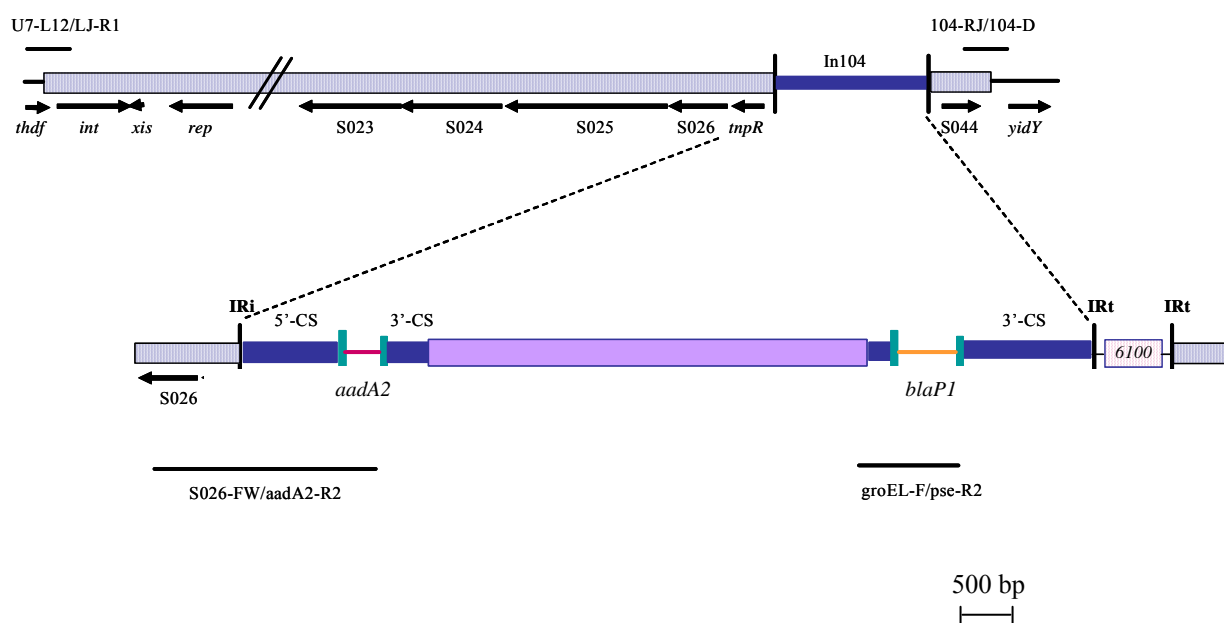


Fig 5.1 Structure of SGI1 region of serovar *S. Typhimurium* DT104 (Genbank Accession no. AF261825) drawn to scale. Different segments are represented by open boxes and lines of differing thicknesses. Arrows indicate the position and orientation of genes and open reading frames (orfs). In104 boundary IR's (inverted repeats) are indicated by vertical bars and are labelled IRi and IRt. SGI1 is flanked by chromosomal genes *thdF* and *yidY*. Primer pairs used in this studied are clearly labelled and fragments amplified are indicated by thin lines. Diagram modified from Levings et al. (2006a).

5.2.3 Pulse field gel electrophoresis (PFGE) of *XbaI* digested *S. Paratyphi* BdT⁺ isolates

Salmonella colonies were grown overnight at 37°C on MacConkey agar plates and resuspended in 100 µl of sterile TE buffer (10 mM Tris-HCl and 0.1 mM EDTA [pH 7.5]). The PFGE agarose plugs were made by mixing 100 µl of pre-warmed 2% w/v certified low melt agarose (BioRad, Hercules, CA, USA) to the bacterial cell suspension. The mixture was immediately poured into PFGE plug moulds and

allowed to set at 4°C for 30 min. After plugs were set they were placed in 1.5 ml Eppendorf tubes to which 400 µl of lysis buffer was added (6 mM Tris-HCl [pH7.6], 1 mM NaCl, 100 mM EDTA [pH 8.5], 0.5 % Brij 35, 0.5 % sodium lauryl sarcosine, 0.2 % deoxycholic acid and 0.5 mg of lysozyme/ml) and the tubes were incubated at 37°C for a minimum of 2 h on a dry cell heating block (Thermoline). After incubation the lysis solution was removed and 400 µl of deproteinisation solution (500 mM EDTA [pH 8.5], 1 % sodium lauryl sarcosine and 1mg of proteinase K/ml) was added and the tubes incubated overnight at 50°C. The deproteinisation solution was removed from the tubes and the plugs were washed three times for 30 min in sterile TE buffer with constant rotation. The plugs were then stored at 4°C in 500 µl of sterile 1 M EDTA (pH 8.5).

Prior to restriction enzyme digestion solution being added to the plugs, 3 X 3.5 mm slices were carefully cut from the plugs and equilibrated for 1 h in 200 µl of 1 X restriction enzyme buffer (supplied by the manufacturer). The plug pieces were then incubated overnight in fresh 1 X restriction enzyme buffer (30 µl) containing 30 U of *Xba*I (Fermentas Inc, Hanover, MD, USA). Restriction enzyme fragments were then separated via a 1% w/v pulse field certified agarose (BioRad) gel in 0.5 X TBE buffer using the Gene NavigatorTM electrophoresis system (Pharmacia LKB). The running buffer was kept at a constant temperature of 10°C throughout the electrophoresis using a water cooler. The total run time was 24 h, with a voltage of 200 V and a linearly ramped pulse time of 4 to 35 s. Low range PFGE markers (New England BioLabs, Beverly, MA, USA) were included as a DNA size standard and are composed of concatamers of bacteriophage lambda DNA. Gels were stained with gentle agitation for 30 min in 0.5 X TBE buffer containing 5 µg/ml of ethidium

bromide and destained for 10 min in 0.5 X TBE buffer only. The gels were visualised under UV illumination using the GelDoc 1000 image analysis system (BioRad).

5.2.4 Southern hybridisation of *Pst*I digested whole cell *S. Paratyphi* DNA probed with DIG labelled IS200

5.2.4.1 Whole cell DNA digestion with *Pst*I and membrane preparation/transfer

Approximately 50-100 ng of whole cell DNA (phenol/chloroform extracted) was digested in a total reaction volume of 50 µl containing 5 µl of 1 X restriction enzyme buffer (supplied by manufacturer), 10 U of *Pst*I restriction enzyme and. *Pst*I digested chromosomal DNA of each of the *S. Paratyphi* isolates were loaded along with DIG labelled molecular mass marker II (Roche Diagnostics) on a 1% w/v agarose gel and electrophoresed at 110 V for 3 h. The agarose gel was then stained and visualised as mentioned previously. The DNA was transferred onto a nitrocellulose membrane as described in Chapter 2.

5.2.4.2 IS200 DIG-labelled DNA probe preparation

IS200 DIG-labelled DNA probe preparation was carried out using conditions described in detail in Chapter 3.

5.2.4.3 Hybridisation and development of southern membrane

Southern hybridisation and membrane development was carried out according to methods described in Chapter 2.

5.3 Results

5.3.1 PCR identification of SGI1 containing strains and the cassettes they contain

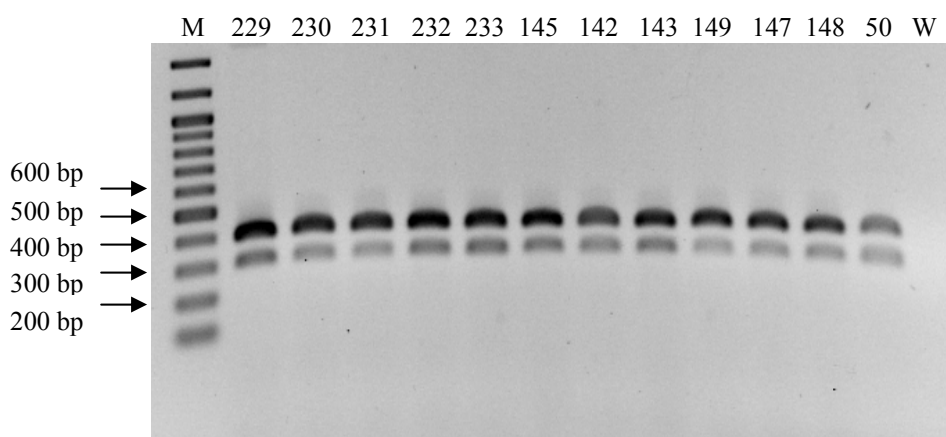


Fig 5.2 L2/L3 multiplex PCR on MR *S. Paratyphi* isolates. The 100 bp Plus DNA ladder is loaded in the well labelled (M) and sizes are indicated. The 389 bp PCR amplicon represents *Salmonella* control product and the 298 bp amplicon represents the L2/L3 integrase product. A negative water control can be seen in the well labelled (W).

The L2/L3 multiplex PCR was performed on the collection of *S. Paratyphi* strains and they were all positive for both the *intI1* integrase and the *Salmonella* specific *invA* gene (see Fig 5.2). The L1/R1 PCR for the strains produced two amplicons (1.0 and 1.2 kb) and identical *RsaI* restriction enzyme profiles consistent with control *S. Paratyphi* strain SRC50 which is known to contain the *aadA2* and *blaP1* as seen in Chapter 4 (see Fig 5.3 and 5.4).

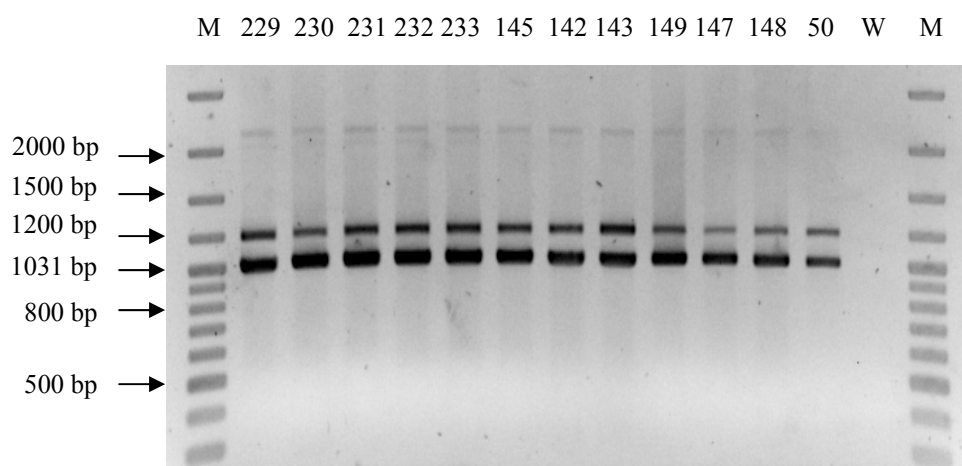


Fig 5.3 L1/R1 gene cassette PCR on the MR *S. Paratyphi* isolates. The 100 bp Plus DNA ladder is loaded in the well labelled (M) and sizes are indicated. The *S. Paratyphi* control strain SRC50 is included.

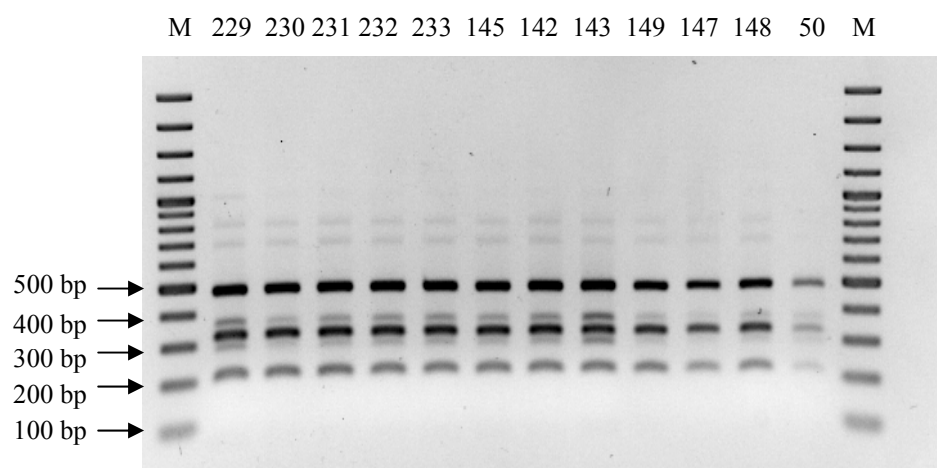


Fig 5.4 *RsaI* digestions of L1/R1 gene cassette PCR amplicons obtained for the MR *S. Paratyphi* isolates. The 100 bp Plus DNA ladder is loaded in the well labelled (M) and sizes are indicated. The *S. Paratyphi* control strain SRC50 is also represented.

All of the strains (excluding SRC233A) were positive for the left and right SG11-chromosomal junctions, each producing the amplicons of the expected sizes for primer sets U7-L12/LJ-R1 and 104-RJ/104-D. The two cassettes were also found to be located in the same positions as in SRC50 using linkage PCR primer pairs S026-FW/aadA2-R2 (expected size 3.7 kb) (see Fig 5.5) and groEL-F/pse-R2 (expected size 1.6kb) (see Fig 5.6), with amplicons of the expected size being obtained for all *S.*

Paratyphi isolates. The *aadA2* cassette in the left and the *blaP1* in the right hand *attI1* site. The DB-T1/MDR-B PCR in conjunction with the S026-FW/*aadA2*-R2 indicated that the integron in all these strains, is inserted in the normal position in the SGI1 backbone between *tnpR* and S044.

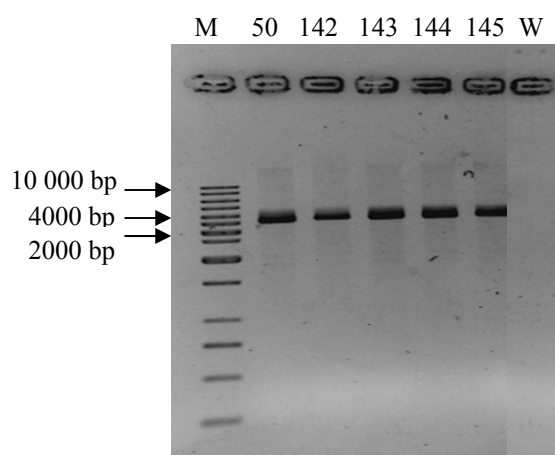


Fig 5.5 The S026-FW/*aadA2*-R2 PCR for a representative number of *S. Paratyphi* isolates. The expected 3.6 kb amplicon, identical in size to control SRC50 is present in all strains. The 1kb DNA molecular marker is loaded in the gel labelled (M) and the negative water control is labelled (W).

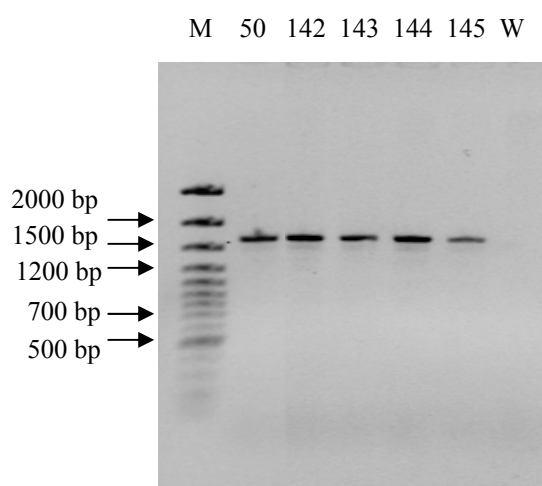


Fig 5.6 The *groEL*-F/*pse*-R2 PCR for a representative number of *S. Paratyphi* isolates. The expected 1.6 kb amplicon, identical in size to control SRC50 is present in all strains. The 100 bp DNA Plus molecular marker is loaded in the gel labelled (M) and the negative water control is labelled (W).

5.3.2 PFGE analysis of *Xba*I digested chromosomal DNA for *S. Paratyphi* BdT⁺ isolates

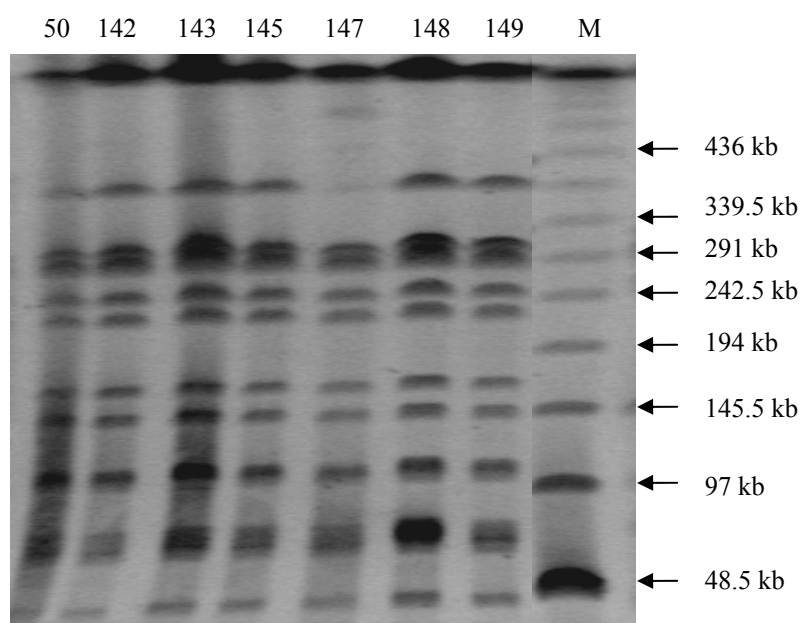


Fig 5.7 Pulsed field gel electrophoresis (PFGE) of *Xba*I digested whole cell DNA for a set of representatives of SG11 containing *Salmonella* enterica serovar Paratyphi B dT⁺ isolates. Molecular mass markers (lane M) are low range PFGE markers and are in kilobases (kb). The high mass molecular band absent for isolate SRC147 in this gel was present in subsequent gels.

From PFGE analysis (as seen for a representative number of isolates in Fig 5.7), it became apparent that all the *S. Paratyphi* BdT⁺ examined in this study produced identical PFGE profiles when digested with *Xba*I. In Fig 5.7, the high mass molecular band absent for SRC147 but appears in all other strains was found to be present in subsequent gels and its absence in this gel was probably as a result of low DNA concentration for this particular plug.

5.3.3 IS200 profiles of *Pst*I digested chromosomal DNA for *S. Paratyphi* BdT⁺ isolates

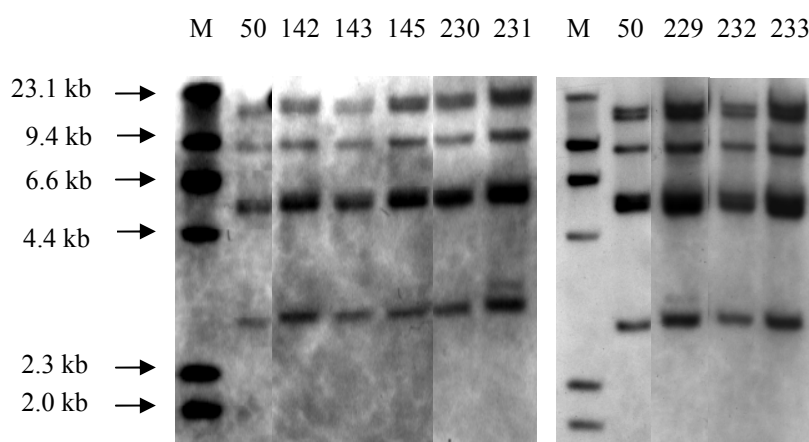


Fig 5.8 Profiles from Southern hybridisation of *Pst*I digested whole cell DNA from *S. Paratyphi* isolates containing SGI1 hybridised with IS200 digoxigenin (DIG) labelled DNA probe. Molecular markers (lane M) are DIG molecular mass marker II.

IS200 profiles were identical for all *S. Paratyphi* BdT⁺ examined in this study, with each strain containing 5 copies of IS200. The highest band seen in Fig 5.8 is in fact two separate bands of very similar molecular weight which is more easily observed in the second panel of the figure.

5.4 Discussion

From this study it was found that the *S. Paratyphi* BdT⁺ isolates (ApCmSmSpSuTc) associated with disease outbreaks in ACT and Victoria, all contained the SGI1 (with *aadA2* and *blaP1* cassettes) previously characterised in Chapter 4. Matched isolates from humans and their respective fish tanks were genetically indistinguishable using PFGE and IS200 profiling. Interestingly, isolates in this study with differing phage types (namely Aus2 and Aus3) along with control strain SRC50 also displayed

identical profiles for each of the typing methods, suggesting that they all represent a clonal cluster and variation in phage type must have occurred within a single clone (Levings et al., 2006a). Variation in phage type among multi-drug resistant *S. Paratyphi* BdT⁺ isolates has been reported in other studies (Mulvey et al., 2004; Threlfall et al., 2005; Weill et al., 2005), however, a number of related but slightly different *Xba*I PFGE profiles were observed between the different phage types. These findings suggest that the multi-drug resistant *S. Paratyphi* BdT⁺ strains (ApCmSmSpSuTc) found worldwide all have a single origin and that variation seen amongst them is probably due to the incorporation of different plasmids and temperate phages over time. Further epidemiological studies including strains from around the world are needed to confirm this hypothesis.

Antimicrobial treatment of fish pathogens in most cases is not administered directly to an infected fish. Worldwide, infected fish are often treated as groups through feed and in many instances the ill fish do not receive the required dosage due to loss of appetite, instead the healthy fish receive unnecessarily higher levels of the antimicrobial agent (Stuart, 1983). Water is an incredibly indiscriminate medium in which antimicrobial agents can be dispersed. Often in the case of aquaculture, not only are the fish within the farm subjected to the antimicrobial agent, the surrounding environment, for example, soil and water supplies that receive run off from the aquaculture ponds are also affected (Akinbowale, et al., 2006). These kinds of practices potentially expose other biological systems, including bacteria and wild fish to these agents (Bondad-Reantaso, et al., 2005). The acquisition of SGI1 by Gram-negative fish pathogens such as *Aeromonas* spp. and *Vibrio* spp. would be financially devastating to the industry. As mentioned earlier the ornamental fish industry is

growing rapidly and has an estimated value of A\$350 million here in Australia (Tilzey, 2005). Unlike other industries, the pathogens and disease risks associated with this particular trade have been minimally and poorly documented (Whittington and Chong, 2007).

Unfortunately, bacteria cultured directly from the fish digestive system were not collected as part of this study. This was due to the fact that sample retrieval would have meant that fish from the suspected tanks would have had to have been euthanised and dissected. Linking the MR *S. Paratyphi* BdT⁺ directly to the fish rather than just the aquatic environment would ultimately provide the scientific evidence required to conclude that ornamental fish are in fact the carriers of this bacterial pathogen and not just their environment. Future studies planned by the laboratory include a detailed screening project, which aims to culture and characterise bacteria directly associated with ornamental fish digestive systems, focussing heavily on fish sourced from overseas sources.

As *Salmonella* is non-pathogenic to ornamental fish and is thought to be excreted in their faeces, its presence within a batch of fish may go unnoticed until the owner of the aquarium becomes ill. What is particularly concerning is the fact that the *Salmonella* Paratyphi B dT⁺ isolated in this study, contain the SGI1 MR region and potential virulence genes associated with it, making medical treatment difficult. This study highlights the need for a surveillance program that monitors not only the fish being imported but the surrounding environments that ornamental fish are transported in.

Understanding the genetic basis of resistant phenotypes is very important in tracking and monitoring the spread of infection and finding the primary source of bacteria of interest. Chromosomal markers such as SGI1 and the genes it contains, IS200 profiling and PFGE together are particularly useful in identifying bacteria involved in outbreaks. By employing these techniques, this study successfully linked cases of disease with a direct source and is the first definitive report showing that ornamental fish tanks are a reservoir for MR *Salmonella* Paratyphi B dT⁺ (Levings et al., 2006a). Molecular tools described in this study would be highly useful in detecting SGI1 from aquacultural sources and would be particularly useful in future surveillance type programs if undertaken.

With increasing numbers of ornamental fish being imported from countries with less stringent regulations on antimicrobial usage and very little monitoring, there appears to be a real threat to our domestic industry. Interestingly, here in Australia, we in fact may be selecting for MR *Salmonella* Paratyphi B dT⁺ containing SGI1. An example can be seen with Tetras, imported from Indonesia which upon arrival, are treated prophylactically here in Australia by adding chloromycetin, tetracycline, metronidazole and sulphadiazine (Musto et al., 2006). Surveillance studies not only aim to protect the Australian ornamental fish industry but also protect fish owners from a potentially dangerous bacterial pathogen. In Australia 12-14% (Lehane and Rawlin, 2000) of households own an aquarium and together with the young age of most affected patients (Levings et al., 2006a; Musto et al., 2006), MR *S. Paratyphi* BdT⁺ is becoming a serious public health issue around the world.

Chapter 6: General Discussion and Future Studies

In Australia, there is limited molecular information on antimicrobial resistance among bacterial pathogens such as *Salmonella* spp. The study on the first collection of diverse MR *Salmonella* spp. from various sources aimed to identify and characterise class 1 integrons and the gene cassettes they contain, in order to gain a better understanding of the evolution of the predominant antibiotic resistance regions, isolated from Australian sources.

Of the 136 strains from the first collection, 81 (59.6%) were positive for the class 1 integrase (*intI1*), 70 of these contained gene cassettes, which upon sequencing representatives were found to confer resistance to trimethoprim and/or aminoglycosides, with only one exception. The most commonly isolated gene cassette among the collection was the *dfrA5* trimethoprim resistance gene cassette. The PCR techniques and subsequent restriction enzyme analysis involved in this study, successfully identified known gene cassette arrays, which included *dfrA12-orfF-aadA2*, *dfrA17-aadA5*, *dfrA1-aadA1* and *aadA4* as well identifying 3 new gene cassettes namely, *aacCA5*, *dfrB6* and *linG* (Levings et al., 2005a; Levings et al., 2006b; Levings et al., 2006c) (See chapter 3).

A variant of a commonly encountered gene cassette array, generated by a possible homologous recombination event, was the Δ orfF-*aadA2* array (see Chapter 3). These types of infrequent events and the acquisition of rare gene cassettes such as the *dfrB6-aadA1* array can be useful markers for the rapid identification of phylogenetically related strains and/or plasmids they may contain. In hospital settings, integrons and

the gene cassettes they contain have proven to be useful epidemiological markers of cross-infection and/or identification of contaminating sources of clinically important strains (Ribot et al., 2002; Severino and Magalhaes, 2004). Many cassette arrays found in integrons contain ORFs whose functions have not yet been determined. By analysing gene cassettes, scientists may also be tapping into an important evolutionary resource that could provide some light on primitive bacterial proteins, previously not identified.

The genetic make-up of the 3'CS is known to vary, with IS elements (such as *IS6100*) playing a major role in deleting and rearranging genes commonly associated with this region (Partridge et al., 2001b; Levings et al., 2007). When looking at the genes present in the 3'CS (namely the *sulI* gene) of the class 1 integrons identified in this study, it became apparent that there was some variability. 21 of the 70 (30%) *intI1*, gene cassette containing isolates within this collection do not appear to contain the *sulI* gene using southern hybridisation techniques. Detailed molecular analysis investigating the reasons for the lack of *sulI* in these strains is currently underway in the laboratory and preliminary data suggests that IS26 has played an important role (J. Liu, unpublished data). Other studies have also noted the involvement of IS26 in 3'CS variability in RSF1010 derived plasmids (Daly et al., 2005).

Identified in the initial PCR screening experiments was the presence of a 1.0 and 1.2 kb gene cassette amplicon for some of the strains within the collection (see Chapter 3). From the literature, the 1.0 and 1.2 kb amplicons were characteristic of the *aadA2* and *blaP1* gene cassettes commonly associated with SGI1 (Briggs and Fratamico, 1999; Boyd et al., 2001). Using this information, PCRs targeting SGI1-chromosomal

boundaries on the entire collection, identified 10 SGI1 containing experimental strains, of which 9 were completely characterised. The existence of two new SGI1 variants namely SGI1-I and SGI1-J were revealed. A third variant from the collection, not discussed in detail here, SGI1-K, has since been characterised for SRC73 and described in the literature (Levings et al., 2007). Using PCRs that target the chromosomal junction of SGI1, in conjunction with gene cassette PCRs have proven to be a more accurate way of identifying SGI1 and variants of it. Previous methodologies looking only for the presence of the characteristic 1.0 and 1.2 kb gene cassette amplicons, will have failed to detect SGI1 variants. This study also highlighted the diverse range of serovars into which SGI1 and variants of it have been incorporated.

Another interesting observation was the insertion of the integron (In104) into a different region within the SGI backbone for SRC19 (*S. Emek*). PCRs designed to link *S026-tnpR* and *IS6100-S044* failed to amplify a product for this strain (Levings et al., 2005b) (see Chapter 4). A series of primers designed along the backbone heading out to the left of *S026* were used in conjunction with a primer in *intI1*. A 1.4 kb amplicon was obtained using SGI19 (F3) in *S023*, suggesting that the In104 in the *Emek* strain had been inserted into a different position. Assuming that In104*Emek* had inserted into *S023*, primers in *IS6100* (*IS6100-F*) and *S024* (*S024-RV*) were then used to link the right hand side of In104 to the backbone for SRC19 for which a 2.5 kb amplicon was obtained. DNA sequencing of these products revealed the presence of a 5 bp duplication CCATG adjacent to both the IR_i and IR_t repeats of In104. This boundary sequence across the IR's provides further evidence that strengthens the hypothesis that In104 is inserted via transposition. Due to the insertion of the integron

into a different location, the SGI variant for this strain has been named SGI2. It is believed that SGI2 evolved independently from SGI1. Further epidemiological studies are being carried out in the laboratory on a number of MR *S. Emek* strains with similar phenotypes from Australia and the United Kingdom to see whether or not these strains are clonal and whether In104 in all these strains is inserted into this alternate position in the SGI backbone.

Based on information in the literature and our findings for SGI1, a second collection of *S. Paratyphi* isolates was examined (see Chapter 5). Data (non-molecular) from overseas suggested a possible association between human illness attributed to *S. Paratyphi* BdT⁺ (containing SGI1) and contact with domestic aquariums housing ornamental fish (Gaulin et al., 2000; Senanayake et al., 2004). This study aimed to genetically link using PFGE and IS200 profiling, MR *S. Paratyphi* BdT⁺ strains from infected individuals with strains collected from their home aquariums. All strains contained the original SGI1 described for *Salmonella* Typhimurium DT104 which harbours the *aadA2* and *blaP1* gene cassettes (Briggs and Fratamico, 1999; Boyd et al., 2001). Strains from infected individuals and their respective fish tanks were indistinguishable using IS200 profiling and PFGE of *Xba*I digested DNA. From this it can be concluded that ornamental fish tanks are a reservoir for SGI1 containing MR *S. Paratyphi* BdT⁺ (Levings et al., 2006a).

12.2 million fish are kept as pets here in Australia (Magnosi, 2003). With statistics like these and the rapid rise of imported ornamental fish into Australia from countries with less stringent restrictions on antibiotic usage, future work within the laboratory is planned to analyse bacterial samples not only from imported ornamental fish aquatic

environments but also samples taken directly from fish digestive systems, in order to monitor SGI1 containing MR *S. Paratyphi* BdT⁺ coming into the country. These types of surveillance programs not only aim to protect the health of Australian citizens but also attempt to protect the ornamental fish industry here in Australia. If a fish pathogen such as *Aeromonas* spp. was to acquire a resistance region such as SGI1, the costs to the industry could be devastating. Tetracycline is a major antimicrobial used in the treatment of fish pathogens, and resistance to this antibiotic is conferred by the *tetA*(G) located in the island.

The reason why antibiotic resistance genes are often clustered within a single region made up of transposable elements and integrons is not fully understood. However, it may simply reflect the fact that there are few places to reside without causing problems to vital functions of the bacterial chromosome or plasmid (Partridge and Hall, 2004). Insertion of any additional antibiotic resistance genes into a transposon already present would not affect vital functions and with the selective pressure of antibiotic usage, these large multi-resistance regions (MRR's) will be retained due to the survival advantage held by the organism. Of particular concern is the acquisition of virulence genes in these large multi-resistance regions (Fluit, 2005).

Given that bacteria are constantly exchanging genetic information and the strains in this study were isolated from Australian residents and farm animals, there is a real potential for any of these multi-drug resistant *Salmonella* to cause problems in the future. The ability to track and monitor the antibiotic resistance genes present in the genetic environments of humans and food-producing animals may aid scientists and minimise the effects of future outbreaks of multi-drug resistant (MR) bacteria.

As outlined in the JETACAR report in 1999 and with increasing global trade and travel, in order to gain some control over increasing antimicrobial resistance a worldwide responsibility to minimise and manage the use of antimicrobials is needed. The ability to rapidly identify strains based on the molecular make-up of antibiotic resistance regions will help in designing appropriate antibiotic treatment regimes that specifically target the bacteria causing the problem, rather than randomly administering an array of antibiotics, which the bacteria may have acquired resistance to. Technologies such as PFGE, IS200 profiling, PCR and DNA micro-chip arrays may prove useful tools in combating the worldwide problem of antimicrobial resistance, once scientists have a better idea of the multi-drug resistance regions circulating within bacterial populations.

References

- Aarestrup, F. M., F. Bager, N. E. Jensen, M. Madsen, A. Meyling, and H. C. Wegener. (1998) Surveillance of antimicrobial resistance in bacteria isolated from food animals to antimicrobial growth promoters and related therapeutic agents in Denmark. *Apmis* **106**:606-22.
- Ahmed, A. M., T. Nakagawa, E. Arakawa, T. Ramamurthy, S. Shinoda, and T. Shimamoto. (2004) New aminoglycoside acetyltransferase gene, *aac(3)-Id*, in a class 1 integron from a multiresistant strain of *Vibrio fluvialis* isolated from an infant aged 6 months. *J Antimicrob Chemother* **53**:947-51.
- Ahmed, A. M., A. I. Hussein, and T. Shimamoto. (2007) *Proteus mirabilis* clinical isolate harbouring a new variant of *Salmonella* genomic island 1 containing the multiple antibiotic resistance region. *J Antimicrob Chemother* **59**:184-90.
- Akinbowale, O. L., H. Peng, and M. D. Barton. (2006) Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. *J Appl Microbiol* **100**:1103-13.
- Alonso, H., and J. E. Gready. (2006) Integron-sequestered dihydrofolate reductase: a recently redeployed enzyme. *Trends Microbiol* **14**:236-242.
- Amabile-Cuevas, C. F., and M. E. Chicurel. (1992) Bacterial plasmids and gene flux. *Cell* **70**:189-99.
- Amavisit, P., P. F. Markham, D. Lightfoot, K. G. Whithear, and G. F. Browning. (2001) Molecular epidemiology of *Salmonella* Heidelberg in an equine hospital. *Vet Microbiol* **80**:85-98.
- Arakawa, Y., M. Murakami, K. Suzuki, H. Ito, R. Wacharotayankun, S. Ohsuka, N. Kato, and M. Ohta. (1995) A novel integron-like element carrying the metallo-beta-lactamase gene *blaIMP*. *Antimicrob Agents Chemother* **39**:1612-5.
- Ashraf, M. M., Z. U. Ahmed, and D. A. Sack. (1991) Unusual association of a plasmid with nalidixic acid resistance in an epidemic strain of *Shigella dysenteriae* type 1 from Asia. *Can J Microbiol* **37**:59-63.
- Baquar, N., E. J. Threlfall, B. Rowe, and J. Stanley. (1994) Phage type 193 of *Salmonella* typhimurium contains different chromosomal genotypes and multiple IS200 profiles. *FEMS Microbiol Lett* **115**:291-5.
- Baggesen, D. L., D. Sandvang, and F. M. Aarestrup. (2000) Characterization of *Salmonella enterica* serovar typhimurium DT104 isolated from Denmark and comparison with isolates from Europe and the United States. *J Clin Microbiol* **38**:1581-6.

- Barlow, R. S., J. M. Pemberton, P. M. Desmarchelier, and K. S. Gobius. (2004) Isolation and characterization of integron-containing bacteria without antibiotic selection. *Antimicrob Agents Chemother* **48**:838-42.
- Barza, M., and K. Travers. (2002) Excess infections due to antimicrobial resistance: the "Attributable Fraction". *Clin Infect Dis* **34 Suppl 3**:S126-30.
- Bettelheim, K. A., M. A. Hornitzky, S. P. Djordjevic, and A. Kuzevski. (2003) Antibiotic resistance among verocytotoxigenic *Escherichia coli* (VTEC) and non-VTEC isolated from domestic animals and humans. *J Med Microbiol* **52**:155-62.
- Bisercic, M., and H. Ochman. (1993) Natural populations of *Escherichia coli* and *Salmonella typhimurium* harbor the same classes of insertion sequences. *Genetics* **133**:449-54.
- Bissonnette, L., S. Champetier, J. P. Buisson, and P. H. Roy. (1991) Characterization of the nonenzymatic chloramphenicol resistance (*cmlA*) gene of the In4 integron of Tn1696: similarity of the product to transmembrane transport proteins. *J Bacteriol* **173**:4493-502.
- Bito, A., and M. Susani. (1994) Revised analysis of *aadA2* gene of plasmid pSa. *Antimicrob Agents Chemother* **38**:1172-5.
- Bondad-Reantaso, M. G., R. P. Subasinghe, J. R. Arthur, K. Ogawa, S. Chinabut, R. Adlard, Z. Tan, and M. Shariff. (2005) Disease and health management in Asian aquaculture. *Vet Parasitol* **132**:249-72.
- Boyd, D., A. Cloeckaert, E. Chaslus-Dancla, and M. R. Mulvey. (2002) Characterization of variant *Salmonella* genomic island 1 multidrug resistance regions from serovars Typhimurium DT104 and Agona. *Antimicrob Agents Chemother* **46**:1714-22.
- Boyd, D., G. A. Peters, A. Cloeckaert, K. S. Boumedine, E. Chaslus-Dancla, H. Imberechts, and M. R. Mulvey. (2001) Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *J Bacteriol* **183**:5725-32.
- Boyd, D. A., G. A. Peters, L. Ng, and M. R. Mulvey. (2000) Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica* Typhimurium DT104. *FEMS Microbiol Lett* **189**:285-91.
- Boyd, D. A., X. Shi, Q. H. Hu, L. K. Ng, B. Doublet, A. Cloeckaert, and M. R. Mulvey. (2008) *Salmonella* Genomic Island 1 (SGI1), Variant SGI1-I, and New Variant SGI1-O in *Proteus mirabilis* Clinical and Food Isolates from China. *Antimicrob Agents Chemother* **52**:340-4.

- Bozdogan, B., L. Berrezouga, M. S. Kuo, D. A. Yurek, K. A. Farley, B. J. Stockman, and R. Leclercq. (1999) A new resistance gene, *linB*, conferring resistance to lincosamides by nucleotidylation in *Enterococcus faecium* HM1025. *Antimicrob Agents Chemother* **43**:925-9.
- Briggs, C. E., and P. M. Fratamico. (1999) Molecular characterization of an antibiotic resistance gene cluster of *Salmonella* typhimurium DT104. *Antimicrob Agents Chemother* **43**:846-9.
- Brisson-Noel, A., P. Delrieu, D. Samain, and P. Courvalin. (1988) Inactivation of lincosaminide antibiotics in *Staphylococcus*. Identification of lincosaminide O-nucleotidyltransferases and comparison of the corresponding resistance genes. *J Biol Chem* **263**:15880-7.
- Brisson-Noel, A., and P. Courvalin. (1986) Nucleotide sequence of gene *linA* encoding resistance to lincosamides in *Staphylococcus haemolyticus*. *Gene* **43**:247-53.
- Brown, H. J., H. W. Stokes, and R. M. Hall. (1996) The integrons In0, In2, and In5 are defective transposon derivatives. *J Bacteriol* **178**:4429-37.
- Burka, J. F., K. L. Hammell, T. E. Horsberg, G. R. Johnson, D. J. Rainnie, and D. J. Speare. (1997) Drugs in salmonid aquaculture--a review. *J Vet Pharmacol Ther* **20**:333-49.
- Burnens, A. P., J. Stanley, R. Sack, P. Hunziker, I. Brodard, and J. Nicolet. (1997) The flagellin N-methylase gene *fliB* and an adjacent serovar-specific IS200 element in *Salmonella* typhimurium. *Microbiology* **143** (Pt 5):1539-47.
- Carattoli, A., E. Filetici, L. Villa, A. M. Dionisi, A. Ricci, and I. Luzzi. (2002) Antibiotic resistance genes and *Salmonella* genomic island 1 in *Salmonella enterica* serovar Typhimurium isolated in Italy. *Antimicrob Agents Chemother* **46**:2821-8.
- Carlson, S. A., V. K. Sharma, Z. P. McCuddin, M. A. Rasmussen, and S. K. Franklin. (2007) Involvement of a *Salmonella* Genomic Island 1 Gene in the Rumen Protozoan-Mediated Enhancement of Invasion for Multiple-Antibiotic-Resistant *Salmonella enterica* Serovar Typhimurium. *Infect. Immun.* **75**:792-800.
- Chan, K., S. Baker, C. C. Kim, C. S. Detweiler, G. Dougan, and S. Falkow. (2003) Genomic comparison of *Salmonella enterica* serovars and *Salmonella bongori* by use of an *S. enterica* serovar typhimurium DNA microarray. *J Bacteriol* **185**:553-63.
- Chang, C. Y., L. L. Chang, Y. H. Chang, T. M. Lee, and S. F. Chang. (2000) Characterisation of drug resistance gene cassettes associated with class 1 integrons in clinical isolates of *Escherichia coli* from Taiwan, ROC. *J Med Microbiol* **49**:1097-102.

- Chart, H. (2003) The pathogenicity of strains of *Salmonella* paratyphi B and *Salmonella* java. J Appl Microbiol **94**:340-8.
- Chiew, Y. F., and L. M. Hall. (1998) Comparison of three methods for the molecular typing of Singapore isolates of enterococci with high-level aminoglycoside resistances. J Hosp Infect **38**:223-30.
- Chiou, C. S., and A. L. Jones. (1993) Nucleotide sequence analysis of a transposon (Tn5393) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram-negative bacteria. J Bacteriol **175**:732-40.
- Chopra, I., and M. Roberts. (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev **65**:232-60 ; second page, table of contents.
- Christensen, H., S. Nordentoft, and J. E. Olsen. (1998) Phylogenetic relationships of *Salmonella* based on rRNA sequences. Int J Syst Bacteriol **48 Pt 2**:605-10.
- Cloekaert, A., K. Praud, B. Doublet, M. Demartin, and F. X. Weill. (2006) Variant *Salmonella* genomic island 1-L antibiotic resistance gene cluster in *Salmonella enterica* serovar Newport. Antimicrob Agents Chemother **50**:3944-6.
- Collis, C. M., G. Grammaticopoulos, J. Briton, H. W. Stokes, and R. M. Hall. (1993) Site-specific insertion of gene cassettes into integrons. Mol Microbiol **9**:41-52.
- Collis, C. M., and R. M. Hall. (1992) Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. J Bacteriol **174**:1574-85.
- Collis, C. M., and R. M. Hall. (1995) Expression of antibiotic resistance genes in the integrated cassettes of integrons. Antimicrob Agents Chemother **39**:155-62.
- Crump, J. A., P. M. Griffin, and F. J. Angulo. (2002) Bacterial contamination of animal feed and its relationship to human foodborne illness. Clin Infect Dis **35**:859-65.
- Dalsgaard, A., A. Forslund, O. Serichantalergs, and D. Sandvang. (2000) Distribution and content of class 1 integrons in different *Vibrio cholerae* O-serotype strains isolated in Thailand. Antimicrob Agents Chemother **44**:1315-21.
- Dalton, C. B., J. Gregory, M. D. Kirk, R. J. Stafford, R. Givney, E. Kraa, and D. Gould. (2004) Foodborne disease outbreaks in Australia, 1995 to 2000. Commun Dis Intell **28**:211-24.
- Daly, M., L. Villa, C. Pezzella, S. Fanning, and A. Carattoli. (2005) Comparison of multidrug resistance gene regions between two geographically unrelated *Salmonella* serotypes. J Antimicrob Chemother **55**:558-61.

- Davies, J. E. (1997) Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Found Symp* **207**:15-27; discussion 27-35.
- Davis, M. A., D. D. Hancock, and T. E. Besser. (2002) Multiresistant clones of *Salmonella enterica*: The importance of dissemination. *J Lab Clin Med* **140**:135-41.
- Dhawan, V. K., and H. Thadepalli. (1982) Clindamycin: a review of fifteen years of experience. *Rev Infect Dis* **4**:1133-53.
- Doublet, B., D. Boyd, M. R. Mulvey, and A. Cloeckaert. (2005) The *Salmonella* genomic island 1 is an integrative mobilizable element. *Mol Microbiol* **55**:1911-24.
- Doublet, B., P. Butaye, H. Imberechts, D. Boyd, M. R. Mulvey, E. Chaslus-Dancla, and A. Cloeckaert. (2004a). *Salmonella* genomic island 1 multidrug resistance gene clusters in *Salmonella enterica* serovar Agona isolated in Belgium in 1992 to 2002. *Antimicrob Agents Chemother* **48**:2510-7.
- Doublet, B., G. R. Golding, M. R. Mulvey, and A. Cloeckaert. (2007) Potential integration sites of the *Salmonella* genomic island 1 in *Proteus mirabilis* and other bacteria. *J Antimicrob Chemother* **59**:801-3.
- Doublet, B., R. Lailier, D. Meunier, A. Brisabois, D. Boyd, M. R. Mulvey, E. Chaslus-Dancla, and A. Cloeckaert. (2003) Variant *Salmonella* genomic island 1 antibiotic resistance gene cluster in *Salmonella enterica* serovar Albany. *Emerg Infect Dis* **9**:585-91.
- Doublet, B., F. X. Weill, L. Fabre, E. Chaslus-Dancla, and A. Cloeckaert. (2004b) Variant *Salmonella* genomic island 1 antibiotic resistance gene cluster containing a novel 3'-N-aminoglycoside acetyltransferase gene cassette, *aac(3)-Id*, in *Salmonella enterica* serovar Newport. *Antimicrob Agents Chemother* **48**:3806-12.
- Ebner, P., K. Garner, and A. Mathew. (2004) Class 1 integrons in various *Salmonella enterica* serovars isolated from animals and identification of genomic island SGI1 in *Salmonella enterica* var. Meleagridis. *J Antimicrob Chemother* **53**:1004-9.
- Enne, V. I., D. M. Livermore, P. Stephens, and L. M. Hall. (2001) Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. *Lancet* **357**:1325-8.
- Evans, S., and R. Davies. (1996) Case control study of multiple-resistant *Salmonella* typhimurium DT104 infection of cattle in Great Britain. *Vet Rec* **139**:557-8.
- Ferretti, R., I. Mannazzu, L. Cocolin, G. Comi, and F. Clementi. (2001) Twelve-hour PCR-based method for detection of *Salmonella* spp. in food. *Appl Environ Microbiol* **67**:977-8.

- Fey, P. D., T. J. Safranek, M. E. Rupp, E. F. Dunne, E. Ribot, P. C. Iwen, P. A. Bradford, F. J. Angulo, and S. H. Hinrichs. (2000) Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. *N Engl J Med* **342**:1242-9.
- Fisher, I., Y. Andersson, B. de Jong, K. O'Grady, and J. Powling. 9 August 2001. *Eurosurveillance Wkly.* 5. (www.eurosurveillance.org/ew/2001/010809.asp).
- Fling, M. E., J. Kopf, and C. Richards. (1985) Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3''(9)-O-nucleotidyltransferase. *Nucleic Acids Res* **13**:7095-106.
- Fluit, A. C. (2005) Towards more virulent and antibiotic-resistant *Salmonella*? *FEMS Immunol Med Microbiol* **43**:1-11.
- Fluit, A. C., M. R. Visser, and F. J. Schmitz. (2001) Molecular detection of antimicrobial resistance. *Clin Microbiol Rev* **14**:836-71, table of contents.
- Francia, M. V., F. de la Cruz, and J. M. Garcia Lobo. (1993) Secondary-sites for integration mediated by the Tn21 integrase. *Mol Microbiol* **10**:823-8.
- Gallego, L., and K. J. Towner. (2001) Carriage of class 1 integrons and antibiotic resistance in clinical isolates of *Acinetobacter baumannii* from northern Spain. *J Med Microbiol* **50**:71-7.
- Gassama, A., A. Aidara-Kane, D. Chainier, F. Denis, and M. C. Ploy. 2004. Integron-associated antibiotic resistance in enteroaggregative and enteroinvasive *Escherichia coli*. *Microb Drug Resist* **10**:27-30.
- Gaulin, C., C. Vincent, L. Alain, and J. Ismail. (2002) Outbreak of *Salmonella* Paratyphi B linked to aquariums in the province of Quebec, 2000. *Can Commun Dis Rep* **28**:89-93, 96.
- Gestal, A. M., H. W. Stokes, S. R. Partridge, and R. M. Hall. (2005) Recombination between the *dfrA12*-orfF-*aadA2* cassette array and an *aadA1* gene cassette creates a hybrid cassette, *aadA8b*. *Antimicrob Agents Chemother* **49**:4771-4.
- Gibb, A. P., C. Tribuddharat, R. A. Moore, T. J. Louie, W. Krulicki, D. M. Livermore, M. F. Palepou, and N. Woodford. 2002. Nosocomial outbreak of carbapenem-resistant *Pseudomonas aeruginosa* with a new bla(IMP) allele, bla(IMP-7). *Antimicrob Agents Chemother* **46**:255-8.
- Gibreel, A., and O. Skold. (1998) High-level resistance to trimethoprim in clinical isolates of *Campylobacter jejuni* by acquisition of foreign genes (*dfr1* and *dfr9*) expressing drug-insensitive dihydrofolate reductases. *Antimicrob Agents Chemother* **42**:3059-64.

- Golding, G. R., A. B. Olson, B. Doublet, A. Cloeckert, S. Christianson, M. R. Graham, and M. R. Mulvey. (2007) The effect of the *Salmonella* genomic island 1 on in vitro global gene expression in *Salmonella enterica* serovar Typhimurium LT2. *Microbes Infect* **9**:21-7.
- Goldstein, C., M. D. Lee, S. Sanchez, C. Hudson, B. Phillips, B. Register, M. Grady, C. Liebert, A. O. Summers, D. G. White, and J. J. Maurer. (2001) Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. *Antimicrob Agents Chemother* **45**:723-6.
- Grape, M., L. Sundstrom, and G. Kronvall. (2003a) New *dfr2* gene as a single-gene cassette in a class 1 integron from a trimethoprim-resistant *Escherichia coli* isolate. *Microb Drug Resist* **9**:317-22.
- Grape, M., L. Sundstrom, and G. Kronvall. (2003b) Sulphonamide resistance gene *sul3* found in *Escherichia coli* isolates from human sources. *J Antimicrob Chemother* **52**:1022-4.
- Grinsted, J., F. de la Cruz, and R. Schmitt. (1990) The Tn21 subgroup of bacterial transposable elements. *Plasmid* **24**:163-89.
- Guardabassi, L., and P. Courvalin. 2006. Modes of Antimicrobial Action and Mechanisms of Bacterial Resistance, p. 1-18. *In* F. M. Aarestrup (ed.), *Antimicrobial Resistance in Bacteria of Animal and Human origin*. ASM Press.
- Guerra, B., E. Junker, and R. Helmuth. (2004) Incidence of the recently described sulfonamide resistance gene *sul3* among German *Salmonella enterica* strains isolated from livestock and food. *Antimicrob Agents Chemother* **48**:2712-5.
- Guerra, B., S. Soto, S. Cal, and M. C. Mendoza. (2000) Antimicrobial resistance and spread of class 1 integrons among *Salmonella* serotypes. *Antimicrob Agents Chemother* **44**:2166-9.
- Hacker, J., and J. B. Kaper. (2000) Pathogenicity islands and the evolution of microbes. *Annu Rev Microbiol* **54**:641-79.
- Hall, R. M., D. E. Brookes, and H. W. Stokes. (1991) Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. *Mol Microbiol* **5**:1941-59.
- Hall, R. M., H. J. Brown, D. E. Brookes, and H. W. Stokes. (1994) Integrons found in different locations have identical 5' ends but variable 3' ends. *J Bacteriol* **176**:6286-94.
- Hall, R. M., and C. M. Collis. (1995) Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol Microbiol* **15**:593-600.

- Hall, R. M., and C. M. Collis. (1998) Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. *Drug Resist Updat* **1**:109-19.
- Hall, R. M., and C. Vockler. (1987) The region of the IncN plasmid R46 coding for resistance to beta-lactam antibiotics, streptomycin/spectinomycin and sulphonamides is closely related to antibiotic resistance segments found in IncW plasmids and in Tn21-like transposons. *Nucleic Acids Res* **15**:7491-501.
- Hall, R. M., G. D. Recchia, C. M. Collis, H. J. Brown, and H. W. Stokes. (1996) Gene Cassettes and Integrons: moving antibiotic resistance genes in Gram-negative bacteria, p. 19-33. *In* C. F. Amabile-Cuevas (ed.), *Antibiotic Resistance: from molecular basics to therapeutic options*.
- Hampton, M. D., L. R. Ward, B. Rowe, and E. J. Threlfall. (1998) Molecular fingerprinting of multidrug-resistant *Salmonella enterica* serotype Typhi. *Emerg Infect Dis* **4**:317-20.
- Hansson, K., O. Skold, and L. Sundstrom. (1997) Non-palindromic *attI* sites of integrons are capable of site-specific recombination with one another and with secondary targets. *Mol Microbiol* **26**:441-53.
- Hansson, K., L. Sundstrom, A. Pelletier, and P. H. Roy. (2002) *IntI2* integron integrase in Tn7. *J Bacteriol* **184**:1712-21.
- Heir, E., B. A. Lindstedt, T. M. Leegaard, E. Gjernes, and G. Kapperud. (2004) Prevalence and characterization of integrons in blood culture *Enterobacteriaceae* and gastrointestinal *Escherichia coli* in Norway and reporting of a novel class 1 integron-located lincosamide resistance gene. *Ann Clin Microbiol Antimicrob* **3**:12.
- Hochhut, B., Y. Lotfi, D. Mazel, S. M. Faruque, R. Woodgate, and M. K. Waldor. (2001) Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. *Antimicrob Agents Chemother* **45**:2991-3000.
- Howell, E. E. (2005) Searching sequence space: two different approaches to dihydrofolate reductase catalysis. *Chembiochem* **6**:590-600.
- Hochhut, B., and M. K. Waldor. (1999) Site-specific integration of the conjugal *Vibrio cholerae* SXT element into *prfC*. *Mol Microbiol* **32**:99-110.
- Huovinen, P., L. Sundstrom, G. Swedberg, and O. Skold. (1995) Trimethoprim and sulfonamide resistance. *Antimicrob Agents Chemother* **39**:279-89.
- JETACAR. (1999) The Use of antibiotics in food-producing animals; antibiotic resistant bacteria in animals and humans. A Report by The Joint Expert Advisory

Committee on Antibiotic Resistance.
<http://www.health.gov.au/internet/wcms/Publishing.nsf/Content/health-pubs-jetacar-cnt.htm>

- Jones, B. D. (2005) *Salmonella* invasion gene regulation: a story of environmental awareness. *J Microbiol* **43 Spec No**:110-7.
- Jones, M. E., E. Peters, A. M. Weersink, A. Fluit, and J. Verhoef. (1997) Widespread occurrence of integrons causing multiple antibiotic resistance in bacteria. *Lancet* **349**:1742-3.
- Kadlec, K., C. Kehrenberg, and S. Schwarz. (2005) Molecular basis of resistance to trimethoprim, chloramphenicol and sulphonamides in *Bordetella bronchiseptica*. *J Antimicrob Chemother* **56**:485-90.
- Kamali-Moghaddam, M., and L. Sundstrom. (2000) Transposon targeting determined by resolvase. *FEMS Microbiol Lett* **186**:55-9.
- Kamali-Moghaddam, M., and L. Sundstrom. (2001) Arrayed transposase-binding sequences on the ends of transposon Tn5090/Tn402. *Nucleic Acids Res* **29**:1005-11.
- Kholodii, G. Y., S. Z. Mindlin, I. A. Bass, O. V. Yurieva, S. V. Minakhina, and V. G. Nikiforov. (1995) Four genes, two ends, and a res region are involved in transposition of Tn5053: a paradigm for a novel family of transposons carrying either a mer operon or an integron. *Mol Microbiol* **17**:1189-200.
- L'Abée-Lund, T. M., and H. Sorum. (2001) Class 1 integrons mediate antibiotic resistance in the fish pathogen *Aeromonas salmonicida* worldwide. *Microb Drug Resist* **7**:263-72.
- Lawson, A. J., M. U. Dassama, L. R. Ward, and E. J. Threlfall. (2002) Multiply resistant (MR) *Salmonella enterica* serotype Typhimurium DT 12 and DT 120: a case of MR DT 104 in disguise? *Emerg Infect Dis* **8**:434-6.
- Lawson, A. J., M. Desai, S. J. O'Brien, R. H. Davies, L. R. Ward, and E. J. Threlfall. (2004) Molecular characterisation of an outbreak strain of multiresistant *Salmonella enterica* serovar Typhimurium DT104 in the UK. *Clin Microbiol Infect* **10**:143-7.
- Leclercq, R. (2002) Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clin Infect Dis* **34**:482-92.
- Lehane, L., and G. T. Rawlin. (2000) Topically acquired bacterial zoonoses from fish: a review. *Med J Aust* **173**:256-9.

- Le Minor, L., M. Veron, and M. Popoff. (1982) A proposal for *Salmonella* nomenclature. *Ann Microbiol (Paris)* **133**:245-54.
- Leverstein-Van Hall, M. A., A. Paauw, A. T. Box, H. E. Blok, J. Verhoef, and A. C. Fluit. (2002) Presence of integron-associated resistance in the community is widespread and contributes to multidrug resistance in the hospital. *J Clin Microbiol* **40**:3038-40.
- Levesque, C., S. Brassard, J. Lapointe, and P. H. Roy. (1994) Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integrons. *Gene* **142**:49-54.
- Levesque, C., L. Piche, C. Larose, and P. H. Roy. (1995) PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob Agents Chemother* **39**:185-91.
- Levesque, C., and P. H. Roy. (1993) PCR analysis of integrons, p. 590-594, D. H. Persing, T. F. Smith, F. C. Tenover and T. J. White (ed.), *Diagnostic Molecular Microbiology: principles and applications*. Mayo Foundation, Rochester.
- Levings, R. S., R. M. Hall, D. Lightfoot, and S. P. Djordjevic. (2006c) *linG*, a new integron-associated gene cassette encoding a lincosamide nucleotidyltransferase. *Antimicrob Agents Chemother* **50**:3514-5.
- Levings, R. S., D. Lightfoot, R. M. Hall, and S. P. Djordjevic. (2006a) Aquariums as reservoirs for multidrug-resistant *Salmonella* Paratyphi B. *Emerg Infect Dis* **12**:507-10.
- Levings, R. S., D. Lightfoot, S. R. Partridge, R. M. Hall, and S. P. Djordjevic. (2005b) The genomic island SGI1, containing the multiple antibiotic resistance region of *Salmonella enterica* serovar Typhimurium DT104 or variants of it, is widely distributed in other *S. enterica* serovars. *J Bacteriol* **187**:4401-9.
- Levings, R. S., D. Lightfoot, L. D. Elbourne, S. P. Djordjevic, and R. M. Hall. (2006b) New integron-associated gene cassette encoding a trimethoprim-resistant DfrB-type dihydrofolate reductase. *Antimicrob Agents Chemother* **50**:2863-5.
- Levings, R. S., S. R. Partridge, S. P. Djordjevic, and R. M. Hall. (2007) SGI1-K, a variant of the SGI1 genomic island carrying a mercury resistance region, in *Salmonella enterica* serovar Kentucky. *Antimicrob Agents Chemother* **51**:317-23.
- Levings, R. S., S. R. Partridge, D. Lightfoot, R. M. Hall, and S. P. Djordjevic. (2005a) New integron-associated gene cassette encoding a 3-N-aminoglycoside acetyltransferase. *Antimicrob Agents Chemother* **49**:1238-41.
- Liebert, C. A., R. M. Hall, and A. O. Summers. (1999) Transposon Tn21, flagship of the floating genome. *Microbiol Mol Biol Rev* **63**:507-22.

- Lillehaug, A., B. T. Lunestad, and K. Grave. (2003) Epidemiology of bacterial diseases in Norwegian aquaculture--a description based on antibiotic prescription data for the ten-year period 1991 to 2000. *Dis Aquat Organ* **53**:115-25.
- Linton, A. H. (1977) Antibiotic resistance: the present situation reviewed. *Vet Rec* **100**:354-60.
- Lolans, K., A. M. Queenan, K. Bush, A. Sahud, and J. P. Quinn. (2005) First nosocomial outbreak of *Pseudomonas aeruginosa* producing an integron-borne metallo-beta-lactamase (VIM-2) in the United States. *Antimicrob Agents Chemother* **49**:3538-40.
- Love, G., D. Lagenkamp, D. Galeano (2004). Australian aquaculture statistics-information sources for status and trends reporting. ABARE Report; 04.1vi + 27 p.
- Magnosi, S. (2003) Contribution of the pet care industry to the Australian economy. Report to the Australian Companion Animal Council Inc. Sydney, Australia.
- Maguire, A. J., D. F. Brown, J. J. Gray, and U. Desselberger. (2001) Rapid screening technique for class 1 integrons in *Enterobacteriaceae* and nonfermenting gram-negative bacteria and its use in molecular epidemiology. *Antimicrob Agents Chemother* **45**:1022-9.
- Martin, C., J. Timm, J. Rauzier, R. Gomez-Lus, J. Davies, and B. Gicquel. (1990) Transposition of an antibiotic resistance element in mycobacteria. *Nature* **345**:739-43.
- Martinez-Freijo, P., A. C. Fluit, F. J. Schmitz, J. Verhoef, and M. E. Jones. (1999) Many class I integrons comprise distinct stable structures occurring in different species of *Enterobacteriaceae* isolated from widespread geographic regions in Europe. *Antimicrob Agents Chemother* **43**:686-9.
- Mastroeni, P. (2002) Immunity to systemic *Salmonella* infections. *Curr Mol Med* **2**:393-406.
- Mazel, D., B. Dychinco, V. A. Webb, and J. Davies. (1998) A distinctive class of integron in the *Vibrio cholerae* genome. *Science* **280**:605-8.
- McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson. (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **413**:852-6.

- McManus, P. S., V. O. Stockwell, G. W. Sundin, and A. L. Jones. (2002) Antibiotic use in plant agriculture. *Annu Rev Phytopathol* **40**:443-65.
- Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. (1999) Food-related illness and death in the United States. *Emerg Infect Dis* **5**:607-25.
- Mercier, J., J. Lachapelle, F. Couture, M. Lafond, G. Vezina, M. Boissinot, and R. C. Levesque. (1990) Structural and functional characterization of *tnpI*, a recombinase locus in Tn21 and related beta-lactamase transposons. *J Bacteriol* **172**:3745-57.
- Meunier, D., D. Boyd, M. R. Mulvey, S. Baucheron, C. Mammina, A. Nastasi, E. Chaslus-Dancla, and A. Cloeckaert. (2002) *Salmonella enterica* serotype Typhimurium DT 104 antibiotic resistance genomic island I in serotype Paratyphi B. *Emerg Infect Dis* **8**:430-3.
- Miko, A., B. Guerra, A. Schroeter, C. Dorn, and R. Helmuth. (2002) Molecular characterization of multiresistant d-tartrate-positive *Salmonella enterica* serovar Paratyphi B isolates. *J Clin Microbiol* **40**:3184-91.
- Millemann, Y., M. C. Lesage, E. Chaslus-Dancla, and J. P. Lafont. (1995) Value of plasmid profiling, ribotyping, and detection of IS200 for tracing avian isolates of *Salmonella typhimurium* and *S. enteritidis*. *J Clin Microbiol* **33**:173-9.
- Miller, M., P. Roche, K. Yohannes, J. Spencer, M. Bartlett, J. Brotherton, J. Hutchinson, M. Kirk, A. McDonald, and C. Vadjic. (2005) Australia's notifiable diseases status, 2003 annual report of the National Notifiable Diseases Surveillance System. *Commun Dis Intell* **29**:1-61.
- Mitsunashi, S. (1971) The problems of drug-resistant pathogenic bacteria. Epidemiology and genetics of R factors. *Ann N Y Acad Sci* **182**:141-52.
- Moellering, R. C., Jr. (1991) The Garrod Lecture. The enterococcus: a classic example of the impact of antimicrobial resistance on therapeutic options. *J Antimicrob Chemother* **28**:1-12.
- Mulvey, M. R., D. Boyd, A. Cloeckaert, R. Ahmed, and L. K. Ng. (2004) Emergence of multidrug-resistant *Salmonella* Paratyphi B dT+, Canada. *Emerg Infect Dis* **10**:1307-10.
- Mulvey, M. R., D. A. Boyd, A. B. Olson, B. Doublet, and A. Cloeckaert. (2006) The genetics of *Salmonella* genomic island 1. *Microbes Infect* **8**:1915-22.

- Musto, J., M. Kirk, D. Lightfoot, B. G. Combs, and L. Mwanri. (2006) Multi-drug resistant *Salmonella* Java infections acquired from tropical fish aquariums, Australia, 2003-04. *Commun Dis Intell* **30**:222-7.
- Nakaya, R., A. Nakamura, and Y. Murata. (1960) Resistance transfer agents in *Shigella*. *Biochem Biophys Res Commun* **3**:654-9.
- NEPSS. (2001) NEPSS Human Annual Report, 2001. MDU, The University of Melbourne.
- Ng, L. K., M. R. Mulvey, I. Martin, G. A. Peters, and W. Johnson. (1999) Genetic characterization of antimicrobial resistance in Canadian isolates of *Salmonella* serovar Typhimurium DT104. *Antimicrob Agents Chemother* **43**:3018-21.
- Nield, B. S., A. J. Holmes, M. R. Gillings, G. D. Recchia, B. C. Mabbutt, K. M. Nevalainen, and H. W. Stokes. (2001) Recovery of new integron classes from environmental DNA. *FEMS Microbiol Lett* **195**:59-65.
- Oelschlaeger, T. A., and J. Hacker. (2004) Impact of pathogenicity islands in bacterial diagnostics. *Apmis* **112**:930-6.
- Parry, C. M. (2003) Antimicrobial drug resistance in *Salmonella enterica*. *Curr Opin Infect Dis* **16**:467-72.
- Parry, C. M., T. T. Hien, G. Dougan, N. J. White, and J. J. Farrar. (2002) Typhoid fever. *N Engl J Med* **347**:1770-82.
- Partridge, S. R., H. J. Brown, and R. M. Hall. (2002) Characterization and movement of the class 1 integron known as Tn2521 and Tn1405. *Antimicrob Agents Chemother* **46**:1288-94.
- Partridge, S. R., H. J. Brown, H. W. Stokes, and R. M. Hall. (2001a) Transposons Tn1696 and Tn21 and their integrons In4 and In2 have independent origins. *Antimicrob Agents Chemother* **45**:1263-70.
- Partridge, S. R., and R. M. Hall. (2003) In34, a complex In5 family class 1 integron containing orf513 and *dfrA10*. *Antimicrob Agents Chemother* **47**:342-9.
- Partridge, S. R., and R. M. Hall. (2004) Complex Multiple Antibiotic and Mercury Resistance Region Derived from the r-det of NR1 (R100). *Antimicrob Agents Chemother* **48**:4250-5.
- Partridge, S. R., G. D. Recchia, C. Scaramuzzi, C. M. Collis, H. W. Stokes, and R. M. Hall. (2000) Definition of the *attI1* site of class 1 integrons. *Microbiology* **146**(Pt 11):2855-64.

- Partridge, S. R., G. D. Recchia, H. W. Stokes, and R. M. Hall. (2001b) Family of class 1 integrons related to In4 from Tn1696. *Antimicrob Agents Chemother* **45**:3014-20.
- Paulsen, I. T., T. G. Littlejohn, P. Radstrom, L. Sundstrom, O. Skold, G. Swedberg, and R. A. Skurray. (1993) The 3' conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. *Antimicrob Agents Chemother* **37**:761-8.
- Peirano, G., Y. Agerso, F. M. Aarestrup, E. M. dos Reis, and D. dos Prazeres Rodrigues. (2006) Occurrence of integrons and antimicrobial resistance genes among *Salmonella enterica* from Brazil. *J Antimicrob Chemother* **58**:305-9.
- Pelkonen, S., E. L. Romppanen, A. Siitonen, and J. Pelkonen. (1994) Differentiation of *Salmonella* serovar infantis isolates from human and animal sources by fingerprinting IS200 and 16S rrn loci. *J Clin Microbiol* **32**:2128-33.
- Perreten, V., and P. Boerlin. (2003) A new sulfonamide resistance gene (*sul3*) in *Escherichia coli* is widespread in the pig population of Switzerland. *Antimicrob Agents Chemother* **47**:1169-72.
- Phillips, I., M. Casewell, T. Cox, B. De Groot, C. Friis, R. Jones, C. Nightingale, R. Preston, and J. Waddell. (2004) Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* **53**:28-52.
- Ploy, M. C., F. Denis, P. Courvalin, and T. Lambert. (2000) Molecular characterization of integrons in *Acinetobacter baumannii*: description of a hybrid class 2 integron. *Antimicrob Agents Chemother* **44**:2684-8.
- Poirel, L., G. F. Weldhagen, C. De Champs, and P. Nordmann. (2002) A nosocomial outbreak of *Pseudomonas aeruginosa* isolates expressing the extended-spectrum beta-lactamase GES-2 in South Africa. *J Antimicrob Chemother* **49**:561-5.
- Popoff, M. Y., J. Bockemuhl, and L. L. Gheesling. (2003) Supplement 2001 (no. 45) to the Kauffmann-White scheme. *Res Microbiol* **154**:173-4.
- Prats, G., B. Mirelis, T. Llovet, C. Munoz, E. Miro, and F. Navarro. (2000) Antibiotic resistance trends in enteropathogenic bacteria isolated in 1985-1987 and 1995-1998 in Barcelona. *Antimicrob Agents Chemother* **44**:1140-5.
- Prescott, J. F. (2006) History of Antimicrobial Usage in Agriculture, p. 19-28. In F. M. Aarestrup (ed.), *Antimicrobial Resistance in bacteria of human origin*. ASM Press, Washington D. C., USA.

- Rabsch, W., H. L. Andrews, R. A. Kingsley, R. Prager, H. Tschape, L. G. Adams, and A. J. Baumler. (2002) *Salmonella enterica* serotype Typhimurium and its host-adapted variants. *Infect Immun* **70**:2249-55.
- Radstrom, P., O. Skold, G. Swedberg, J. Flensburg, P. H. Roy, and L. Sundstrom. (1994) Transposon Tn5090 of plasmid R751, which carries an integron, is related to Tn7, Mu, and the retroelements. *J Bacteriol* **176**:3257-68.
- Randall, L. P., S. W. Cooles, M. K. Osborn, L. J. Piddock, and M. J. Woodward. (2004) Antibiotic resistance genes, integrons and multiple antibiotic resistance in thirty-five serotypes of *Salmonella enterica* isolated from humans and animals in the UK. *J Antimicrob Chemother* **53**:208-16.
- Rasmussen, M. A., S. A. Carlson, S. K. Franklin, Z. P. McCuddin, M. T. Wu, and V. K. Sharma. (2005) Exposure to rumen protozoa leads to enhancement of pathogenicity of and invasion by multiple-antibiotic-resistant *Salmonella enterica* bearing SGI1. *Infect Immun* **73**:4668-75.
- Recchia, G. D., and R. M. Hall. (1995) Gene cassettes: a new class of mobile element. *Microbiology* **141** (Pt 12):3015-27.
- Recchia, G. D., and R. M. Hall. (1997) Origins of the mobile gene cassettes found in integrons. *Trends Microbiol* **5**:389-94.
- Recchia, G. D., H. W. Stokes, and R. M. Hall. (1994) Characterisation of specific and secondary recombination sites recognised by the integron DNA integrase. *Nucleic Acids Res* **22**:2071-8.
- Ribot, E. M., R. K. Wierzb, F. J. Angulo, and T. J. Barrett. (2002) *Salmonella enterica* serotype Typhimurium DT104 isolated from humans, United States, 1985, 1990, and 1995. *Emerg Infect Dis* **8**:387-91.
- Riccio, M. L., J. D. Docquier, E. Dell'Amico, F. Luzzaro, G. Amicosante, and G. M. Rossolini. (2003) Novel 3-N-aminoglycoside acetyltransferase gene, *aac(3)-Ic*, from a *Pseudomonas aeruginosa* integron. *Antimicrob Agents Chemother* **47**:1746-8.
- Ridley, A., and E. J. Threlfall. (1998) Molecular epidemiology of antibiotic resistance genes in multiresistant epidemic *Salmonella* typhimurium DT 104. *Microb Drug Resist* **4**:113-8.
- Rosser, S. J., and H. K. Young. (1999) Identification and characterization of class 1 integrons in bacteria from an aquatic environment. *J Antimicrob Chemother* **44**:11-8.
- Russell, P. J. (1998) *Genetics*. Harper Collins Publishers, New York.

- Sallen, B., A. Rajoharison, S. Desvarenne, and C. Mabilat. (1995) Molecular epidemiology of integron-associated antibiotic resistance genes in clinical isolates of *Enterobacteriaceae*. *Microb Drug Resist* **1**:195-202.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. (1989) *Molecular Cloning: A Laboratory Manual*, **2nd ed.** Cold Spring Harbor Laboratory Press, New York.
- Sandvang, D., and F. M. Aarestrup. (2000) Characterization of aminoglycoside resistance genes and class 1 integrons in porcine and bovine gentamicin-resistant *Escherichia coli*. *Microb Drug Resist* **6**:19-27.
- Sandvang, D., F. M. Aarestrup, and L. B. Jensen. (1998) Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS Microbiol Lett* **160**:37-41.
- Schwarz, S., and E. Chaslus-Dancla. (2001) Use of antimicrobials in veterinary medicine and mechanisms of resistance. *Vet Res* **32**:201-25.
- Schwocho, L. R., C. P. Schaffner, G. H. Miller, R. S. Hare, and K. J. Shaw. (1995) Cloning and characterization of a 3-N-aminoglycoside acetyltransferase gene, *aac(3)-Ib*, from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **39**:1790-6.
- Senanayake, S. N., M. J. Ferson, S. J. Botham, and R. T. Belinfante. (2004) A child with *Salmonella enterica* serotype Paratyphi B infection acquired from a fish tank. *Med J Aust* **180**:250.
- Severino, P., and V. D. Magalhaes. (2004) Integrons as tools for epidemiological studies. *Clin Microbiol Infect* **10**:156-62.
- Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller. (1993) Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev* **57**:138-63.
- Silver, S., and L. T. Phung. (1996) Bacterial heavy metal resistance: new surprises. *Annu Rev Microbiol* **50**:753-89.
- Stanley, J., N. Baquar, and E. J. Threlfall. (1993) Genotypes and phylogenetic relationships of *Salmonella* typhimurium are defined by molecular fingerprinting of IS200 and 16S *rrn* loci. *J Gen Microbiol* **139 Pt 6**:1133-40.
- Stanley, J., C. S. Jones, and E. J. Threlfall. (1991) Evolutionary lines among *Salmonella enteritidis* phage types are identified by insertion sequence IS200 distribution. *FEMS Microbiol Lett* **66**:83-9.

- Stokes, H. W., and R. M. Hall. (1989) A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol* **3**:1669-83.
- Stokes, H. W., and R. M. Hall. (1991) Sequence analysis of the inducible chloramphenicol resistance determinant in the Tn1696 integron suggests regulation by translational attenuation. *Plasmid* **26**:10-9.
- Stokes, H. W., D. B. O'Gorman, G. D. Recchia, M. Parsekhian, and R. M. Hall. (1997) Structure and function of 59-base element recombination sites associated with mobile gene cassettes. *Mol Microbiol* **26**:731-45.
- Stokes, H. W., C. L. Nesbo, M. Holley, M. I. Bahl, M. R. Gillings, and Y. Boucher. (2006) Class 1 integrons potentially predating the association with Tn402-like transposition genes are present in a sediment microbial community. *J Bacteriol* **188**:5722-30.
- Stokes, H. W., C. Tomaras, Y. Parsons, and R. M. Hall. (1993) The partial 3'-conserved segment duplications in the integrons In6 from pSa and In7 from pDGO100 have a common origin. *Plasmid* **30**:39-50.
- Stuart, N. C. (1983) Treatment of fish disease. *Vet Rec* **112**:173-7.
- Sunde, M. (2005) Prevalence and characterization of class 1 and class 2 integrons in *Escherichia coli* isolated from meat and meat products of Norwegian origin. *J Antimicrob Chemother* **56**:1019-24.
- Sundstrom, L., C. Jansson, K. Bremer, E. Heikkila, B. Olsson-Liljequist, and O. Skold. (1995) A new dhfrVIII trimethoprim-resistance gene, flanked by IS26, whose product is remote from other dihydrofolate reductases in parsimony analysis. *Gene* **154**:7-14.
- Sundstrom, L., P. Radstrom, G. Swedberg, and O. Skold. (1988) Site-specific recombination promotes linkage between trimethoprim- and sulfonamide resistance genes. Sequence characterization of *dhfrV* and *sulI* and a recombination active locus of Tn21. *Mol Gen Genet* **213**:191-201.
- Sundstrom, L., P. H. Roy, and O. Skold. (1991) Site-specific insertion of three structural gene cassettes in transposon Tn7. *J Bacteriol* **173**:3025-8.
- Sundstrom, L., and O. Skold. (1990) The *dhfrI* trimethoprim resistance gene of Tn7 can be found at specific sites in other genetic surroundings. *Antimicrob Agents Chemother* **34**:642-50.
- Swartz, M. N. (1994) Hospital-acquired infections: diseases with increasingly limited therapies. *Proc Natl Acad Sci U S A* **91**:2420-7.

- Swift, G., B. J. McCarthy, and F. Heffron. (1981) DNA sequence of a plasmid-encoded dihydrofolate reductase. *Mol Gen Genet* **181**:441-7.
- Szczepanowski, R., S. Braun, V. Riedel, S. Schneiker, I. Krahn, A. Puhler, and A. Schluter. (2005) The 120 592 bp IncF plasmid pRSB107 isolated from a sewage-treatment plant encodes nine different antibiotic-resistance determinants, two iron-acquisition systems and other putative virulence-associated functions. *Microbiology* **151**:1095-111.
- Talmadge, K., S. Stahl, and W. Gilbert. (1980) Eukaryotic signal sequence transports insulin antigen in *Escherichia coli*. *Proc Natl Acad Sci U S A* **77**:3369-73.
- Taylor, D. E., and E. C. Brose. (1985) Characterization of incompatibility group HII plasmids from *Salmonella typhi* by restriction endonuclease digestion and hybridization of DNA probes for Tn3, Tn9, and Tn10. *Can J Microbiol* **31**:721-9.
- Tennstedt, T., R. Szczepanowski, S. Braun, A. Puhler, and A. Schluter. (2003) Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. *FEMS Microbiol Ecol* **45**:239-252.
- Threlfall, E. J. (2000) Epidemic *Salmonella* Typhimurium DT 104--a truly international multiresistant clone. *J Antimicrob Chemother* **46**:7-10.
- Threlfall, J., B. Levent, K. L. Hopkins, E. de Pinna, L. R. Ward, and D. J. Brown. (2005) Multidrug-resistant *Salmonella* Java. *Emerg Infect Dis* **11**:170-1.
- Threlfall, E. J., L. R. Ward, J. A. Frost, and G. A. Willshaw. (2000) The emergence and spread of antibiotic resistance in food-borne bacteria. *Int J Food Microbiol* **62**:1-5.
- Threlfall, E. J. (2002) Antimicrobial drug resistance in *Salmonella*: problems and perspectives in food- and water-borne infections. *FEMS Microbiol Rev* **26**:141-8.
- Threlfall, E. J., I. S. Fisher, C. Berghold, P. Gerner-Smidt, H. Tschape, M. Cormican, I. Luzzi, F. Schnieder, W. Wannet, J. Machado, and G. Edwards. (2003) Trends in antimicrobial drug resistance in *Salmonella enterica* serotypes Typhi and Paratyphi A isolated in Europe, 1999-2001. *Int J Antimicrob Agents* **22**:487-91.
- Thungapathra, M., Amita, K. K. Sinha, S. R. Chaudhuri, P. Garg, T. Ramamurthy, G. B. Nair, and A. Ghosh. (2002) Occurrence of antibiotic resistance gene cassettes *aac(6')-Ib*, *dfrA5*, *dfrA12*, and *ereA2* in class I integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. *Antimicrob Agents Chemother* **46**:2948-55.

- Tietze, E., J. Brevet, and H. Tschape. (1987) Relationships among the streptothricin resistance transposons Tn1825 and Tn1826 and the trimethoprim resistance transposon Tn7. *Plasmid* **18**:246-9.
- Tilzey, R. (2005) A strategic approach to the management of ornamental fish in Australia. Bureau of Rural Sciences, The Commonwealth of Australia, Canberra.
- van Pelt, W., H. van der Zee, W. J. Wannet, A. W. van de Giessen, D. J. Mevius, N. M. Bolder, R. E. Komijn, and Y. T. van Duynhoven. (2003) Explosive increase of *Salmonella* Java in poultry in the Netherlands: consequences for public health. *Euro Surveill* **8**:31-5.
- Varma, J. K., K. D. Greene, J. Ovitt, T. J. Barrett, F. Medalla, and F. J. Angulo. (2005) Hospitalization and antimicrobial resistance in *Salmonella* outbreaks, 1984-2002. *Emerg Infect Dis* **11**:943-6.
- Velge, P., A. Cloeckaert, and P. Barrow. (2005) Emergence of *Salmonella* epidemics: the problems related to *Salmonella enterica* serotype Enteritidis and multiple antibiotic resistance in other major serotypes. *Vet Res* **36**:267-88.
- Vo, A. T., E. van Duijkeren, A. C. Fluit, W. J. Wannet, A. J. Verbruggen, H. M. Maas, and W. Gaastra. (2006) Antibiotic resistance, integrons and *Salmonella* genomic island 1 among non-typhoidal *Salmonella* serovars in The Netherlands. *Int J Antimicrob Agents* **28**:172-9.
- Vo, A. T., E. van Duijkeren, A. C. Fluit, and W. Gaastra. (2007) A novel *Salmonella* genomic island 1 and rare integron types in *Salmonella* Typhimurium isolates from horses in The Netherlands. *J Antimicrob Chemother* **59**:594-9.
- Wain, J., L. T. Diem Nga, C. Kidgell, K. James, S. Fortune, T. Song Diep, T. Ali, O. G. P, C. Parry, J. Parkhill, J. Farrar, N. J. White, and G. Dougan. (2003) Molecular analysis of incHI1 antimicrobial resistance plasmids from *Salmonella* serovar Typhi strains associated with typhoid fever. *Antimicrob Agents Chemother* **47**:2732-9.
- Waldor, M. K., H. Tschape, and J. J. Mekalanos. (1996) A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. *J Bacteriol* **178**:4157-65.
- Wall, P. G., D. Morgan, K. Lamden, M. Ryan, M. Griffin, E. J. Threlfall, L. R. Ward, and B. Rowe. (1994) A case control study of infection with an epidemic strain of multiresistant *Salmonella* typhimurium DT104 in England and Wales. *Commun Dis Rep CDR Rev* **4**:R130-5.
- Ward, L. R., J. D. de Sa, and B. Rowe. (1987) A phage-typing scheme for *Salmonella* enteritidis. *Epidemiol Infect* **99**:291-4.

- Watanabe, T. (1963) Infective heredity of multiple drug resistance in bacteria. *Microbiol. Mol. Biol. Rev.* **27**:87-115.
- Wegener, H. C. (2003) Antibiotics in animal feed and their role in resistance development. *Curr Opin Microbiol* **6**:439-45.
- Weill, F. X., L. Fabre, B. Grandry, P. A. Grimont, and I. Casin. (2005) Multiple-antibiotic resistance in *Salmonella enterica* serotype Paratyphi B isolates collected in France between 2000 and 2003 is due mainly to strains harboring *Salmonella* genomic islands 1, 1-B, and 1-C. *Antimicrob Agents Chemother* **49**:2793-801.
- White, P. A., C. J. McIver, and W. D. Rawlinson. (2001) Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrob Agents Chemother* **45**:2658-61.
- White, D. G., S. Zhao, S. Simjee, D. D. Wagner, and P. F. McDermott. (2002) Antimicrobial resistance of foodborne pathogens. *Microbes Infect* **4**:405-12.
- Whittington, R. J., and R. Chong. (2007) Global trade in ornamental fish from an Australian perspective: the case for revised import risk analysis and management strategies. *Prev Vet Med* **81**:92-116.
- WHO (World Health Organisation) (2002). Use of antimicrobials outside human medicine and resultant antimicrobial resistance in humans. Fact sheet number **268**, p.2. www.who.int/mediacentre/factsheets/fs268/en/print.html
- WHO (World Health Organisation) (2005). Drug Resistant *Salmonella*. Fact sheet number **139**, p.1. www.who.int/mediacentre/factsheets/fs139/en/print.html
- Witte, W. (1998) Medical consequences of antibiotic use in agriculture. *Science* **279**:996-7.
- Wohlleben, W., W. Arnold, L. Bissonnette, A. Pelletier, A. Tanguay, P. H. Roy, G. C. Gamboa, G. F. Barry, E. Aubert, J. Davies, and et al. (1989) On the evolution of Tn21-like multiresistance transposons: sequence analysis of the gene (*aacCI*) for gentamicin acetyltransferase-3-I(AAC(3)-I), another member of the Tn21-based expression cassette. *Mol Gen Genet* **217**:202-8.
- Womble, D. D., and R. H. Rownd. (1988) Genetic and physical map of plasmid NR1: comparison with other IncFII antibiotic resistance plasmids. *Microbiol Rev* **52**:433-51.
- Woodcock, D. M., P. J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. S. Smith, M. Z. Michael, and M. W. Graham. (1989) Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res* **17**:3469-78.

- Yu, H. S., J. C. Lee, H. Y. Kang, Y. S. Jeong, E. Y. Lee, C. H. Choi, S. H. Tae, Y. C. Lee, S. Y. Seol, and D. T. Cho. (2004) Prevalence of *dfr* genes associated with integrons and dissemination of *dfrA17* among urinary isolates of *Escherichia coli* in Korea. J Antimicrob Chemother **53**:445-50.
- Zhao, S., D. G. White, P. F. McDermott, S. Friedman, L. English, S. Ayers, J. Meng, J. J. Maurer, R. Holland, and R. D. Walker. (2001) Identification and expression of cephamycinase bla(CMY) genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob Agents Chemother **45**:3647-50.

A.1 Appendix 1

General working solutions and buffers

Culture Reagents

LB Media

Tryptone	10g/l
Yeast	5g/l
NaCl	5g/l

LB Agar

Tryptone	10g/l
Yeast	5g/l
NaCl	5g/l
Agar	15g/l

Harvesting Bacterial Pellets

PBS (1 L)

NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄	1.15 g
KH ₂ PO ₄	0.31 g

Phenol/Chloroform DNA Extractions

TE Buffer (1 L)

Tris	10 mM
EDTA	1 mM
pH 8.0	

0.5 M EDTA (200 ml)

EDTA	37.224 g
pH 8.0	

NaOH pellets were added to adjust the pH the EDTA would not go into solution before the pH was adjusted.

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

phenol pH 8.0	150 ml
chloroform	144 ml
isoamylalcohol	6 ml

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

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

chloroform	288 ml
isoamylalcohol	12 ml

The above solution was mixed thoroughly and 100 ml of TE buffer (pH 8.0) was added. The bottle was stored in the fridge.

Agarose Gel Electrophoresis

5 X TBE Buffer (1 L) (stock solution)

Tris	54 g
Boric acid	27.5 g
0.5 M EDTA pH 8.0	20 ml

0.5 X TBE Running Buffer (2 L) (working solution)

5 X TBE Buffer	200 ml
MilliQ water	1800 ml

100bp⁺ molecular weight markers (GeneRuler™)

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

DNA Sequence Analysis

TE Buffer (1 L)

Tris-HCl	0.3152 g
EDTA	0.0744 g
pH 8.0	

Loading Buffer

deionised formamide	83.3%
25 mM EDTA (pH 8.0)	16.7%
with 50 mg/ml blue dextran	

A.2 Appendix 2

DNA sequencing primers

Isolate No	Primer Name	Primer Sequence (5'- 3')
SRC01	1L1FA	GTT CAC GTT GAA GTC GAA GG
	1L1RA	CCT TCG ACT TCA ACG TGA AC
	1R1FA	GCA GAG TCG GAT TGG AAG GG
	1R1RA	CCC TTC CAA TCC GAC TCT GC
SRC17	17L1FA	GTG GCG GGG AGA TTT ACA GAG
	17R1RA	GAG TAA CGA CCG CGT ATT TCC
SRC19	19L1FA	CTA CAA TAG ACA TCG AGC CGG
	19L1RA	CCG GCT CGA TGT CTA TTG TAG
	19R1FA	GGC AAT CGA GCG GCA TGA GCG
	19R1RA	CGC TCA TGC CGC TCG ATT GCC
SRC22	22L1FA	CCG CGC TAT AGA AGT CAC CC
	22L1RA	GGG TGA CTT CTA TAG CGC GG
	22R1FA	GCT TAG CTT CAA GTA AGA CGG
	22R1RA	CCG TCT TAC TTG AAG CTA AGC
	22orf(R)	AAG CCG ACC GCA GAA TGC
SRC24	24L1FA	CGC TTT GGC ATC CGA ACT CGG
	24L1RA	CCG AGT TCG GAT GCC AAA GCG
	24R1FA	CCT TGA AGC TAT GGA ACT CGC
	24R1RA	GCG AGT TCC ATA GCT TCA AGG
	24L1FB	GCA AGC GCA AGC AGC TAT CG
	24L1RB	CGA TAG CTG CTT GCG CTT GC
	24R1FB	GGT TAC TGT GGC CGT AAA GC
	24R1RB	GCT TTA CGG CCA CAG TAA CC
SRC45	45L1FA	GCT CTG CTA GGT CCA TCC G
	45L1RA	CGG ATG GAC CTA GCA GAG C
	45R1RA	GCC ACT CAC CGA ACT GAA GC
	45L1FB	GCA TCG ACC GGC GCC CAG GC
SRC54	54L1FB	GCG AGA TCA TCA AGT CAG TGG
	54R1RB	GGT CAT TGA GCA ATG CTC GC
	54L1FA(2)	GCG CTG AGG GAA ACC TTG
	54R1RB(2)	GCT GAT GAG GTT CAA CAC
	54R1FA(2)	CAC AAC TTG GAG TAG TCG
	54F1	CCT TGA AGC TAT GGA ACT CGC
	54F2	CAA GGC TAT GGG TGG TTT CC
	LinF(F1)	GCG CTC GTG GCT TAA TGC
	LinF(R1)	GCA TTA AGC CAC GAG CGC
	LinF(F2)	CTG AAT ATC GAG CTT CCG AG
	LinF(R2)	CTC GGA AGC TCG ATA TTC AG
SRC70	70L1FA	GCC ATC TCG AAC CGA CGT TGC
	70R1RB	GCT GTA CCA CCC GAC AAT CTG
	70R1RA	GCC ACG GAA TGA TGT CGT CG
	70R1FA	CGA CGA CAT CAT TCC GTG GC
SRC73	73L1FA	GCG TAG AAG ACC AAC CAG C
	73R1RB	GCT TGT GAT AGT TGC ACC G
	73R1RA	GCG GCG AGT TCC ATA GCT TC
	73R1FA	GAA GCT ATG GAA CTC GCC GC
	73R1RB(2)	GGA AGT GAC CAT CGT CG
	73L1FA(2)	GCC GAC GGC TTT GCC TTC G
	73R1RA(2)	CGA AGG CAA AGC CGT CGG C
	73L2F1	TTA CGT CCA AGC TGA TAA AGG
	73QS-R1	GTG GCA GCA ACA TCC TTT GG

A.3 Appendix 3

Table A.3.1 Summary of the L1/R1 variable gene cassette PCR amplicon and *RsaI* digestion profiles.

Gene Cassette Array	Approx amplicon size	RFLP profile	SRC numbers of isolates containing cassette array (total number)	Serovars	Sources
<i>dfrA5</i>	750 bp	A	17*, 21, 23, 26, 29, 30, 32, 60, 61, 62, 77, 79, 80, 97, 98, 100, 108, 111, 112, 115, 117, 118, 120, 121, 122, 123, 125, 127, 129, 135, 136 (31)	Bovismorbificans, Kiambu, Seftenberg, Typhimurium (PT1var2, PT29, PT44, PT68, PT135, PT141, 141v4, RDNC), Sofia, Bovismorbificans (PT11, PT13, PT14, PT24, PT32), Stanley, unknown	bovine, chicken, porcine, unknown
<i>aadA2</i>	1 kb	B	46* (1)	Infantis	human
<i>aadA4</i>	1 kb	D	45* (1)	Havana	human
Δ <i>orfF</i> - <i>aadA2</i>	1.1 kb	C	22*, 69*, 74, 83, 91, 102, 119 (7)	Ohio, Havana, Bovismorbificans u, Seftenberg, Typhimurium PT208 v1	porcine, chicken
<i>dfrA1</i> - <i>orfC</i>	1.2 kb	E	19* (1)	Emek	waste effluent
Potential SGI1 (Chpt 4)	1.2 kb	F	5 [#] , 38 [#] (2)	Cerro, Dusseldorf	human
<i>dfrB6</i> - <i>aadA1</i>	1.4 kb	G	70*, 71, 72, 92, 93, 94, 95, 96 (8)	Infantis	feline, chicken, canine
<i>aacCA5</i> - <i>aadA7</i>	1.6 kb	I	73* (1)	Kentucky	imported spice
<i>dfrA1</i> - <i>aadA1</i>	1.6 kb	J	116* (1)	Typhimurium PT135	bovine
<i>dfrA17</i> - <i>aadA5</i>	1.7 kb	H	1*, 87, 90 (3)	Singapore, Derby, Havana	human, porcine
<i>dfrA12</i> - <i>orfF</i> - <i>aadA2</i>	1.8kb	K	16, 24*, 27, 28, 34, 56 (6)	Typhimurium (PTu, 104L, PT135) Sofia, , Agona	human, chicken, equine
<i>aadA2</i> - <i>linG</i>	2 kb	L	54* (1)	Stanley	human
Potential SGI1 (Chpt 4)	1 & 1.2kb	M	10, 11, 49, 50, 99, 101 ^Y (6)	Kiambu, Paratyphi B dT ⁺	human, chicken, fish tank water
Potential SGI1 (Chpt 4)	1 & 1.2kb	N	4 ^Y (1)	Derby	human

* indicates isolates that were sequenced as representatives from each restriction enzyme profile.

Y isolates of the multiple PCR product and *RsaI* profile were not sequenced due to the presence of multiple PCR products.

amplicons could not be sequenced.

NB. Further information on potential SGI1 containing strains can be found in chapters listed.

Table A.3.2 A summary of *intI1*, *sulI* and gene cassette results for *intI1* negative strains.

SRC #	Serovar	Source/Yr	R Phen	<i>intI1/sulI</i> hyb	L1/R1
35	Agona	human, M Malay/ (01)	SmSuTc (Na)	-/-	-
57	Agona	porcine / (01)	ApGmTc	-/-	-
58	Agona	porcine/ (01)	Tc	-/-	-
78	Anatum	porcine / (00)	Tc	-/-	-
02	Blockley	human, F Bali / (99)	KmSmTc (NaCp')	-/-	-
03	Blockley	human, M Thai / (99)	CmKmSmSpSuTcTp (NaCp')	-/-	-
36	Bovismorbificans PT13	human, F/ (01)	ApKmSmSuTcTp	-/-	-
59	Bovismorbificans PT13	bovine/ (01)	ApKmSmSuTcTp	-/-	-
81	Bovismorbificans 24	abattoir effluent/ (00)	ApGmTc	-/-	-
84	Bredeney	porcine/ (00)	SuTcTp	-/-	-
37	Derby	human, F/ (01)	SuTc	-/-	-
18	Derby	porcine/ (99)	ApTc	-/-	-
85	Derby	porcine/ (00)	SmTc	-/-	-
86	Derby	porcine/ (00)	ApCmTc	-/-	-
88	Derby	porcine / (00)	ApGmTc	-/-	-
63	Derby	porcine/ (01)	SmTc	-/-	-
06	Enteritidis PT1	human, F HK/ (99)	SmSuTc (NaCp')	-/-	-
07	Hadar	human, M Indo / (99)	ApSmTc	-/-	-
08	Hadar PT10	human, M o/s/ (99)	ApTc (NaCp')	-/-	-
09	Hadar PT2	human, F/ (99)	SmTc	-/-	-
40	Hadar PT11	human, F Bali/ (01)	Tc (NaCp')	-/-	-
42	Hadar PT14	human, F Bali/ (01)	Tc (Na)	-/-	-
39	Hadar PT10	human, M Indo/ (01)	SmTc (NaCp')	-/-	-
43	Hadar PT2	human, F/ (01)	SmTc (Na)	-/-	-
44	Hadar PT22	human, F/ (01)	SmTc (Na)	-/-	-
64	Hadar PT11	duck/ (01)	SmTc	-/-	-
65	Hadar PT14	duck/ (01)	SmTc	-/-	-
66	Hadar PT2	duck/ (01)	SmTc	-/-	-
67	Hadar PT22	duck/ (01)	SmTc	-/-	-
68	Hadar PT33	duck/ (01)	SmTc	-/-	-
47	Infantis	human, M/ (01)	ApTcTp	-/-	-
20	Infantis	chicken/ (99)	SmSpSuTc	-/-	-

SRC #	Serovar	Source/Yr	R Phen	<i>intI1/ sulI</i> hyb	L1/R1
48	Montevideo	human, M/ (01)	GmKmSmSpTc (NaCp')	-/-	-
104	Seftenberg	meat, bone meal/ (00)	Sm	-/-	-
52	Sofia	human,M/ (01)	Tp	-/-	-
105	Sofia	canine/ (00)	Tc	-/-	-
25	Sofia	chicken/ (99)	ApTc	-/-	-
106	Sofia	chicken/ (00)	ApSmSuTc	-/-	-
107	Sofia	chicken/ (00)	SmSu	-/-	-
109	Sofia	chicken/ (00)	SmSuTcTp	-/-	-
110	Sofia	chicken / (00)	ApSmSuTc	-/-	-
75	Sofia	chicken/ (01)	Tp	-/-	-
76	Sofia	chicken/ (01)	SuTp	-/-	-
55	Stanley	human, F Thai /(01)	SmSuTc	-/-	-
12	Typhimurium PT135	human, F/ (99)	ApSu	-/-	-
13	Typhimurium PT135	human, F (99)	ApSmSuTcTp	-/-	-
15	Typhimurium PT9	human, F/ (99)	SmSu	-/-	-
114	Typhimurium PT135	avian/ (00)	Sm	-/-	-
33	Typhimurium PT9	bovine/ (99)	SmSu	-/-	-
113	Typhimurium PT12	bovine/ (00)	Tc	-/-	-
130	Typhimurium PT64	bovine/ (00)	SmSu	-/-	-
132	Typhimurium PT9	bovine/ (00)	ApSmSu	-/-	-
133	Typhimurium PT9	bovine /(00)	ApKmSmSu	-/-	-
134	Typhimurium PT99	pheasant/ (00)	Tc	-/-	-
137	Typhimurium	??/??	Tc	-/-	-

Table A.3.3 A summary of *intI1*, *sulI* and gene cassette results for *intI1* containing strains with no L1/R1 PCR product.

SRC #	Serovar	Source/Yr	R Phen	<i>intI1/sulI</i> hyb	L1/R1
82	Bovismorbificans 32	bovine /(00)	ApSmSuTp	+/-	-
41	Hadar PT11	human, M (01)	CmKmSmSpSuTc (NaCp')	+/-	-
103	Seftenberg	porcine (00)	ApGmKmSmSpSuTc	+/+	-
51	Singapore	human, M (01)	CmSmSpSuTcTp (NaCp')	+/+	-
53	Sofia	human, F/ (01)	Km	+/-	-
14	Typhimurium PT44	human, F (99)	ApSmSuTcTp	+/-	-
31	Typhimurium PT44	bovine (99)	ApSmSuTcTp	+/-	-
131	Typhimurium PT9	bovine (00)	ApSmSuTp	+/-	-
124	Typhimurium PT44	bovine (00)	ApSmSuTcTp	+/-	-
126	Typhimurium PT44	bovine (00)	ApSmSuTp	+/-	-
128	Typhimurium PT44	bovine (00)	ApSmSuTcTp	+/-	-

Table A.3.4 A summary of *intI1*, *sulI* and gene cassette results for *intI1* positive strains not containing *sulI*.

SRC #	Serovar	Source/Yr	R Phen	<i>intI1</i> / <i>sulI</i> hyb	L1/R1
80	Bovismorbificans PT24	bovine /(00)	ApCmKmSmSuTcTp	+/-	<i>dfrA5</i>
61	Bovismorbificans PT11	bovine /(01)	ApKmSmSuTcTp	+/-	<i>dfrA5</i>
91	Havana	porcine (00)	GmSmSpSuTc	+/-	Δ <i>orfF-aadA2</i>
69	Havana	porcine (01)	ApGmKmSmSpTc	+/-	Δ <i>orfF-aadA2</i>
23	Seftenberg	porcine (99)	ApCmSmSpSuTcTp	+/-	<i>dfrA5</i>
24	Sofia	chicken (99)	SmSpSuTp	+/-	<i>dfrA12-orfF-aadA2</i>
32	Typhimurium PT68	bovine (99)	ApKmSmSuTcTp	+/-	<i>dfrA5</i>
129	Typhimurium PT44	bovine (00)	ApKmSmSuTcTp	+/-	<i>dfrA5</i>
116	Typhimurium PT135	bovine (00)	ApSmSuTcTp	+/-	<i>dfrA1-aadA1</i>
115	Typhimurium PT135	chicken/ (00)	TcTp	+/-	<i>dfrA5</i>
29	Typhimurium PT141	porcine (99)	ApGmKmSmSuTcTp	+/-	<i>dfrA5</i>
30	Typhimurium PT141 v4	porcine (99)	ApGmKmSmSuTcTp	+/-	<i>dfrA5</i>
120	Typhimurium PT29	porcine (00)	ApKmSmSuTp	+/-	<i>dfrA5</i>
122	Typhimurium PT29	porcine (00)	ApKmSmSuTp	+/-	<i>dfrA5</i>
123	Typhimurium PT29	porcine (00)	ApKmSmSuTcTp	+/-	<i>dfrA5</i>
118	Typhimurium PT141	porcine (00)	ApGmKmSmSuTcTp	+/-	<i>dfrA5</i>
136	Typhimurium RDNC	porcine (00)	ApKmSmSuTp	+/-	<i>dfrA5</i>
117	Typhimurium PT141	porcine (??)	ApGmKmSmSuTp	+/-	<i>dfrA5</i>
135	Typhimurium RDNC	porcine (??)	ApKmSmSuTcTp	+/-	<i>dfrA5</i>
27	Typhimurium PT135	equine (99)	ApCmGmKmSmSpSuTcTp	+/-	<i>dfrA12-orfF-aadA2</i>
121	??	porcine (00)	ApGmKmSmSuTp	+/-	<i>dfrA5</i>

Table A.3.5 A summary of *intI1*, *sulI* and gene cassette results on isolates containing *intI1*, gene cassette arrays and *sulI*.

SRC #	Serovar	Source/Yr	R Phen	<i>intI1/ sulI</i> hyb	L1/R1
34	Agona	human, F Africa (01)	CmSmSpSuTcTp	+/+	<i>dfrA12-orfF-aadA2</i>
17	Bovismorbificans	bovine / (99)	ApCmSmSuTcTp	+/+	<i>dfrA5</i>
79	Bovismorbificans PT14	bovine /(00)	ApCmKmSmSuTcTp	+/+	<i>dfrA5</i>
60	Bovismorbificans PT13	bovine /(01)	ApCmKmSmSuTcTp	+/+	<i>dfrA5</i>
62	Bovismorbificans PT32	bovine /(01)	ApCmKmSmSuTcTp	+/+	<i>dfrA5</i>
83	Bovismorbificans u	chicken / (00)	CmSmSpSuTc	+/+	Δ <i>orfF-aadA2</i>
05	Cerro	human, F Thai (99)	ApCmSuTcTp (NaCp')	+/+	see Chapter 4
04	Derby	human, M Malay (99)	CmGmSmSpSuTcTp	+/+	see Chapter 4
87	Derby	porcine (00)	SmTcTp	+/+	<i>dfrA17-aadA5</i>
38	Dusseldorf	human, M Malay (01)	ApCmSuTcTp (Na)	+/+	see Chapter 4
19	Emek	waste effluent (99)	CmSuTcTp (NaCp')	+/+	see Chapter 4
45	Havana	human, M (01)	SmSpSuTc	+/+	<i>aadA4</i>
90	Havana	porcine (00)	ApCmSuTcTp	+/+	<i>dfrA17-aadA5</i>
46	Infantis	human, F (01)	SmSpSuTp	+/+	see Chapter 4
92	Infantis	canine (00)	SmSpTcTp	+/+	<i>dfrB6-aadA1</i>
93	Infantis	chicken (00)	ApCmSpSuTc	+/+	<i>dfrB6-aadA1</i>
94	Infantis	chicken (00)	CmSmSpSuTc	+/+	<i>dfrB6-aadA1</i>
95	Infantis	chicken (00)	SmSpSuTc	+/+	<i>dfrB6-aadA1</i>
96	Infantis	chicken (00)	SmSuTc	+/+	<i>dfrB6-aadA1</i>
71	Infantis	chicken (01)	SmSpSuTc	+/+	<i>dfrB6-aadA1</i>
72	Infantis	chicken (01)	ApSmSpSuTc	+/+	<i>dfrB6-aadA1</i>
70	Infantis	feline (01)	SmSpSuTc	+/+	<i>dfrB6-aadA1</i>
73	Kentucky	imported spice India (01)	ApGmSmSpSuTc (Na)	+/+	<i>aacCA5-aadA7</i>
10	Kiambu	human, M (99)	ApCmSmSpSuTcTp	+/+	see Chapter 4
11	Kiambu	human, M (99)	ApCmSmSpSuTcTp (NaCp')	+/+	see Chapter 4
21	Kiambu	chicken (99)	SuTcTp	+/+	<i>dfrA5</i>
97	Kiambu	chicken/ (00)	SuTp	+/+	<i>dfrA5</i>
98	Kiambu	chicken (00)	SuTcTp	+/+	<i>dfrA5</i>
99	Kiambu	chicken (00)	ApCmSmSpSuTcTp	+/+	see Chapter 4
100	Kiambu	chicken (00)	SmSuTcTp	+/+	<i>dfrA5</i>

SRC #	Serovar	Source/Yr	R Phen	<i>intI1/ sulI</i> hyb	L1/R1
22	Ohio	porcine (99)	CmGmKmSmSpSuTc	+/+	$\Delta orfF$ - <i>aadA2</i>
74	Ohio	porcine (01)	ApCmGmKmSmSpSuTc	+/+	$\Delta orfF$ - <i>aadA2</i>
49	Paratyphi B dT ⁺	human, F (01)	ApCmSmSpSuTc	+/+	see Chapter 4
50	Paratyphi B dT ⁺	human, M (01)	ApCmSmSpSuTc	+/+	see Chapter 4
101	Paratyphi B dT ⁺	fish tank water (00)	ApCmSmSpSuTc	+/+	see Chapter 4
102	Seftenberg	porcine (00)	ApGmKmSmSpSuTcTp	+/+	$\Delta orfF$ - <i>aadA2</i>
01	Singapore	human, F (01)	CmSmSpSuTcTp (Na)	+/+	<i>dfrA17</i> - <i>aadA5</i>
108	Sofia	chicken (00)	SuTcTp	+/+	<i>dfrA5</i>
111	Sofia	chicken (00)	SuTcTp	+/+	<i>dfrA5</i>
77	Sofia	chicken (01)	SuTcTp	+/+	<i>dfrA5</i>
54	Stanley	human, M Thai (01)	CmGmKmSmSpSuTc (Cp')	+/+	<i>aadA2</i> - <i>linG</i>
112	Stanley	porcine (00)	ApGmSuTcTp	+/+	<i>dfrA5</i>
16	Typhimurium Pt	human, M (99)	ApCmGmSpSuTcTp	+/+	<i>dfrA12</i> - <i>orfF</i> - <i>aadA2</i>
56	Typhimurium 104L	human, M Timor (??)	ApCmGmKmSmSpSuTcTp	+/+	<i>dfrA12</i> - <i>orfF</i> - <i>aadA2</i>
26	Typhimurium PT1var2	bovine (99)	ApKmSmSuTcTp	+/+	<i>dfrA5</i>
125	Typhimurium PT44	bovine (00)	KmSuTcTp	+/+	<i>dfrA5</i>
127	Typhimurium PT44	bovine (00)	KmSuTcTp	+/+	<i>dfrA5</i>
119	Typhimurium PT208 v1	porcine (00)	ApCmGmKmSmSpSuTc	+/+	$\Delta orfF$ - <i>aadA2</i>
28	Typhimurium PT135	equine (99)	ApCmGmKmSmSpSuTcTp	+/+	<i>dfrA12</i> - <i>orfF</i> - <i>aadA2</i>